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Fractional Polymerization of a Suspended Planar Bilayer Creates a Fluid, Highly Stable Membrane for Ion Channel Recordings

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Abstract: Suspended planar lipid membranes (or black lipid membranes (BLMs)) are widely used for studying reconstituted ion channels, although they lack the chemical and mechanical stability needed for incorporation into high-throughput biosensors and biochips. Lipid polymerization enhances BLM stability but is incompatible with ion channel function when membrane fluidity is required. Here, we demonstrate the preparation of a highly stable BLM that retains significant fluidity by using a mixture of polymerizable and nonpolymerizable phospholipids. Alamethicin, a voltage-gated peptide channel for which membrane fluidity is required for activity, was reconstituted into mixed BLMs prepared using bis-dienoyl phosphatidylcholine (bis-DenPC) and diphytanoyl phosphatidylcholine (DPhPC). Polymerization yielded BLMs that retain the fluidity required for alamethicin activity yet are stable for several days as compared to a few hours prior to polymerization. Thus, these polymerized, binary composition BLMs feature both fluidity and long-term stability.

Introduction

Artificial lipid membranes have been widely used to reconstitute and study isolated membrane proteins.[1−5] Suspended planar lipid bilayers, also known as black lipid membranes (BLMs), are frequently utilized to characterize ion channel (IC) proteins as they allow electrochemical access to both sides of the ion channel−membrane complex.[6−9] In a fluid BLM, however, the lipids are associated solely via relatively weak intermolecular interactions, and therefore, rupture usually occurs within a few hours after membrane formation.[5,10,11] The inherent instability is a significant limitation to BLM use for long-term monitoring of IC activity and in biosensing devices and arrays.[12−14]

A number of approaches have been used to stabilize BLMs including miniaturized apertures[15,16] and hydrogel supports.[17−21] BLMs formed on nanoporous alumina substrates have lifetimes upward of 1 day.[15] BLMs suspended across a glass nanopore can sustain activity of incorporated α-hemolysin (αHL) channels for up to 1 month,[16,22] although 1 day is more typical. The enhanced longevity in these cases is attributable to the decrease in membrane surface area susceptible to disruptions. Alternatively, encapsulation in a hydrogel matrix increases membrane stability. For example, sandwiching a BLM between two agarose layers increased its lifetime to ca. 2 days while also supporting the activity of reconstituted alamethicin and human glycine receptor.[17] Kang et al.[21] utilized agarose gel layers to encapsulate a bilayer containing a single αHL channel, extending its lifetime to 3 weeks. In another approach, BLMs were encapsulated in a poly(ethylene glycol) dimethacrylate hydrogel that was covalently anchored to the glass aperture resulting in membrane lifetimes up to 12 days.[19,20] However, encapsulation has some

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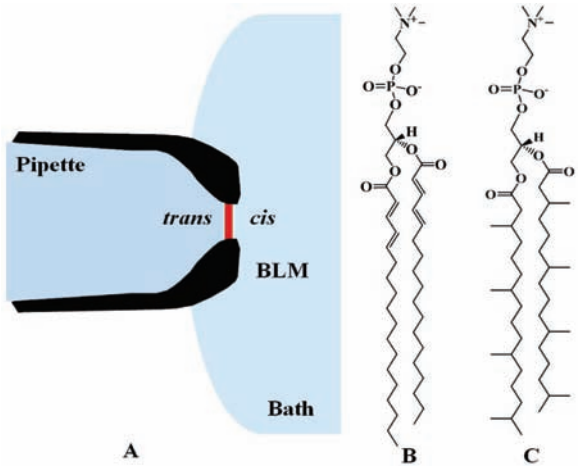

Figure 1. (A) Experimental configuration of recording pipettes. The recording buffer on the *cis* and *trans* sides of the BLM (red) was 5 mM HEPES, pH 7.5, containing 0.1 M KCl. Alamethicin was added to the *cis* compartment. Structures of (B) bis-DenPC and (C) DPhPC.

significant disadvantages: (i) the hydrogel matrix may restrict fluid flow adjacent to the BLM, slowing and/or preventing insertion of ion channels and introduction/removal of channel effectors, and (ii) after rupture, the BLM cannot be reformed across the aperture.

Lipid polymerization has also been explored as a strategy to enhance BLM stability.[23−25] Shenoy et al.[23] formed BLMs using a monodiacetylenic lipid, inserted αHL channels, and partially polymerized the membrane via UV irradiation. Sensing of single poly(nucleotides) driven electrophoretically through single αHL channels was demonstrated. In a subsequent study, polymerization of BLMs composed of a mixture of monodiacetylenic and nonpolymerizable lipids resulted in a slightly longer lifetime (increasing from 13 min before to 31 min after polymerization), and these membranes supported the activity of incorporated gramicidin.[24] A more recent paper described BLMs formed from bis-dienoyl phosphatidylcholine (bis-DenPC, Figure 1).[25] Cross-linking polymerization increased the lifetime of bis-DenPC BLMs from a few hours to 3 weeks, and the activity of αHL was maintained for 1 week, suggesting that this approach has considerable potential for preparation of robust IC-based sensors and arrays. However, in a cross-linked lipid bilayer, lateral lipid diffusion is significantly attenuated relative to an unpolymerized bilayer.[26] The lack of fluidity does not appear to adversely affect the activity of αHL,[25] but for other types of ICs such as gramicidin and alamethicin, a fluid membrane is required for function[27−31] (also see data below).

A possible strategy to prepare a highly stable yet fluid BLM is to use a mixture of polymerizable and nonpolymerizable lipids. Polymerization should promote phase segregation, creating a membrane composed of domains of nonpolymerizable lipid dispersed in a poly(lipid) network.[32−34] This strategy has been explored in several studies involving bacteriorhodopsin and bovine rhodopsin reconstituted into liposomes prepared from mixtures of polymerizable and nonpolymerizable lipids.[32,35−37] UV photopolymerization before reconstitution of proteins into the liposomes resulted in retention of rhodopsin activity, presumably because the proteins inserted into the unpolymerized, fluid domains. A more recent example is the work on BLMs composed of diacetylenic and nonpolymerizable lipids described above.[24] Gramicidin activity was observed after the bilayer was photopolymerized, indicating that the membrane retained some degree of fluidity, although as noted the gain in stability afforded by lipid polymerization was minimal.

In this paper, we describe mixed BLMs prepared using bis-DenPC and diphytanoyl phosphatidylcholine (DPhPC, Figure 1), a nonpolymerizable, fluid lipid that is frequently used for IC recordings. We show that upon photopolymerization, these mixed BLMs exhibit greatly enhanced stability relative to unpolymerized BLMs, and they maintain the IC activity of alamethicin for which membrane fluidity is required.

Experimental Section

Reagents. Ion channel recording buffer (0.1 M KCl, 5 mM HEPES, pH 7.5) was prepared with components obtained from Sigma-Aldrich dissolved in deionized (DI) water (18 MΩ·cm) and filtered through a 0.2 μm filter. 3-Cyanopropyldimethylchlorosilane and *n*-decane were purchased from Alfa Aesar and used as received. Acetonitrile and ethanol were purchased from EMD. Alamethicin was purchased from Alexis Biochemicals, diluted to 10 μg/mL in ethanol, and used as stock solution. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased in chloroform from Avanti Polar Lipids. Bis-dienoylphosphatidylcholine$_{18,18}$ (bis-DenPC) was synthesized according to Dorn et al.[38] and was always handled under yellow light to avoid inadvertent photopolymerization.

Fabrication and Modification of Pipettes. Borosilicate capillary tubing with a 1.5 mm outer diameter and 1.0 mm inner diameter was purchased from Sutter Instrument Co. or World Precision Instruments. Pipettes were pulled to a sharp tip using a Sutter P-97 micropipet puller and then broken to an open diameter of ca. 200 μm and fire polished using a microforge (Narishige MF-900) to obtain an aperture ranging from 5 to 20 μm in diameter. Pipettes having an aperture diameter of ca. 10 μm were selected by visual inspection for use in the experiments described below. Prior to BLM formation, pipettes were modified with 3-cyanopropyldimethylchlorosilane according to published procedures,[16,25] rinsed with acetonitrile, ethanol, and water to remove excess silane, and then air-dried and stored until use.

BLM Formation. The lipid or lipid mixture dissolved in chloroform was dried under Ar in a glass vial, followed by vacuum for a minimum of 4 h. Dried lipids were resuspended to a

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Table 1. Electrical Properties of Non-Irradiated and UV-Irradiated BLMs in the Absence of Alamethicin[a]

	non-irradiated			UV-irradiated		
lipid composition	specific current[b] ($\times 10^{-2}$ pA/μm^2)	specific conductance[c] ($\times 10^{-2}$ pS/μm^2)	specific capacitance[d] (μF/cm^2)	specific current[b] ($\times 10^{-2}$ pA/μm^2)	specific conductance[c] ($\times 10^{-2}$ pS/μm^2)	specific capacitance[d] (μF/cm^2)
DPhPC	1.3 ($\pm$0.1)	8.5 ($\pm$0.3)	0.54 ($\pm$0.01)	1.1 ($\pm$0.1)	7.3 ($\pm$0.3)	0.52 ($\pm$0.03)
bis-DenPC	0.6 ($\pm$0.2)	4.2 ($\pm$0.3)	0.71 ($\pm$0.04)	0.5 ($\pm$0.2)	3.8 ($\pm$0.3)	0.66 ($\pm$0.03)
1:1 DPhPC/bis-DenPC	1.0 ($\pm$0.1)	6.8 ($\pm$0.4)	0.63 ($\pm$0.04)	1.0 ($\pm$0.3)	6.7 ($\pm$0.4)	0.59 ($\pm$0.02)

[a] The standard deviations were computed from $n = 20$ trials for each lipid composition and irradiation condition. [b] Currents were recorded at an applied potential of +150 mV vs Ag/AgCl and normalized to a membrane area of 1 μm^2. [c] Conductance values were obtained from the slope of the i vs V plots for the respective BLMs (see the Supporting Information) and normalized to a membrane area of 1 μm^2. [d] Capacitance values were obtained from the lifetimes of the transient decays measured upon application of a 50 ms square pulse of 100 mV potential across the respective BLMs and normalized to a membrane area of 1 cm^2.

concentration of 10 mg/mL in *n*-decane. Formation and subsequent analysis of BLMs, both with and without alamethicin, was performed at 22 $\pm$ 2 °C. BLMs were formed by applying a small volume (ca. 3 μL) of lipid solution to the tip of the silanized pipet and allowing the solvent to evaporate. The pipet was then backfilled with recording buffer, and the tip was recoated with lipid solution and then placed in a bath containing recording buffer. A clean, disposable pipet tip was dragged across the glass pipet tip while monitoring resistance at an applied potential of +150 mV versus a Ag/AgCl reference electrode (instrumentation is described further below), until an increase to greater than 15 GΩ was observed, signifying BLM formation. If no increase in resistance was observed, the tip dragging process was repeated until the increase occurred. BLM formation was further verified by applying a 1 V potential across the pipet and observing rupture of the BLM as indicated by a return (decrease) to open pipet resistance. The BLM was then easily reformed by dragging a clean tip across the pipet.

Current−voltage curves ($i-$V) were measured to characterize the electrical properties of BLMs as a function of lipid composition and alamethicin concentration (see the Supporting Information for experimental procedures). Resistance values were monitored throughout the experiments described below to verify that the BLM remained intact by measuring the current in response to application of a 5 mV square-wave pulse. Capacitance values were determined by applying a 50 ms square pulse of 100 mV to the BLM while recording the current response.[39−41] Pipettes were used for multiple experiments by cleaning the pipet and bath to remove residual lipid and peptide from the previous trial.

UV Polymerization of BLMs. BLMs containing bis-DenPC were polymerized by irradiation with a UV pen lamp (UVP, Upland, CA, model 90-0012-01) placed a few centimeters from the pipet tip for 15 min. The manufacturer's specified intensity is 4750 μW/cm^2 at 254 nm at a distance of 0.75 in. from the lamp; the actual intensity was not measured. In control experiments, BLMs composed of DPhPC were irradiated under identical conditions.

Measurements of Alamethicin Activity. After a BLM was formed and a resistance value >15 GΩ was obtained, alamethicin was added to the bath solution to a final concentration of 15, 30, 60, or 120 ng/mL and the solution incubated for 15 min at −150 mV. Measurement of IC activity during the incubation period showed that no further changes in the frequency of IC activity and the magnitude of the associated current occurred after 10 min. Thus, the 15 min incubation time was deemed sufficient to achieve a steady-state concentration of peptide associated with the membrane. Following incubation, the alamethicin solution was replaced with fresh, peptide-free recording buffer.

Current recordings for alamethicin-functionalized BLMs were obtained from −150 to +150 mV, starting at −150 mV. Measurements were made at 50 mV intervals for a minimum of 2 min each. The potential was returned to 0 mV between each measurement. Recordings were acquired using a HEKA Electronik EPC-8 patch clamp amplifier configured with an ITC-16 A/D converter controlled with HEKA Pulse data acquisition software. Data were acquired at 5 kHz and filtered at 500 Hz for a monitoring period up to 1 h, from which representative samples of recordings were stored. Recordings were processed using HEKA PulseFit software. In some cases, progressively higher potentials (up to +1000 mV) were applied to determine breakdown voltages of BLMs. The incubation time and measurement procedures were identical for all experiments regardless of whether the BLM was unpolymerized, polymerized prior to alamethicin functionalization, or polymerized after functionalization. A minimum of five trials was performed for each set of experimental conditions, except when the alamethicin concentration was 120 ng/mL for which three trials were performed. Background currents were measured for unpolymerized, UV polymerized, alamethicin-free, and alamethicin-functionalized BLMs. In the latter case, short periods devoid of IC activity occurred at all but the highest alamethicin concentrations that were tested, which allowed the background current to be monitored continuously. In all cases, the background currents were less than 1 pA and showed little variability over the lifetime of experiments.

Results and Discussion

Electrical Properties of BLMs Lacking Alamethicin. A series of experiments was performed to characterize the electrical properties of BLMs composed of DPhPC, bis-DenPC, and equimolar DPhPC/bis-DenPC in the absence of alamethicin. $i-$V curves were measured before and after irradiation of BLMs with UV light (see the Supporting Information for experimental procedures). Irradiation for 15 min was determined to be sufficient to convert >95% of dienoyl functionalities in bis-DenPC to polymer,[35] based on experiments with liposomes that were polymerized using an identical optical geometry (see the Supporting Information). The degree of polymerization (X_n) was not measured in BLMs; however, previous studies of liposomes composed of dienoyl lipids showed that UV irradiation generates oligomers (X_n of 3−10).[42]

In all cases, linear $i-$V curves were obtained (see data plots in the Supporting Information) from which the specific conductance data listed in Table 1 were determined. The conductance of DPhPC BLMs is comparable to literature values[43] and did not change upon UV irradiation. The conductance of bis-DenPC BLMs was ca. 50% of that measured for DPhPC BLMs and decreased ca. 10% after photopolymerization. The conductance of mixed BLMs was slightly lower than that of DPhPC BLMs and did not change upon UV irradiation. Specific current values obtained at +150 mV are also listed in Table 1 and correlate well with conductance data. Specific capacitance values were also determined for all lipid mixtures tested and are listed

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Table 2. Summary of Stability Properties for BLMs Composed of DPhPC, bis-DenPC, and 1:1 bis-DenPC/DPhPC

lipid composition	non-irradiated BLM lifetime[a]	UV-irradiated BLM lifetime[a]	air−water transfers until rupture: non-irradiated BLMs[a]	air−water transfers until rupture: UV-irradiated BLMs[a]
DPhPC	260 (±25) min	240 (±32) min	2.4 (±0.5)	2.4 (±0.5)
bis-DenPC	240 (±29) min	24 (±4) days	3.0 (±0.7)	72 (±28)
1:1 DPhPC/bis-DenPC	250 (±14) min	4.0 (±0.7) days	2.4 (±0.5)	21 (±6)

[a] $n = 9$ for each BLM and for each type of experiment.

in Table 1. The values of unpolymerized membranes were in the range of 0.5−0.7 μF/cm^2, which are consistent with published data,[6,43] and declined slightly after irradiation.

Overall, the data in Table 1 show the following: (a) The presence of the dienoyl groups in bis-DenPC does not create an electrically "leaky" BLM; on the contrary, the conductance of bis-DenPC is less than that of DPhPC. (b) Photopolymerization does not adversely affect the electrical properties of pure bis-DenPC BLMs. (c) If phase segregation of bis-DenPC and DPhPC occurs in mixed, unpolymerized BLMs, it does not cause increased conductance. (d) If photopolymerization induces phase segregation (i.e., domains of DPhPC are dispersed in a poly(bis-DenPC) network)[32−34] the domain boundaries do not cause an increase in leakage current.

BLM Stability. To assess BLM stability, the integrity of unpolymerized and UV polymerized BLMs was monitored as a function of storage time in buffer as well as repeated transfer across the air−buffer interface. BLM conductance was monitored to determine when membrane rupture occurred, as previously described.[25] The results are listed in Table 2. For non-irradiated BLMs, the average lifetime was approximately 4 h for all lipid compositions. After UV irradiation, no change was observed for DPhPC BLMs, whereas the lifetime of poly(bis-DenPC) BLMs increased to about 3 weeks. Polymerization of mixed BLMs increased the mean lifetime to 4 days. Removal of a lipid bilayer from water typically causes significant disruption of its lamellar structure.[44,45] BLM stability was therefore further examined by monitoring conductance during repetitive transfers across the air−buffer interface. Nonirradiated BLMs (all three lipid compositions) and irradiated DPhPC BLMs typically ruptured after only a few transfers (Table 2). In contrast, BLMs composed of pure poly(bis-DenPC) survived an average of 72 transfers, and polymerized, mixed BLMs ruptured after an average of 21 transfers. Overall, these results show that fractional polymerization of a BLM composed of a mixture of polymerizable and nonpolymerizable lipids provides a significant stability enhancement relative to an unpolymerized BLM. Not surprisingly, the stability characteristics of fractionally polymerized, mixed BLMs are intermediate between those of the pure DPhPC and poly(bis-DenPC) BLMs. It should be noted that rupture is the end result of less drastic changes in membrane structure that likely occur throughout its lifetime. An indication that such changes occur is our observation that a significant increase in the conductance of polymerized BLMs is observed shortly before rupture occurs (data not shown).

Alamethicin Activity in Unpolymerized BLMs. Alamethicin is a helical peptide composed of 20 amino acids that binds to lipid bilayers with a partition coefficient of $\sim 10^3-10^5$ (bilayer/solution).[46−48] At relatively low concentrations in a lipid membrane, alamethicin is oriented parallel to the membrane plane, whereas at higher concentrations, it orients perpendicular.[49,50] The most widely accepted model of pore formation is the barrel-stave model in which alamethicin helices form water-filled pores with the hydrophilic residues of the peptide lining the lumen and the hydrophobic portions interacting with the hydrophobic interior of the bilayer.[27] The pores are composed of 3−11 monomers, resulting in multiple conductance states, and the distribution of pore structures varies with the lipid composition of the bilayer.[27,29,51] Alamethicin was chosen as a model IC for this study because a fluid membrane is required to maintain its voltage-gated activity (see evidence below).

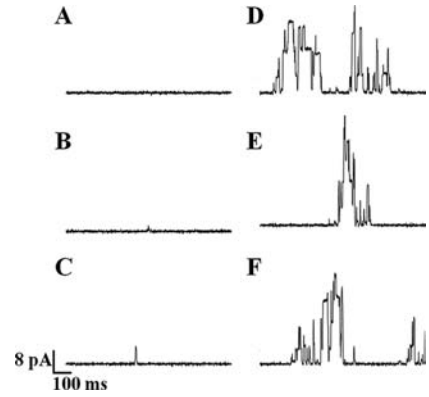

Figure 2. Alamethicin activity in non-irradiated BLMs. Representative recordings from BLMs incubated with alamethicin at 15 ng/mL (A−C) and 30 ng/mL (D−F). The lipid compositions were DPhPC (A, D), bis-DenPC (B, E), and 1:1 DPhPC/bis-DenPC (C, F). The applied potential was +150 mV.

BLMs composed of DPhPC, bis-DenPC, and 1:1 DPhPC/bis-DenPC were incubated with 15, 30, 60, or 120 ng/mL of alamethicin for 15 min, and IC activity was recorded at 50 mV intervals from −150 mV to +150 mV. An example set of recordings over the entire voltage range for DPhPC with 60 ng/mL of alamethicin is shown in the Supporting Information. For all three lipid compositions, both positive and negative voltage gating were observed with no significant differences in voltage dependence. For brevity, recordings at only +150 mV are shown through the remainder of this paper.

Figure 2 shows representative examples of +150 mV recordings at alamethicin concentrations of 15 and 30 ng/mL. At 15 ng/mL (panels A−C), little to no IC activity was observed for all three lipid compositions. Only a few current bursts were observed during each 5 min measurement period, as illustrated by the small current spike in Figure 2C. This indicates that the peptide concentration in these membranes is too low to form conducting pore structures. In contrast, at 30 ng/mL (panels

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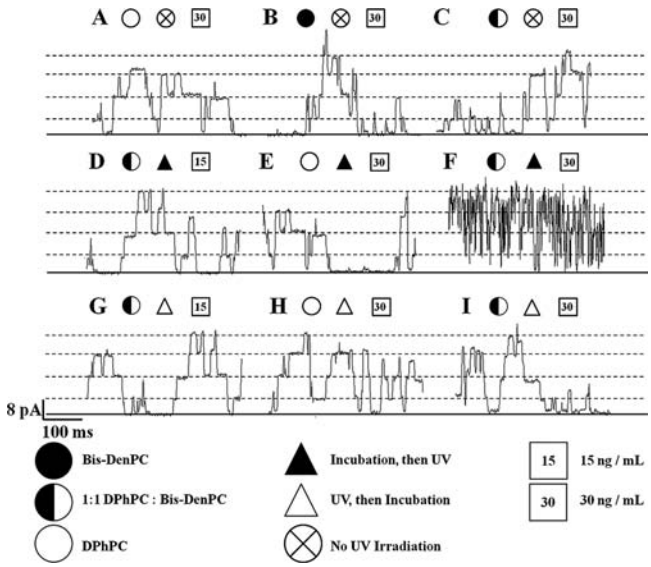

Figure 3. Temporally resolved alamethicin conductance states. BLMs composed of (A) DPhPC, (B) bis-DenPC, and (C) 1:1 DPhPC/bis-DenPC incubated with 30 ng/mL of alamethicin. BLMs incubated with alamethicin and then UV irradiated: (D) 1:1 DPhPC/bis-DenPC, 15 ng/mL, (E) DPhPC, 30 ng/mL, and (F) 1:1 DPhPC/bis-DenPC, 30 ng/mL. BLMs UV irradiated and then incubated with alamethicin: (G) 1:1 DPhPC/bis-DenPC, 15 ng/mL, (H) DPhPC, 30 ng/mL, and (I) 1:1 DPhPC/bis-DenPC, 30 ng/mL. These combinations are indicated by the legend below the data. Dashed lines correspond to currents of 6, 14, 22, and 26 pA above the background current (<1 pA and signified by the solid line); these current levels correspond to the O1−O4 subconductance states centered at 40, 93, 147, and 173 pS, respectively.

D−F), frequent bursts of current characteristic of alamethicin activity were observed. The peptide concentration in these BLMs was therefore high enough to produce several discrete pore structures at moderate frequency. Expanded sections of the recordings in Figures 2D−F are shown in Figures 3A−C and show a series of subconductance states centered at 40, 93, 147, and 173 pS (denoted the O1−O4 states, respectively). No significant differences in the frequency and amplitude of the current bursts among the three lipid compositions were measured, indicating that the lipid structure has no observable effect on the distribution of pore structures and subconductance states.

The effect of lipid composition on IC activity was further examined by analysis of the mean probabilities for occurrence of the closed (nonconducting) state and the several subconductance states at an alamethicin concentration of 30 ng/mL. The results of the analysis for unpolymerized BLMs are listed in the left side of Table 3 (see the footnotes for a description of the analysis procedure). For all three lipid compositions, the probabilities for observation of the closed state (P_C) and the subconductance states centered at 40, 93, 147, and 173 pS (denoted P_{O1}, P_{O2}, P_{O3}, and P_{O4}, respectively) were similar, verifying that the lipid composition has no observable effect on the distribution of conducting pore structures.

Increasing the alamethicin concentration to 60 ng/mL greatly increased the frequency of current bursts, as expected for a greater peptide concentration in the membrane.[52] Representative recordings and all-points histograms for the three lipid compositions at 60 ng/mL alamethicin are presented in the Supporting Information (see Figures S4 and S5). Mean current values, current ranges, and subconductance states were obtained from the plots in the Supporting Information and are listed in the left side of Table 4. When BLMs were incubated with alamethicin at a concentration of 120 mg/mL, continuous IC activity was observed, and as a consequence, discrete subconductance states could not be resolved. An example set of data for DPhPC is summarized in Table 4 (see Figure S6 in the Supporting Information for the respective recording and all-points histogram). The mean current was 96 pA with a range of 60 pA, as compared to a mean of 7 pA and a range of 24 pA at 60 ng/mL. Overall, the data presented in Figure 2 and Table 4 are interpreted as evidence that the distribution of pore structures and their respective conductances depend on the alamethicin concentration in the BLM, which can be adjusted by varying the dissolved peptide concentration. This finding is consistent with published data.[49,53,54]

BLMs Polymerized Following Alamethicin Functionalization. IC activity in BLMs that were incubated with alamethicin and then irradiated with UV light was examined. Representative current recordings at +150 mV for DPhPC BLMs at alamethicin concentrations of 15 and 30 ng/mL are shown in Figure 4 (panels A and D). An expanded section of the recording in Figure 4D is shown in Figure 3E, illustrating the O1−O4 subconductance states. The current recordings are very similar to those obtained from DPhPC BLMs that were not irradiated (Figures 2A,D and 3A). Furthermore, the probabilities for observing the closed state and the open states after UV irradiation (P_C and $P_{O1}-P_{O4}$; listed on the right side of Table 3) are equivalent to the probabilities obtained for non-irradiated BLMs. Thus, the exposure to UV light did not measurably alter the activity of alamethicin in DPhPC BLMs. In addition, no difference in IC activity was observed between non-irradiated and irradiated DPhPC when the alamethicin concentration was increased to 60 ng/mL, as shown in Table 4 (also see the Supporting Information for a representative recording and all-points histogram at 60 ng/mL).

In contrast, UV irradiation of bis-DenPC BLMs completely eliminated alamethicin activity, as shown by a comparison of parts B and E of Figure 4, as well as the probabilities listed in Table 3. Complete loss of activity was also observed at 60 ng/mL (Table 4; also see the Supporting Information for a sample recording and all-points histogram). On the basis of the proposed mechanism for alamethicin channel formation and conductance, a fluid membrane is required,[26,49,55,56] and the lack of fluidity in poly(bis-DenPC) is clearly incompatible with this requirement. A significant increase in membrane thickness upon polymerization would also cause a decrease in alamethicin activity. However, this is unlikely to be the case here on the basis of (a) a previous study[57] in which only a 4−5% increase in optical thickness was measured upon bis-DenPC polymerization and (b) the absence of significant changes in specific capacitance after UV irradiation (Table 1).

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(56) The requirement for fluidity was verified by preparing BLMs from dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) which has a main-phase transition temperature of 41 °C, well above the measurement temperature of 22 °C. No ion channel activity was observed when these gel-phase BLMs were incubated with 60 ng/mL of alamethicin for 15 min (i.e., the data were very similar to that shown in Figure 3 for poly(bis-DenPC)).
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Table 3. Summary of Probabilities Determined for Closed and Open (Conducting) States of Alamethicin Incubated at 30 ng/mL with BLMs before and after UV Irradiation[a,b]

	non-irradiated						UV-irradiated					
lipid composition	P_C	P_{O1}	P_{O2}	P_{O3}	P_{O4}	P_{O5}	P_C	P_{O1}	P_{O2}	P_{O3}	P_{O4}	P_{O5}
DPhPC	0.32 (±0.09)	0.17 (±0.06)	0.20 (±0.08)	0.21 (±0.09)	0.10 (±0.04)	<0.01	0.31 (0.09)	0.15 (±0.07)	0.21 (±0.09)	0.23 (±0.06)	0.10 (±0.06)	<0.01
bis-DenPC	0.30 (±0.08)	0.15 (±0.09)	0.23 (±0.06)	0.20 (±0.08)	0.12 (±0.04)	<0.01	1.00 (±0.00)	0.00	0.00	0.00	0.00	0.00
1:1 DPhPC/ bis-DenPC	0.34 (±0.09)	0.19 (±0.05)	0.17 (±0.07)	0.23 (±0.04)	0.07 (±0.02)	<0.01	0.05 (±0.03)	0.14 (±0.08))	0.23 (±0.09)	0.27 (±0.08)	0.31 (±0.10)	<0.01

[a] P_C is the probability of observing alamethicin in the closed state. P_{O1}, P_{O2}, P_{O3}, and P_{O4} are the probabilities of observing alamethicin in open (conducting) states, denoted O1−O4, centered at 40, 93, 147, and 173 pS, respectively. P_{O5} is the probability of observing alamethicin in conducting states at >200 pS. [b] Five trials were analyzed for each lipid composition and irradiation condition; for each trial, 60 s of data were analyzed. P_C and $P_{O1} - P_{O5}$ were determined by constructing a histogram with six channels corresponding to conductances centered at 0, 40, 93, 147, 173, and >200 pS for each data set. The fractional area in each channel was divided by the total area (sum of six channels) and normalized to a total probability of unity.

Table 4. Summary of Currents and Conductance Values Measured for Pure and Mixed BLMs Incubated with Alamethicin at Concentrations ≥60 ng/mL[a,b]

		non-irradiated			UV-irradiated		
lipid composition	alamethicin concentration (ng/mL)	mean current (pA)	current range (pA)	subconductance levels centered at (pS)	mean current (pA)	current range (pA)	subconductance levels centered at (pS)
DPhPC	60	7 (±6)	24	33, 60, 87, 113, 133	8 (±6)	26	40, 60, 93, 120, 133
bis-DenPC	60	9 (±5)	24	67, 107	0 (±1)	3	no activity
1:1 DPhPC/bis-DenPC	60	6 (±4)	17	20, 53, 80	110 (±30)	241	733[c]
DPhPC	120	96 (±8)	60	640[c]			

[a] The data in this table summarize the raw data presented in Figures S4−S6 in the Supporting Information. All measurements were made at +150 mV. [b] Background currents (see Table 1) were subtracted from the data listed in this table. [c] A single, broad distribution was observed. See histograms in Figures S5 and S6 in the Supporting Information.

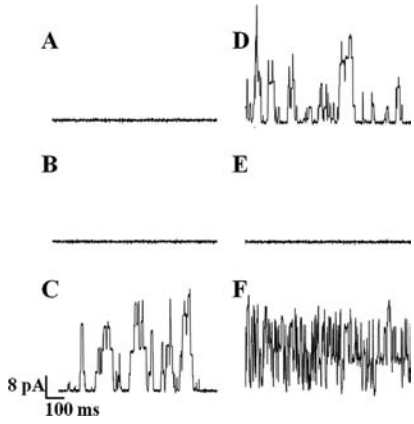

Figure 4. Representative recordings showing the effect of UV irradiation on BLMs incubated with alamethicin. BLMs were incubated with alamethicin at 15 ng/mL (A−C) and 30 ng/mL (D−F) followed by 15 min of UV irradiation. The lipid compositions were DPhPC (A, D), bis-DenPC (B, E), and 1:1 DPhPC/bis-DenPC (C, F). The applied potential was +150 mV.

Very different behavior was observed with 1:1 DPhPC/bis-DenPC, as demonstrated by comparing recordings from BLMs incubated with 15 ng/mL of alamethicin. Before UV irradiation, minimal activity was observed (Figure 2C), whereas significant IC activity was measured after polymerization (Figures 4C and Figure 3D). The frequency and amplitude of current bursts in Figures 4C and 3D are similar to those measured for non-irradiated BLMs incubated at 30 ng/mL (Figures 2F and 3A−C). Similar changes were observed for higher alamethicin concentrations: (a) A typical recording obtained from a 1:1 DPhPC/bis-DenPC BLM that was polymerized after incubation with 30 ng/mL is shown in Figure 4F, with an expanded section shown in Figure 3F. The high frequency of current bursts is comparable to data obtained for non-irradiated BLMs that were functionalized with 60 ng/mL of alamethicin (see Figure S4 in the Supporting Information). (b) The probabilities of observing the closed state and the open states before and after UV irradiation at 30 ng/mL are listed in Table 3. After polymerization, a significant shift in the probability distribution toward higher subconductance states was observed; specifically, P_C and P_{O1} declined whereas $P_{O2}-P_{O4}$ increased. (c) Incubation of a 1:1 DPhPC/bis-DenPC BLM at 60 ng/mL followed by polymerization eliminated discernible conductance levels; instead a single, broad distribution (see Figure S5 in the Supporting Information) with a mean value of 110 ± 30 pA and a range of 241 pA was observed. These current values are much greater than those observed before irradiation (see Table 4) and more closely approximate the values for DPhPC BLMs incubated with 120 ng/mL of alamethicin.

Several major points can be drawn from the results of these experiments. First, alamethicin functionalization of 1:1 DPhPC/bis-DenPC BLMs at ≥15 ng/mL followed by polymerization generates transient currents that are significantly greater than the specific background currents measured for peptide-free BLMs listed in Table 1. Therefore, the possibility that these transient currents may arise due to membrane defects created upon polymerization is eliminated. Second, these data demonstrate that the requisite fluidity required for alamethicin activity is maintained in a fractionally polymerized BLM. This suggests that photopolymerization of 1:1 DPhPC/bis-DenPC generates a phase-segregated membrane composed of fluid DPhPC domains dispersed in a poly(bis-DenPC) matrix. Third, the increased current that accompanies fractional polymerization indicates that the apparent concentration of alamethicin in the mixed BLM has also increased. This could occur if, upon bis-DenPC polymerization, alamethicin is excluded from the polymerized domains and as a consequence becomes concentrated in the fluid DPhPC domains. Assuming that the membrane area is composed of equal amounts of DPhPC and poly(bis-DenPC), the alamethicin concentration in the DPhPC domains would double after polymerization, increasing the probability of transient pore formation and shifting the number of helices per pore to larger values and higher conductances. The prob-

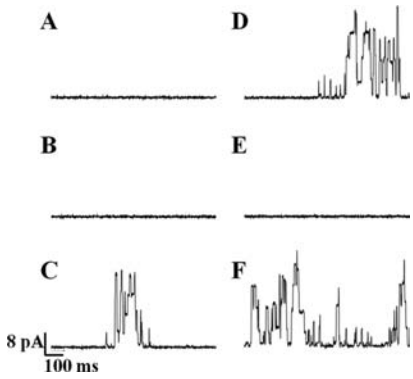

Figure 5. Representative recordings of IC activity in BLMs that were UV irradiated prior to alamethicin insertion. BLMs were UV irradiated for 15 min and then incubated with alamethicin at 15 ng/mL (A−C) and 30 ng/mL (D−F). The lipid compositions were DPhPC (A, D), bis-DenPC (B, E), and 1:1 DPhPC/bis-DenPC (C, F). The applied potential was +150 mV.

ability data (Table 3) support this hypothesis, and further support is provided by an analysis of lifetimes of the subconductance states presented in the Supporting Information.

An assumption inherent in this hypothesis is that alamethicin dissociation from the membrane is relatively slow compared to the time scale of the measurement. To our knowledge, apparent dissociation constants for alamethicin have not been published; however, rates for other α-helical peptides bound to membranes have been determined and in some cases are slow, on the order of a few hours.[58−60] To address this issue, 1:1 DPhPC/bis-DenPC BLMs functionalized with alamethicin were monitored for 2 h after polymerization. No change in IC activity was observed, verifying that peptide concentration in the membrane remained constant over this time period.

BLMs Polymerized before Alamethicin Functionalization. The properties of BLMs that were incubated with alamethicin after UV irradiation were also examined. Figures 5 and 3G−I show typical results at peptide concentrations of 15 and 30 ng/mL. For pure DPhPC and bis-DenPC BLMs, the recordings were similar to those obtained when incubation was performed before irradiation: IC activity is retained in DPhPC, whereas in poly(bis-DenPC), no activity is observed (i.e., compare the upper and middle panels in Figures 4 and 5). Higher alamethicin concentrations were also investigated; summary data are listed in Table 5 with example recordings and all-points histograms presented in the Supporting Information. No significant differences were observed relative to BLMs that were functionalized with alamethicin before irradiation.

In the case of 1:1 DPhPC/bis-DenPC, significant IC activity was measured at alamethicin concentrations of 15 and 30 ng/mL, as illustrated by the recordings in parts C and F, respectively, of Figure 5. The amplitude of the current bursts was similar to those observed when incubation preceded polymerization; however, their frequency was generally less (i.e., compare Figure 4C,F to Figure 5C,F). Expanded sections of the recordings in Figure 5C,F, shown in Figure 3G,I, respectively, verify the presence of the O1−O4 subconductance states centered near 40, 93, 147, and 173 pS. Experiments were also conducted at 60 ng/mL; summary data are listed in Table 4,

Table 5. Summary of Currents and Conductance Values Measured for Pure and Mixed BLMs That Were UV-Irradiated and then Incubated with Alamethicin at Concentrations ≥60 ng/mL[a,b]

lipid composition	alamethicin concentration (ng/mL)	mean current (pA)	range (pA)	subconductance levels centered at (pS)
DPhPC	60	6 (±7)	25	33, 60, 93, 120, 140
bis-DenPC	60	0 (±1)	3	no activity
1:1 DPhPC/bis-DenPC	60	15 (±6)	37	100[b]

[a] The data in this table summarize the raw data presented in Figures S4 and S5 in the Supporting Information. All data were collected at +150 mV. [b] A single, broad distribution was observed. See the histogram in Figure S5 in the Supporting Information.

and example recordings and all-points histograms are presented in the Supporting Information. The IC activity was greater than at 30 ng/mL, as expected, but again it was less than that observed when incubation preceded polymerization (see Figures S4 and S5 in the Supporting Information).

These comparisons show that when a mixed DPhPC/bis-DenPC BLM is incubated with alamethicin after photopolymerization, the peptide inserts into and forms conducting pores in the membrane, presumably in fluid DPhPC domains. However the number, size, and or frequency of conducting pores are less than when incubation is performed before polymerization. The underlying cause of this difference is unknown. However it is important to note that the two procedures may not produce equivalent membrane structures. When incubation precedes irradiation, bis-DenPC is photopolymerized in a bilayer that contains three components (DPhPC, peptide, bis-DenPC), whereas when irradiation precedes incubation, the bilayer is composed solely of lipids during polymerization. The presence of the peptide may alter the bilayer structure[61,62] (e.g., domain size, shape, and registry between leaflets), producing a different distribution of alamethicin pore structures.

Alamethicin Activity after BLM Storage for 1−3 Days. Alamethicin activity in photopolymerized 1:1 DPhPC/bis-DenPC BLMs was investigated during the course of the BLM lifetimes listed in Table 4. In one set of experiments, BLMs were incubated with 15 ng/mL of alamethicin, photopolymerized, and then stored in recording buffer at 4 °C for up to 3 days. Typical recordings after 1 and 3 days of storage are shown in parts A and B, respectively, of Figure 6. These data are very similar to recordings acquired immediately following irradiation (e.g., Figure 4C). In a second set of experiments, BLMs were formed, photopolymerized, and then stored in recording buffer at 4 °C for up to 3 days. After storage, BLMs were incubated with 30 ng/mL of alamethicin, and IC activity was measured. Typical recordings obtained after storage for 1 and 3 days are shown in parts C and D, respectively, of Figure 6. The frequency and amplitude of the current bursts were very similar to those of BLMs that were functionalized immediately following UV irradiation (Figure 5F). Overall, the data presented in Figure 6 show that BLM storage did not measurably alter the activity of incorporated alamethicin, regardless of whether the peptide was incorporated into the membrane before or after polymerization.

Conclusions

We have demonstrated that photopolymerization of mixed lipid bilayers composed of bis-DenPC and DPhPC produces

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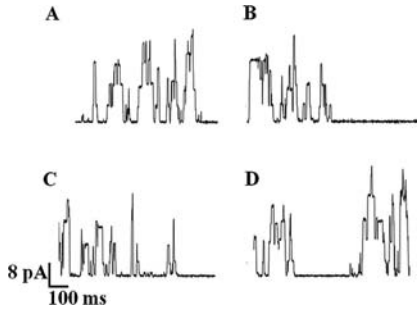

Figure 6. Long-term IC activity in photopolymerized, mixed BLMs. Representative recordings of 1:1 DPhPC/bis-DenPC BLMs that were incubated with 15 ng/mL of alamethicin, photopolymerized, and then stored in buffer for (A) 1 day and (B) 3 days before IC activity was measured. Representative recordings of 1:1 DPhPC/bis-DenPC BLMs that were photopolymerized, stored in buffer, then incubated with 30 ng/mL of alamethicin (C) after storage for 1 day and (D) after storage for 3 days. The applied potential was +150 mV.

highly stable BLMs. A substantial degree of fluidity is maintained in these BLMs, as evidenced by the retention of alamethicin activity (for which fluidity is a requirement). Thus, fractional lipid polymerization is an effective strategy for creating membranes that feature *both stability and fluidity*. Fractional polymerization likely drives phase segregation, generating fluid and poly(lipid) domains. Alamethicin appears to be concentrated in the fluid domains during polymerization, leading to higher measured currents due to increases in the size, number, and/or frequency of pores. Further studies are ongoing to determine the size and spatial distribution of the poly(lipid) and fluid domains, if these and other membrane properties depend on the DPhPC/bis-DenPC molar ratio, and if alamethicin is localized in fluid domains after polymerization. Additional studies are also needed to determine if the activity of other types of ion channel peptides and proteins can be retained in photopolymerized, mixed BLMs.

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Supporting Information Available: (1) Data demonstrating UV-initiated polymerization of bis-DenPC. (2) Representative $i-V$ curves for all three lipid compositions before and after UV irradiation. (3) Example voltage screens of alamethicin in a DPhPC BLM recorded at 50 mV intervals from −150 mV to +150 mV. (4) Representative current recordings and all-points histograms of non-irradiated and UV-irradiated BLMs functionalized at alamethicin concentrations ≥60 ng/mL. (5) Analysis of lifetimes of subconductance states of alamethicin before and after UV irradiation and at various peptide concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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