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Regulated Attachment Method for Reconstituting Lipid Bilayers of Prescribed Size within Flexible Substrates

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A new method called the regulated attachment method (RAM) for reproducibly forming lipid bilayers within flexible substrates has been developed that enables precise control over the size of the bilayer. This technique uses a deformable flexible substrate to open and close an aperture that subdivides aqueous volumes submersed in an organic solvent. Phospholipids incorporated as vesicles in the aqueous phase self-assemble at the oil/water interface to form lipid monolayers that encapsulate each aqueous volume. Controlled attachment of opposing lipid monolayers is achieved by regulating the dimensions of the aperture in the substrate that separates the adjacent aqueous volumes. In this manner, the size of a lipid bilayer formed within a flexible substrate is a function of the substrate and aperture dimensions, and not determined by the sizes or shapes of the aqueous volumes. Lipid bilayers formed within the prototype flexible substrate exhibit DC resistances consistently higher than 10 GΩ and can survive 20−30× changes in area without rupture. Furthermore, RAM permits lipid bilayers to be completely unzipped after thinning by applying sufficient force to fully close the dividing aperture and even allows the introduction of species, such as alamethicin channels, into preformed lipid bilayers via controlled injection through an intersecting channel within the substrate. Controlling the size of the interface through indirect interactions with the supporting substrate offers a new platform for assembling durable lipid bilayers. We envision that this technology can be scaled to higher dimensions consisting of multiple apertures required for creating aqueous networks partitioned by functional lipid bilayers and to smaller length scales to produce very small lipid bilayers capable of hosting single proteins.

The droplet interface bilayer (DIB) emerged in the last several years as a new approach for constructing biomolecular networks using the principles of molecular self-assembly at an oil/water interface.[1] In this method water droplets submersed in oil become encapsulated with phospholipid molecules present in either the aqueous or organic phase.[2,3] Lipid-encased aqueous droplets are then attached to one other, where a lipid bilayer stabilizes the interface between contacting droplets. The droplet interface bilayer marked a transition in constructing biomolecular arrays that utilize proteins for developing functional devices and the works of Bayley's group at Oxford showcased that proteins incorporated strategically into these "cell mimics" contribute collectively to the whole-network response.[1,4]

Forming lipid bilayers from contacting lipid monolayers, however, is not purely novel approach; the principles of self-assembly have been used to form lipid bilayers for nearly 50 years. Takagi et al.[5] and later Montal and Mueller[6] demonstrated a lipid folding technique in which lipid monolayers, assembled at the air/water interfaces of two aqueous volumes, are folded across an aperture in a vertical Teflon partition that separates both chambers. Funakoshi, et al. more-recently laid the foundation for the DIB technique through the formation of a lipid bilayer at the interface of water droplets in oil.[7] These results provided initial validation that crossing streams of aqueous solution and organic solvent containing phospholipids can be used to form a lipid bilayer within a microfluidic lab-on-chip platform. Malmstadt similarly used the swelling properties of polydimethyl siloxane (PDMS) to extract organic solvent separating lipid-encased aqueous volumes within a microchannel to promote lipid bilayer thinning.[8] In addition, several variations of bilayer formation techniques utilizing lipid-encased aqueous droplets have emerged since 2007.[9,10]

The observed[1] and now quantified[11] durability of droplet-interface bilayers is a key advantage of the DIB method. The droplet interface bilayer eliminates the use of a supporting

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substrate in favor of an oil/water interface to host self-assembled phospholipids and, as a result, promotes the formation of a continuous lipid monolayer that completely encapsulates each aqueous volume. The uninterrupted nature of the assembled monolayers is a key delineation from traditional suspended/supported lipid bilayer techniques that feature a "patch" of phospholipid solution spread onto discrete regions of a substrate.[6,12] Consequently, the durability of the assembled lipid bilayers in these techniques is limited, whereas the continuity of lipids that self-assemble at an oil/water interface allows droplet interface bilayers to last for weeks while supporting active biomolecules.

However, further refinement and integration of DIB-based platforms is impeded by four challenges: First, positioning and interrogating droplet interface bilayers requires careful insertion of custom electrodes into droplets, a process that requires skill and the use of a micromanipulator and which can damage the adsorbed lipid monolayer.[13] Second, the contents of each droplet are established prior to bilayer formation, preventing the introduction of species into droplets after network assembly.[3] Third, the size of the interface between two droplets is determined by both the size of the droplets, their relative positions, and the ability of the organic solvent molecule to reside in the hydrocarbon region of the bilayer;[14] therefore, precise control of droplet volume and positioning within the network is required to produce consistent interface sizes. Last, the dispensing and droplet positioning techniques developed by Holden, et al.[1] can be used to easily form and manipulate droplets that are 0.1−100 μL in volume (100−1000 μm in diameter), but constructing small-droplet networks that feature bilayer interfaces less than 1 μm in diameter remains a challenge. Specifically, the need for reducing the size of the droplets in the network to achieve higher volumetric packing, smaller interfacial areas, and additional structural stability poses challenges to accurately dispense sub-nanoliter aqueous volumes, as well as controllably manipulate individual droplets.

In this paper, we present an alternative method for bilayer formation in which the attachment of lipid monolayers is regulated by controlling the dimensions of an aperture formed in a flexible supporting substrate. In this method, which we denote as the regulated attachment method (RAM) because we can regulate both the bilayer formation and the bilayer size, a mechanical force applied to the substrate is used to close an aperture to separate a single aqueous volume submersed in oil into multiple volumes that become encased with continuous lipid monolayers. The mechanical force on the substrate is then reduced to open the aperture and allow the opposing lipid monolayers to come into contact. Regulating the dimensions of the aperture, then, provides direct control over the size of the lipid bilayer which forms spontaneously after initial contact. In this paper, we use the regulated attachment method to form lipid bilayers in a prototype flexible substrate made from PDMS. We also show that the areal size of the bilayer can be precisely prescribed and even modulated after initial formation by regulating the amount of contact between opposing monolayers via the applied force. The results of this study highlight the flexibility of this technique for constructing highly precise biomolecular assemblies and are presented in a format that builds a case for integrating RAM into microfluidic devices for constructing new platforms for molecular assembly and analysis.

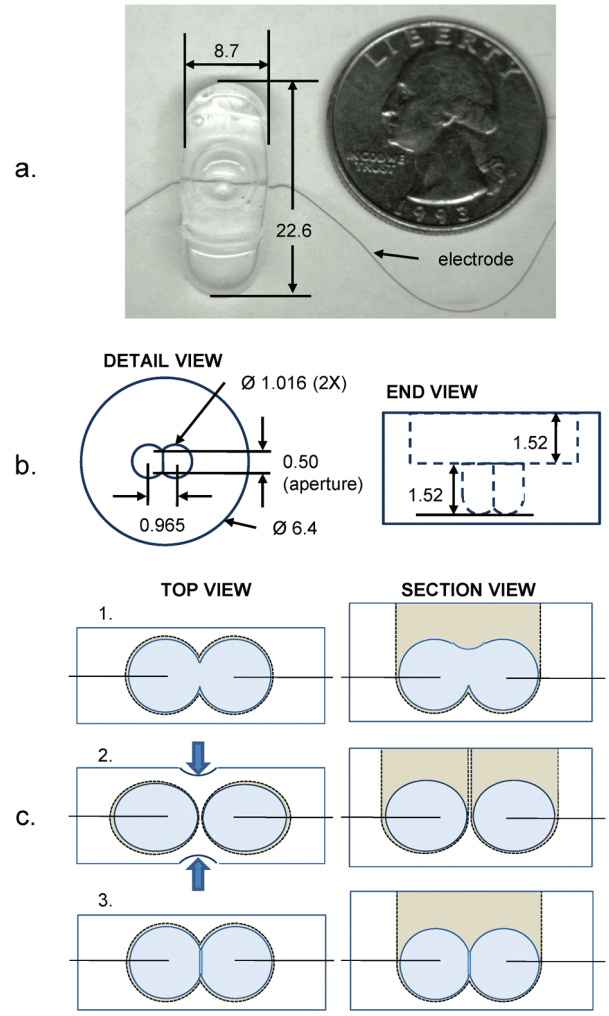

Figure 1. A flexible substrate is used to produce lipid bilayers by the regulated attachment method, which divides a single aqueous volume into multiple volumes and then reattaches them after lipid monolayer adsorption. A prototype substrate made from PDMS provides a soft, flexible, and translucent support for separating and then reattaching lipid-encased aqueous volumes (a, b). 125 μm silver−silver chloride electrodes are inserted through the PDMS substrate such that the tip of each electrode resides within a compartment (a, c).

EXPERIMENTAL METHODS

Prototype Flexible Substrate. The initial prototype shown in Figure 1a features two connected compartments for holding aqueous volumes, where the design of the substrate was inspired by the overlapping droplet compartments used by Funakoshi.[7] The compartments (1.02 mm in diameter and 1.52 mm deep, with hemispherical bottom surfaces) are connected by a 500 μm-wide by 1 mm-tall aperture that spans the window formed between the circular compartments (Figure 1b). The features that define the droplet compartments and aperture dimensions of the substrate are first machined into an acrylic (PMMA) substrate using a vertical end-mill. Uncured Sylgard 184 (Dow-Corning) PDMS (10 : 1 wt-wt ratio of base to curing agent) is poured into the acrylic substrate and cross-linked at 80 − 90 °C for 1 h in a benchtop oven in order to create the geometrical negative of the original

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acrylic substrate. The cured PDMS negative is coated with a silicone lubricant (3M) and then placed into a shallow tray filled with uncured PDMS. After curing in the oven at the same conditions, the PDMS negative is peeled from the flexible PDMS substrate, which is then removed from the acrylic tray. Silver−silver chloride (Ag/AgCl) electrodes made from 125 μm-thick silver wire (Goodfellow) are then pierced through the sides of the PDMS substrate such that the tip of each electrode is positioned approximately in the center of a compartment (Figure 1a, c).

Materials. Aqueous vesicles, consisting of 2 mg/mL 1, 2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) phospholipid vesicles (purchased as lyophilized powder, Avanti Polar Lipids, Inc.) suspended in either 10 mM MOPS (Sigma), 100 mM NaCl (Sigma), pH7 or 10 mM MOPS, 100 mM KCl (Sigma), pH 7 buffer solution, are prepared and stored as described elsewhere.[2] Hexadecane (99%, Sigma) is used without further purification as the oil phase in this study. α-Hemolysin (αHL) channels (Sigma) from *Staphylococcus areus* are stored at 3−8 °C in 10 mM MOPS, 100 mM KCl, pH7 solution and diluted to final concentrations of 1 μg/mL and 100 ng/mL in DPhPC-KCl vesicle solution for gross electrical measurements and single-channel recordings, respectively. Alamethicin channels (A.G. Scientific) from *Trichoderma viride* are stored in ethanol (Sigma) at 0.1% (w/v), and this stock solution is diluted further to a concentration of 100 μg/mL alamethicin in 10 mM MOPS, 100 mM NaCl, pH 7 buffer solution. A 50:2 volume ratio of DPhPC/NaCl vesicle solution to aqueous alamethicin solution provides a final alamethicin concentration of 3.85 μg/mL for tests in which the initial single aqueous volume contains proteins. In tests where alamethicin channels are added through a syringe to a preformed lipid bilayer network, the syringe contains the 100 μg/mL alamethicin solution in buffer with no phospholipids.

Bilayer Formation. Hexadecane (>10 μL) is first pipetted into the droplet compartments to provide the necessary oil/water interface surrounding the aqueous volumes. Then, 1.2 μL of aqueous lipid vesicle solution is pipetted into the droplet compartments. The aqueous volume spans both droplet compartments as shown in Figure 1c.1, and the silver−silver chloride electrodes insert easily into the aqueous phase without the need for a hydrophilic surface treatment, such as the one used by Holden et al.[1,2]

An electrode holder (M3301EH, World Precision Instruments, Inc.) mounted to a 3-axis, motorized micromanipulator (SM325, WPI, Inc.) is used to compress the soft substrate to fully close the aperture between the neighboring compartments. Upon compression the closing aperture divides the single aqueous volume into two separate volumes submersed in hexadecane (Figure 1c.2). Complete separation is maintained for 10−15 min to allow for monolayer adsorption to occur at the oil/water interface surrounding each aqueous volume (phospholipids adsorb from the aqueous phase in this work,[2] though lipids can also adsorb from the oil phase[1]). Lastly, the compression force on the substrate is slowly reduced by moving the electrode holder in the opposite direction to open the aperture. A bilayer forms spontaneously after the aqueous volumes come into contact and excess oil is removed, allowing for the hydrocarbon tails of the lipids to zip together (Figure 1c.3).

Electrical Measurements. Bilayer formation is confirmed with electrical impedance spectroscopy (EIS) performed via the Ag/AgCl electrodes using an Autolab PGSTAT12 with FRA module (Eco Chemie) across the frequency range of 500 kHz to 10 mHz using a 5 mV (rms) sinusoidal voltage. Unfiltered single-channel recordings of α-HL channels in bilayers formed using RAM are measured at a sampling rate of 10 Hz using Autolab. Cyclic voltammetry (CV) measurements of bilayers containing alamethicin or α-hemolysin channels are performed using the Autolab with an applied scan rate of 2.5 mV/s and a fixed step size of 0.15 mV. A Canon PowerShot G6 digital camera connected to an AxioVert 40CFL inverted microscope is used to obtain ditigal images of bilayers formed using RAM in the PDMS substrate. All recordings are performed within a homemade Faraday cage for electrical shielding.

RESULTS AND DISCUSSION

Lipid bilayers are formed using the regulated attachment method within the flexible, PDMS prototype substrate. A motorized manipulator is used to produce incremental compression of the substrate for opening and closing the aperture in this study, though other methods could be used to separate and rejoin the adjacent aqueous volumes including magnetically-controlled valves,[15] electrorheological fluids,[16] and directed electric fields.[17,18] The prototype substrate fabricated for and used in this initial is made of a commercial silicone elastomer that imbibes the oil phase and that may leach out impurities. However, our initial results do not indicate that either the absorption or possible leaching affects the formation or the resulting quality of the bilayers that are formed using RAM. We acknowledge that many different flexible, hydrophobic materials, such as a new class of photocurable and solvent-resistant perfluoropolyethers (PFPEs) developed by DeSimone's group at UNC,[19] could be used instead of PDMS.

Electrical impedance spectroscopy measurements obtained using the inserted electrodes confirm the changes in molecular ordering that occur during each step (Figure 2a). While our own experiences in forming DIBs reveal that monolayer-covered droplets can be difficult to pierce, the insertion of untreated electrodes into the single aqueous volume in the regulated attachment method occurs immediately and simultaneously before the monolayer has time to assemble. The EIS data presented in Figure 2b shows the initial magnitude of the electrical impedance measured for a single aqueous volume is on the order of 0.1−1 MΩ because of the combined electrical conductivity of the aqueous solution and the interactions of this solution with the electrodes (blue circles). Once the aperture is closed by the application of the mechanical force, the magnitude of the electrical impedance increases to a value that indicates that there is no conducting path between the two aqueous volumes (red squares). This response verifies that the single aqueous volume has been completely divided into two separate volumes and ensures that

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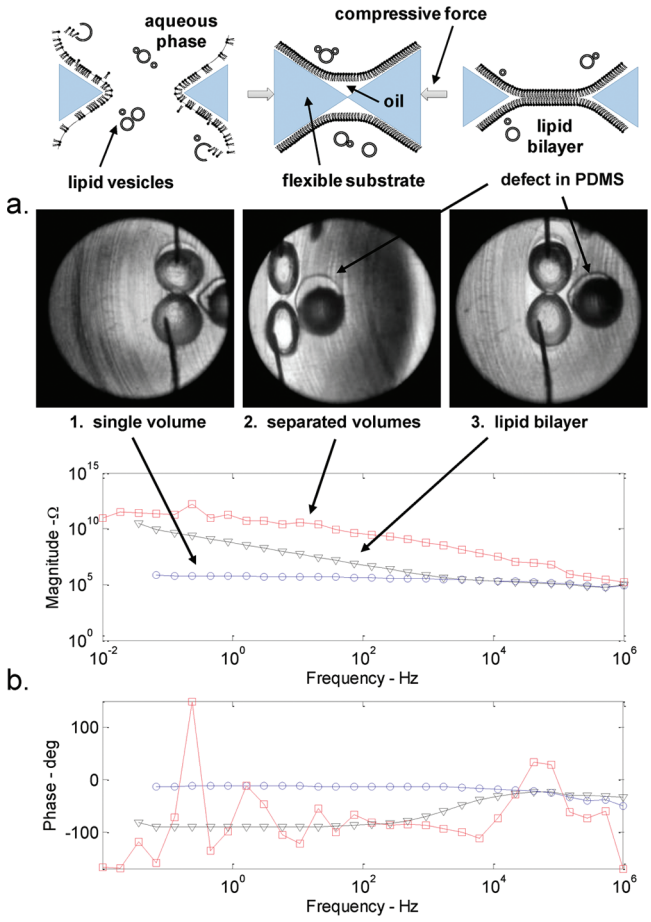

Figure 2. Lipid monolayer adsorption begins immediately when the aqueous volume containing lipid vesicles is injected into the surrounding hexadecane and continues after the aperture is closed (a). Electrical impedance measurements taken at each stage confirm the separation and reattachment of the lipid-encased volumes to form a lipid bilayer (b). Videos showing the. separation and rejoining of the aqueous volumes also show global compression of the PDMS as seen in the lateral movement of the compartments.

lipid monolayer adsorption can occur along the entire oil/water interface surrounding both volumes. In the third stage, a lipid bilayer (black triangles) forms after the compression force is relaxed to promote contact between the adjacent lipid-encased volumes. The EIS measurement of a lipid bilayer demonstrates a DC resistance of more than 10 GΩ because of the hydrocarbon core of the bilayer, which is consistent with previous impedance measurements on DIBs.[13] Estimates of the electrical resistance and capacitance of the lipid bilayer are obtained by fitting the EIS data with an equivalent electrical circuit model[13] to extract information about the quality and size of the bilayer lipid membrane.

The measured electrical properties of bilayers formed using RAM showcase the reproducibility of this method for forming high-quality lipid membranes. Values of electrical resistance for more than 10 different bilayers formed within the same substrate using RAM range from 10−500 GΩ, easily providing the gigaohm seal necessary for measuring single-channel recordings.[20] The area and equivalent diameter of each bilayer are computed using the measured value of capacitance and a normalized capacitance value of 0.6 μF/cm^2 for DPhPC bilayers.[1,13,21] Maximum equivalent diameters for lipid bilayers formed in the uncompressed prototype substrate are estimated to be 350 ± 27 μm (n = 3). Bilayers formed using RAM consistently for lasted more than three to four hours during the course of testing, even under applied potentials as high as 100−120 mV.

The regulated attachment method offers a new approach for prescribing the areal size of a lipid bilayer. For comparison, the size of a traditional supported/suspended lipid bilayer is bound, but not controlled, by the dimensions of the supporting substrate and the volume of the annulus of excess lipid solution that surrounds the thinned region of the bilayer. The dimensions of this annulus are subsequently determined by the surface tension of the lipid monolayer, a function of both the physical properties of the phospholipid molecules and the molecular size of the organic solvent,[14] and the contact angle between the surface of the support and the lipid solution. Moreover, the sizes of unsupported lipid bilayers (DIB, DHB,[9,22,23] etc.) are constrained by the sizes of the droplets and the relative spacing between adjacent lipid-encased aqueous media, which is maintained by precise electrode positioning. The regulated attachment method, however, enables precise control over bilayer size by controlling the amount of mechanical force that is applied to the aperture. This control mechanism for the bilayer size is completely independent of the size or shape of the aqueous volumes, or the type of organic solvent used at the oil−water interface. While the molecular interactions of the organic solvent with the hydrocarbon tails still exist, size control is obtained by merely regulating the extent to which opposing lipid monolayers on neighboring volumes can zip together. A key distinction of this approach from previous methods is that control of the substrate is used to regulate the size of the bilayer even though the lipid monolayers are supported by the oil/water interface (shown in the top diagram of Figure 2). The regulated attachment method is further aided by the hydrophobic nature of the PDMS, which ensures a small amount of the hexadecane remains between the substrate and the lipid monolayer. This separation is crucial for allowing the size of the bilayer to be changed without causing disruption to the monolayers that could result in bilayer failure.

The results of the initial test presented in Figure 3 demonstrate control of bilayer size on a single lipid bilayer, showing that the membrane area (as measured by the bilayer capacitance) can be modulated reversibly by varying the level of compression applied to the substrate. The images in Figure 3a correspond to values of bilayer capacitance plotted in Figure 3b for positions 5−8 (moving from left to right) and show that increases in compression result in both large-scale compression of the substrate indicated by the left-ward shift in the compartment positions and partial aperture closing. The capacitance of the bilayer varied from approximately 125 pF at position 8 to more than 600 pF with the aperture fully open (recorded at position 9), a 4.8-fold change in bilayer area. This same experiment is repeated and the position

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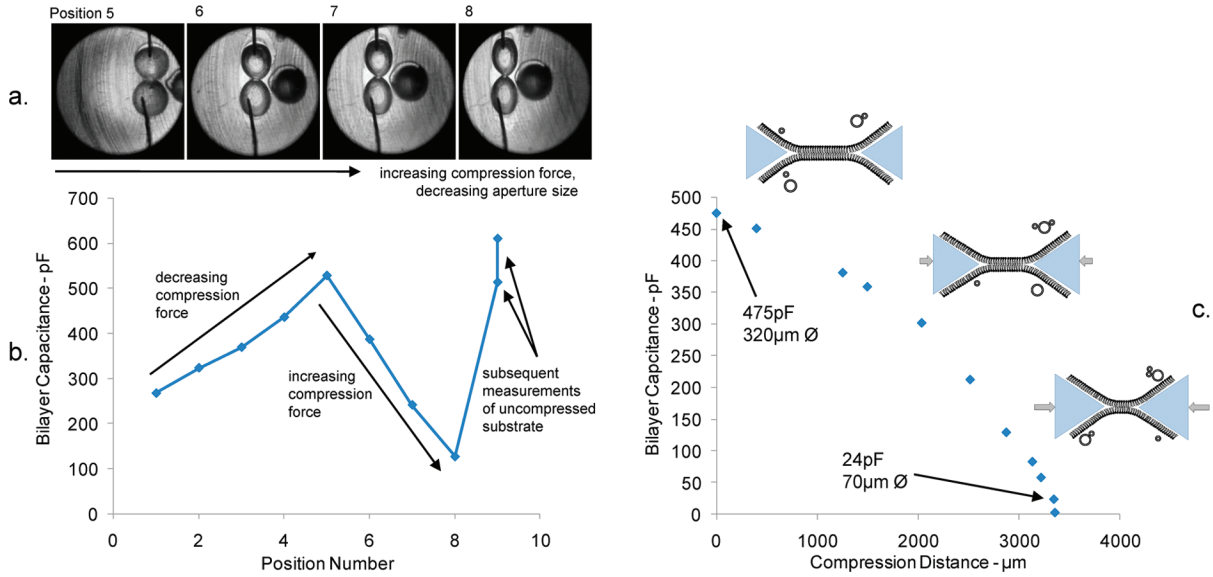

Figure 3. Modulation of the applied compressive force is used to reversibly change the bilayer size after formation. Images taken show the visible changes that occur to the aqueous volumes as the size of the aperture is consecutively reduced (a). The capacitance of the bilayer obtained through EIS measurements at each aperture setting confirms that the area of connection between the two aqueous volumes changes as a result of a mechanical force applied to the supporting substrate (b). The last two data points taken at position 9 reveal that the rate at which a growing bilayer thins is on the order of minutes. Measured bilayer capacitance versus recorded compression distance (measured as the *x*-coordinate of the electrode holder position) showcases the full range of sizes of bilayers that can be formed in the prototype flexible substrate (c).

coordinates for the electrode holder are tracked to correlate the applied deformation of the substrate to the change in the size of the membrane (Figure 3). EIS measurements at each position are again used to estimate bilayer area through measured capacitance.

The data presented in Figure 3c shows the full range of bilayer sizes that can be formed using the regulated attachment method in the prototype flexible substrate. The first measurement taken at a compression distance of 0 μm (i.e., no applied force on the fixture) results in a bilayer with a capacitance of 475 pF. The equivalent diameter for the membrane at this position is approximately 320 μm. At a compression distance of approximately 3350 μm, the smallest sustainable bilayer had a capacitance of 24 pF, which translates to a diameter of 70 μm, a nearly 5-fold decrease in diameter and a more than 30-fold decrease in bilayer area from the maximum state. The last data point taken yielded a capacitance of 2 pF (equivalent to a diameter of only 23 μm), though the volumes separated during the EIS measurement. Complete separation of the bilayer does not result in rupturing of the interface; instead, the lipid monolayers merely unzip. At this point, opening the aperture leads to new contact between the adjacent aqueous volumes that reestablishes a lipid bilayer interface. We believe that this ability to connect, disconnect, and reconnect the lipid monolayers creates a platform where the contents within each aqueous volume can be prescribed and safely maintained without undesired contamination of the opposite volume.

Lipid bilayers formed using the regulated attachment method provide a suitable and tunable environment for studying transmembrane proteins since both the size and composition of the bilayer can be prescribed. Furthermore, proteins can be introduced into RAM bilayer systems in several ways, both before and after bilayer formation. In the first scheme, prior to bilayer formation, separate volumes consisting of different compositions can be placed into adjacent compartments with the dividing aperture closed. Second, with the aperture open, the single volume that spans both compartments can contain proteins to prescribe protein insertion from both sides of the bilayer. The following results illustrate these separate introduction tactics and highlight the ability to form high-quality lipid bilayers and tailor their properties using proteins.

One-sided protein insertion is first demonstrated by pipetting different vesicle solutions into each compartment divided by the closed aperture. Similar to Hwang's demonstration of asymmetric bilayer formation using DIBs,[2] one volume consists of α-hemolysin channels in DPhPC-KCl vesicle solution, while the second volume contains only DPhPC-KCl vesicle solution. The bilayer forms once the aperture is opened, following monolayer formation, and α-HL channels insert into the membrane spontaneously via two mechanisms: through monomer aggregration and insertion directly from the aqueous solution[1,20] or through vesicle fusion events that incorporate channels into the bilayer that had previously inserted into suspended DPhPC vesicles.[24,25] We observe that each of these event types creates measurable transient currents, and the fusion of vesicles containing reconstituted α-HL pores with the bilayer results in multiple α-HL-conductance level increases in current. At low (≤100 ng/mL) final concentrations of α-HL, single insertion and desorption events are easily seen in current traces measured after bilayer formation. Step-wise current variations of 3−5 pA correspond to conductance levels of 80−100 pS (shown in Figure 4a), agreeing well with published conductances for α-hemolysin channels measured in bilayers formed in 100 mM KCl electrolytes.[26,27] The ability capture these single molecule activities further

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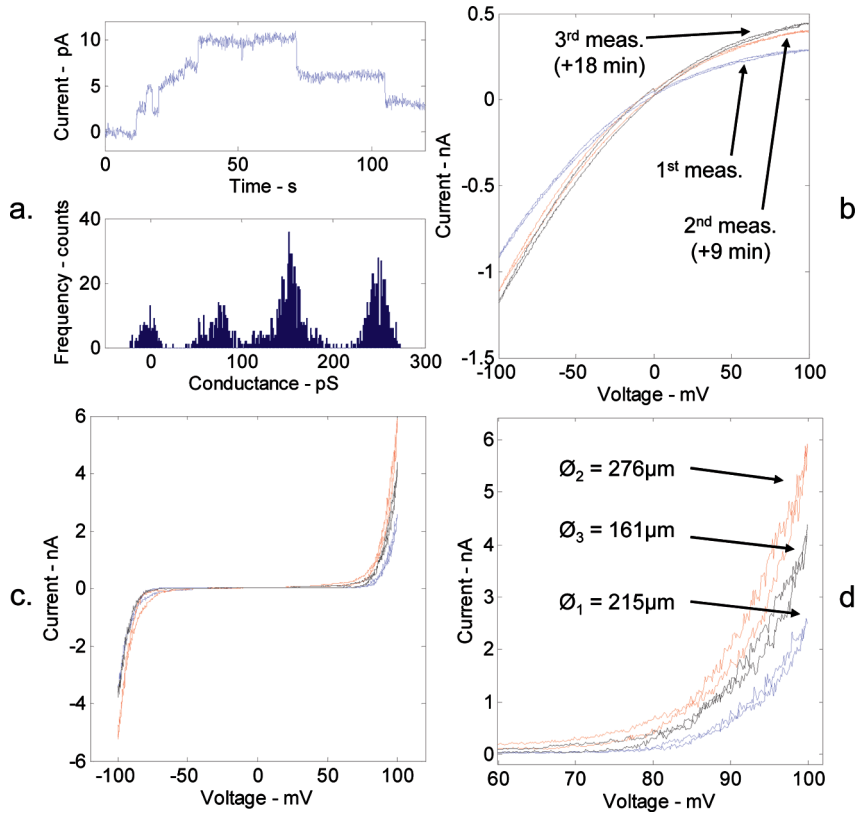

Figure 4. Single-channel recordings of α-HL channels are measured with an applied potential of +40 mV (a) and gross current−voltage relationships of a bilayer containing a larger number of α-HL channels are measured with CV (b). Alamethicin channels incorporated into the initial single aqueous phase that is separated and reattached to form a bilayer results in symmetric voltage-dependent gating at potentials greater than approximately |70 mV| (c) and which is a function of prescribed bilayer size (d).

confirms that the regulated attachment method produces high quality lipid bilayers. At higher concentrations (≥1 μg/mL α-HL), a larger number of channels insert into the bilayer, causing a gradual reduction in the resistance of the membrane (corresponding to the increasing slope of the current−voltage traces shown in Figure 4b) and resulting in partial rectification of ion currents at positive potentials. This gross electrical behavior of a membrane containing α-hemolysin channels agrees well with several published works, where similar concentrations of α-hemolysin were used.[28−30]

Alamethicin channels are used to demonstrate two-sided insertion into bilayers formed using RAM because they demonstrate a well-known voltage-dependent conduction based on direction of insertion. In these tests, a single volume of alamethicin/DPhPC-NaCl solution is pipetted into the flexible substrate. The aperture is subsequently closed and then reopened after monolayer adsorption to form a bilayer. Alamethicin channels spontaneously insert into the resulting bilayer from both sides, following membrane formation. Single-channel measurements of alamethicin channels are not performed because of the rapid gating behavior of these molecules and sampling rate restrictions of the Autolab equipment. Instead, cyclic voltammetry measurements of a bilayer containing alamethicin exhibit the voltage-dependent conductance of a large number of aggregated alamethicin channels residing in a bilayer (Figure 4c), where the increase in the magnitude of the measured current at potentials above |70 mV| indicates that these channels form conductive pores through the bilayer.[31−33] The magnitude of the measured current upon channel opening agrees well with current−voltage traces obtained by Vodyanoy et al. on bilayers containing alamethicin channels from a similar solution concentration of 2 μg/mL.[34] The symmetry of the current−voltage curves is attributed to the membrane containing alamethicin channels that have inserted into the bilayer from both sides. Figure 4d shows that the magnitude of the current flowing through the membrane when the channels are "open" (>|70 mV|) is a function of bilayer size and time. As the force applied to the substrate is decreased, the size of the membrane increases from an initial diameter of 215 to 276 μm and the measured current above the gating potential of 70 mV increases as more proteins insert into the larger interface. This change is reversible, as seen in the reduced magnitude of measured current when the bilayer size is reduced to 161 μm for the third measurement. Some hysteresis between the first and third measurement is observed since alamethicin proteins continue to insert into the membrane area after initial bilayer

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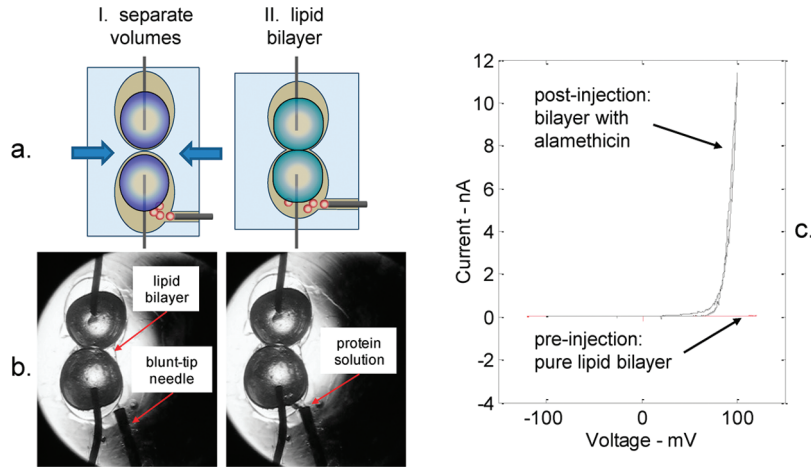

Figure 5. Alamethicin channels dissolved in an aqueous buffer are incorporated into a preformed lipid bilayer formed using RAM, by injection through a 10 μL NanoFil syringe (WPI, Inc.) with a 34-gage blunt-tip NanoFil needle (WPI, Inc.) that was pierced through the soft PDMS substrate. The injection can be performed both before bilayer formation with the aperture closed (a.I.) or as in this case, after bilayer formation (a.II, b.). The insertion of a large number of proteins occurs within 20 min after the injection. A video of the. injection process shows the coalescence of alamethicin solution with the preexisting lipid-encased volume.

formation. We believe further that repeated expansion and shrinking of the interface may also serve to consolidate more proteins into a smaller interface, thus achieving higher protein concentrations within the bilayer.

A distinct advantage of partitioning the aqueous volumes with a controllable aperture is that it allows specific species to be introduced into specific regions of the network even after bilayer formation. This added feature provides the unique capability for merging RAM with microfluidics for introducing species into the connected volumes via microchannels or syringes. Unlike the original droplet interface bilayer where the compositions of the droplets remain fixed, the introduction of species into a preformed bilayer can be studied and used for a variety of high-throughput screening applications.[10,12,35,36] Furthermore, the addition of species can occur at two different times: before the attachment of adjacent aqueous volumes or after bilayer formation (Figure 5a).

A blunt-tip needle (34-gage NanoFil needle, WPI, Inc.) and syringe pump (10 μL NanoFil syringe, WPI, Inc.) are used to incorporate alamethicin proteins into an aqueous volume on one side of a preexisting lipid bilayer. Prior to the test, a small channel routed into the lower droplet compartment is formed by piercing the needle through the PDMS substrate. The needle is then retracted slightly to prevent unwanted contact with the aqueous volume that could result in uncontrolled diffusion of species from within the syringe into the droplet. The first photograph in Figure 5b was taken after bilayer formation with the needle tip located to the lower right of the lower aqueous volume. The syringe contained a 100 μg/mL solution of alamethicin channels dissolved in 10 mM MOPS, 100 NaCl, pH 7 buffer. Lipids were not incorporated into this phase to promote the coalescence and incorporation of contents of the injected volumes into the preexisting lipid-encased volume. During injection, the protein solution flows through the small channel at the end of the syringe and is directed toward the lower droplet shown in the second photograph. The small volumes (<10 nl each) of protein solution coalesce with the large lipid-encased droplet 1−2 s after contact and release of their contents into the larger volume. In total, we estimate that approximately 50−100 nL of protein solution are incorporated into the lower 500 nL aqueous volume consisting of lipid vesicle solution. Electrical measurements confirmed the incorporation of channels into just one droplet when, approximately 10−15 min after injection, the current−voltage behavior of the membrane demonstrated an asymmetric voltage-dependent response. Unlike earlier tests where alamethicin proteins were incorporated into the initial single aqueous volume and resulted in the insertion of channels in both directions into the membrane (see Figure 4c,d), this method successfully incorporates species into just one aqueous volume, thus leading to single-sided insertion evident by the voltage-dependent behavior occurring only at potentials greater than +50 mV. Moreover, we found that we were able to successfully incorporate 100−200 nL of alamethicin solution from the syringe into preformed networks in three out of three separate trials. The importance of this result is that for the first time external species and solutions can be introduced into preformed lipid bilayers to form modified lipid bilayers that can easily be compared with preinjection membrane properties. Simply, this ability allows membranes to be constructed one component at a time.

In this experiment, we added voltage-dependent ion channels to an existing bilayer in a controllable fashion, enabling the study of the roles of specific molecules on the entire assembly. As an example, the electrical impedance measurements, in addition to the differences observed in the current−voltage relationship of the bilayer before and after alamethicin is added, show that the capacitance of the interface decreases by more than 100 pF (three separate trials) upon channel insertion. We attribute this difference to changes in composition, rather than size, of the membrane and estimate, using the postinjection values of capacitance and normalized capacitance for DPhPC bilayers, that approximately 20−50% of the area of the bilayer consists of alamethicin channels. We acknowledge the same technique could be used to transport species into droplet interface bilayers as formed by Holden et al.,

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although the designed partitioning of aqueous volumes that occurs in our prototype substrate aids in directing the injected solution toward the desired aqueous volume and prevents unwanted contamination of the other volumes. Further, because the sizes of bilayers formed using RAM are not determined by the size or shape of each aqueous volume, the addition of species contained in a carrier solvent allows for the composition of the bilayer to be tailored independently from the size of the interface.

CONCLUSION

In this paper, we have demonstrated that a flexible substrate can be used to control the attachment of opposing lipid monolayers which provides a novel and effective method for prescribing bilayer size. Our study used the regulated application of a mechanical force to controllably open and close an aperture that separates two aqueous volumes, permitting large changes in the size of the bilayer (from more than 360 μm in diameter to less than 70 μm in diameter, a change of >5× in equivalent diameter and >30× in area), while retaining bilayers with high-quality electrical seals ($R > 10$ MΩ cm ^{2}) necessary for monitoring single and few protein activities. This approach stands apart from previous bilayer formation methods in that both an immiscible liquid interface and a solid supporting substrate are used in tandem to support molecular assembly and regulate bilayer thinning. Our results provide credible evidence that the regulated attachment method offers the unique capability to control the composition of lipid bilayers independently from the size of the interface, and in this light, we demonstrated controlled incorporation of alamethicin channels into preformed lipid bilayers. Moreover, the ability to partition aqueous volumes, preventing unwanted contamination of species, allows for asymmetric lipid bilayers to be formed much like with droplet interface bilayers.[2]

The platform presented herein establishes a novel methodology for constructing a diverse set of self-assembled material systems, one where phospholipids may be replaced by polymeric analogues,[20] the aqueous solutions could contain whole cells,[37] and where the interfaces can be designed to provide a collective network response.[4] We believe that the natural evolution of this technology is direct integration into a microfluidics platform for building higher-order networks and demonstrating the reliable assembly of very small lipid bilayers (<1 μm in equivalent diameter). With very small apertures and the low capacitance of a polymeric substrate, we further believe that nanosized lipid bilayers formed in this manner will offer extremely low electrical noise levels for precise single channel measurements. We anticipate that these platforms could consist of multiple intersecting fluidic channels arranged in nearly any desired configuration, where each intersection would have a controllable aperture for inducing local solution separation and bilayer formation. Furthermore, each region of the fluidic network would have its own electrode for interrogation, as well as dedicated side channels for performing controlled species addition and solution exchange. Such an idea takes advantage of years of research dedicated to constructing highly precise microfluidic networks and we are of the opinion that the integration of RAM into microfluidics will offer a highly modular and customizable platform for constructing biomolecular networks.

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