Direct Observation of Poloxamer 188 Insertion into Lipid Monolayers

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ABSTRACT P188, a triblock copolymer of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) helps seal electroporated cell membranes, arresting the leakage of intracellular materials from the damaged cells. To explore the nature of the interaction between P188 and cell membranes, we have constructed a model system that assesses the ability of P188 to insert into lipid monolayers. Using concurrent Langmuir isotherm and fluorescence microscopy measurements, we find that P188 changes the phase behavior and morphology of the monolayers. P188 inserts into both dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol monolayers at surface pressures equal to and lower than ~22 mN/m at 30°C; this pressure corresponds to the maximal surface pressure attained by P188 on a pure water subphase. Similar results for the two phospholipids indicate that P188 insertion is not influenced by headgroup electrostatics. Because the equivalent surface pressure of a normal bilayer is on the order of 30 mN/m, the lack of P188 insertion above 22 mN/m further suggests the poloxamer selectively adsorbs into damaged portions of electroporated membranes, thereby localizing its effect. P188 is also found to be "squeezed out" of the monolayers at high surface pressures, suggesting a mechanism for the cell to be rid of the poloxamer when the membrane is restored.

INTRODUCTION

Victims of electrical trauma suffer extensive loss of structural integrity of cell membranes in skeletal tissue (Hannig et al., 2000; Lee and Kolodney, 1987a,b; Tropea and Lee, 1992). Acute membrane damage is clinically characterized as the liberation of intracellular materials into intravascular space (Lee et al., 1988, 1992). Although sophisticated life support systems have reduced deaths due to electrical trauma, large numbers of patients are still left with amputations, rendering them permanently disabled (DiVincenti et al., 1969; Lee et al., 1992). Most high voltage accidents in the United States occur through contact with electrical fields of 6 to 10 kV (Lee et al. 1992). Potentials of this magnitude are large enough to cause both joule heating (Lee et al., 1988, 1992; Lee and Kolodney, 1987b) and electroporation damage (Lee and Kolodney, 1987a), especially in skeletal muscle and peripheral nerve cells. Joule heating, a rise in intracellular temperature caused by the cell's resistance to electric current, had long been considered the main contributor of tissue damage in electrical injury. In 1990 (Bhatt et al., 1990), however, a second mechanism of electrical cell membrane damage, electroporation, was documented as another principle component of membrane disruption. Electroporation accounts for the widespread damage in cells that demonstrates no visible thermally induced impairment.

Because increased cell membrane permeability accounts for the majority of tissue damage in many common clinical conditions after physico-chemical insults such as electrical injuries, burns, frostbite, ischemia-reperfusion injuries, as well as neurotoxic events, it is clear that effective therapy for such ailments must aim to restore cell membrane structural integrity. Exposing electroporated cells to the noncytotoxic nonionic surfactant, Poloxamer 188 (MW ≈ 8400 g/mol; poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) weight ratio is 4:2:4), effectively seals the damaged membranes of skeletal muscle cells, thereby arresting the leakage of intracellular components (Hannig et al., 2000; Lee et al., 1994, 1992; Matsuura et al., 1966; Merchant et al., 1998; Sharma et al., 1996; Terry et al., 1999). Although results of these studies show that P188 is effective against injury, the mechanisms involved in surfactant mediated cell membrane sealing are not known. Consequently, basic physico-chemical research on the interaction between model lipid systems and surfactants is needed so that poloxamer therapy can be improved rationally.

To investigate possible molecular mechanisms responsible for the observed membrane sealing capabilities of triblock copolymer surfactants, we have modeled the outer leaflet of the cell membrane using a lipid monolayer at the air-water interface and examined the ability of P188 to insert into it. Although cell membranes are composed of lipid bilayers, the Langmuir lipid monolayer system serves as a good model for the outer leaflet of the membrane. Langmuir lipid monolayers are two-dimensional surface films that have been extensively used as model biological membranes (Möhwald, 1990). Using surface pressure-area isotherms (Gaines 1966), one can observe that decreasing the lipid's surface area at the interface induces a series of two-dimensional phase transitions (Andelman et al., 1994; Kaganer et al., 1999; Knobler and Desai, 1992; McConnell, 1991; Möhwald, 1990, 1993; Weis, 1991). At very high areas per lipid molecule, the molecules at the air-water

Submitted November 5, 2001 and accepted for publication November 9, 2001.

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0006-3495/02/03/1453/07 $2.00

interface exist in a two-dimensional gas-like (G) state. Upon reduction of surface area by lateral compression, the monolayer condenses from the G state to an isotropic two-dimensional fluid state known as the liquid expanded (LE) phase. A further decrease in surface area causes a transition from the LE phase to the anisotropic liquid condensed (LC) phase. By measuring the extent to which these transitions are affected by P188, we can gain insight into the incorporation of P188 into the monolayer. We have tested how P188 interacts with dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine (DPPC) monolayers when present in pure water at 30°C. Adsorption of P188 into a DPPC monolayer spread at an air-water interface has been reported (Magalhaes, et al. 1991; Sundaram and Stebe, 1997; Weingarten et al., 1991). In this work, we focus on P188 insertion preferences as a function of surface pressure for both DPPC and DPPG monolayers and how P188 insertion affects lipid morphology. By measuring P188 insertion as a function of surface pressure (π), which reports directly on lipid packing density, we determined that P188 inserts into both types of monolayers at $\pi \leq 22$ mN/m, but not at higher pressures. Morphologically, we find that the presence of P188 in the monolayers disorders the packing of the film. Our data suggest that the relatively hydrophobic midsection of P188 inserts into damaged portions of cell membranes, where the lipid packing density is compromised. The damaged membrane has an equivalent surface pressure lower than that of a normal cell membrane. Inability of P188 to remain inserted at the equivalent bilayer surface pressure of a normal membrane further suggests that P188 may be naturally "squeezed out" (Weingarten et al., 1991) from the bilayer of the cell once it has repaired its injuries.

MATERIALS AND METHODS

Lipids and subphase

DPPC and DPPG were purchased in powdered form from Avanti Polar Lipids, Inc (Alabaster, AL). Monolayer spreading solutions were prepared by dissolving the solid lipid in chloroform (high-performance liquid chromatography grade, Fisher Scientific, Pittsburgh, PA) to obtain a concentration of 200 mg/ml. One-mole percent Texas Red, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Molecular Probes Inc., Eugene, OR) was the fluorescent probe used. For all the experiments, ultra-pure water (Resistivity $\geq$ 18 MΩcm) was used as the subphase, made by using a combination of reverse osmosis (RiOs/Elix-10) and ultra-purification (Milli-Q, A-cogradient, Millipore, Bedford, MA).

P188 solution

Solutions of 200 milligrams of P188 (BASF, Parisippany, NJ) per mL of ultra-pure water were prepared by adding the poloxamer and water to a vial containing a magnetic stir bar, and then left to mix on a stirplate for 1/2 h to ensure complete dissolution. The poloxamer solution was stored at 4°C before use, and made fresh weekly.

Equipment

All surface pressure-area isotherms were collected using a Teflon Langmuir trough equipped with a Wilhelmy plate (Reigler and Kirstein, Berlin, Germany). The home-built Teflon Langmuir trough (27.5 cm $\times$ 6.25 cm $\times$ 0.63 cm) equipped with two identical mobile Teflon barriers (l = 6.25 cm) enables compression or expansion of monolayers spread at the air-water interface, thereby increasing or reducing the surface pressure, respectively. The water subphase volume used was ~95 mL and the maximal working surface area was 145 cm^2. The subphase temperature was maintained within 0.5°C of the desired temperature through the use of a homebuilt control station comprised of thermoelectric units (Omega Engineering Inc, Stamford, CT) joined to a heat sink held at 20°C by a Neslab RTE-100 water circulator (Portsmouth, NH). A resistively heated indium tin oxide coated glass plate (Delta Technologies, Dallas, TX) was placed over the trough to minimize dust contamination, air currents, evaporative losses, and to prevent condensation of water on the microscope objective.

Our Langmuir trough is positioned on translation stages that permit scanning along the air-water interface in the *x*, *y*, and *z* directions. This assembly is fixed to a custom-built microscope stage for simultaneous fluorescence microscopy with a 50$\times$ long working distance objective lens (Nikon Y-FL, Fryer Co., Huntly, IL). Excitation between 530 and 590 nm and emission between 610 and 690 nm were gathered through the use of a filter cube (Nikon HYQ Texas Red, Fryer Co., Huntly, IL). Images from the fluorescence microscope were collected at a video rate of 30 frames/s using a silicon intensified target camera (Hamamatsu Corporation, Bridgewater, NJ), and recorded on Super-VHS formatted videotape with a recorder (JVC HR-S4500U, JVC Co. of America, Wayne, NJ). This assembly permits the monolayer morphology to be observed over a large lateral area while isotherm data are obtained concurrently.

The entire apparatus is set on a vibration isolation table (Newport, Irvine, CA) and controlled using a custom software interface designed using LabView 4.1(National Instruments, Dallas, TX).

Critical micelle concentration experiments

We first determined the critical micelle concentration (CMC) of P188 by examining its surface activity at the air-water interface. Twelve separate experiments were performed, each with increasing increments of a 200-mg/mL P188 solution into the water subphase at 30°C. P188 was added to the subphase and left undisturbed for 30 min, and the rise in surface pressure was noted. No additional increase in surface pressure above a certain concentration of P188 signifies an established equilibrium between the monolayer at the surface and micelles in the subphase. This concentration of P188 in the subphase is termed the CMC for P188. Subsequent experiments were performed at a P188 concentration well beneath the CMC to avoid micellization.

Lateral compression experiments

All experiments were performed on pure water at 30°C. The monolayer was spread by gentle dropwise addition of the monolayer spreading solution to the water surface, and the solvent was allowed to evaporate for 15 min. The barrier compression was commenced and isotherm measurements in the form of surface pressure (mN/m) versus area per lipid molecule (Å^2/molecule) were obtained automatically at 1-s intervals until the system had reached its compression limit. Two different lateral compression experiments were performed: injection experiments were carried out to identify under what packing conditions P188 would insert into the monolayer, whereas pretreatment experiments were performed to observe if P188 could be eliminated from the monolayer should the lipid packing density of the membrane be restored.

Injection experiments and fluorescence microscopy

For injection experiments, the lipid films were compressed to either a desired surface pressure or surface area before P188 was introduced to the subphase. For constant pressure experiments, the monolayer was first compressed to a surface pressure of 30 mN/m to mimic the packing density of a normal bilayer. When this surface pressure was attained, the barriers were switched from a linear compression mode to a computerized mode that held the pressure constant by adjusting the surface area via a feedback mechanism. P188 was then injected into the subphase when the desired surface pressure was reached. If no change in area per molecule was observed at this designated pressure after 10 min, the surface pressure was lowered by 2 mN/m and held steady for observation. This surface pressure lowering procedure was continued until P188 insertion was noted, and the barriers expanded to their original precompression position. Between each pressure decrease, the system was given 10 min to allow for insertion. During each experiment, fluorescence microscopy (FM) images were recorded on Super-VHS tape as mentioned previously.

When P188 was injected underneath monolayers at a desired surface pressure with the area held constant, the barriers were immobilized to allow for changes in surface pressure as a result of the administration of P188. The system was left to equilibrate for 1 h to allow for a maximal surface pressure to be achieved. The change in surface pressure was monitored with respect to time.

Pretreatment experiments

Pretreatment experiments were performed to detect if P188 could remain incorporated in the lipid film at high lipid packing densities. P188 was introduced to the subphase before the lipid monolayer was compressed. As in all previous experiments, the subphase was prepared with appropriate volume and temperature, and the monolayer material was spread at the interface at a low surface density ($\pi \approx 0$ mN/m) and left undisturbed for 15 min. P188 was then injected into the system, and the entire assembly was left undisturbed for 5 min allowing the surface pressure of the system to rise to ~20 mN/m before the lateral compression commenced.

Image grabbing and handling

Static images were transferred from the Super-VHS tape as 640 pixel × 480 pixel bitmap images using an 8-MB All-in-Wonder Pro Card (ATI Technologies, Thornhill, ON, Canada). These images were subsequently resized and enhanced in brightness and contrast for visual clarity.

RESULTS

CMC results

To perform all experiments for polymer insertion into lipid monolayers below the CMC of P188, experiments to identify the CMC were first completed (Fig. 1). The CMC for P188 was found to be 1.25×10^{-4} M (equivalent to 500 μL of the 200 mg/mL P188 solution) for our trials. Thus, the concentration of P188 used in all subsequent trials was 50 μM, 40% of the obtained CMC. By adding only 200 μL of the 200 mg/mL P188 solution, it can be assumed that all of the poloxamer goes to the surface and that the subphase volume change on injection is negligible.

Lateral compression results

Isotherm and surface morphology measurements were first performed on pure DPPC and DPPG monolayers to form the basis for observing phase and morphological changes as a result of P188 insertion (Fig. 2). Any deviations from the characteristics observed in DPPC and DPPG isotherms obtained in the absence of P188 are attributed to the presence of P188 at the air-water interface.

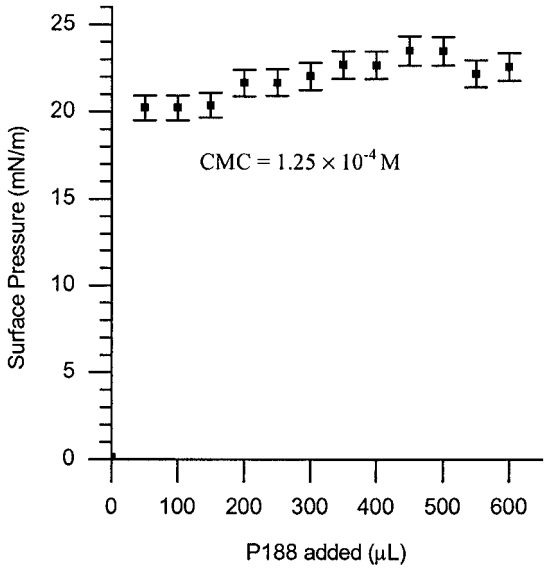

FIGURE 1 CMC results of P188 in a water subphase at 30°C. Surface pressures increase with corresponding increasing amounts of P188, until ~500 μL of 200 mg/mL P188 solution is added. At this point, a plateau in surface pressures is observed.

Fig. 2 displays isotherms of pure DPPC and pure DPPG on pure water at 30°C. Both isotherms show lift-off areas between 90 and 110 Å^2 per molecule, indicating the formation of a uniform LE phase from the G-LE phase coexist-

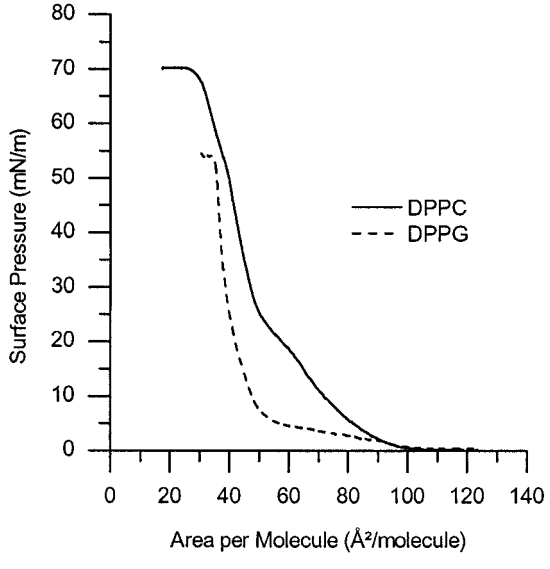

FIGURE 2 Lateral compression isotherms of DPPC and DPPG in a water subphase at 30°C.

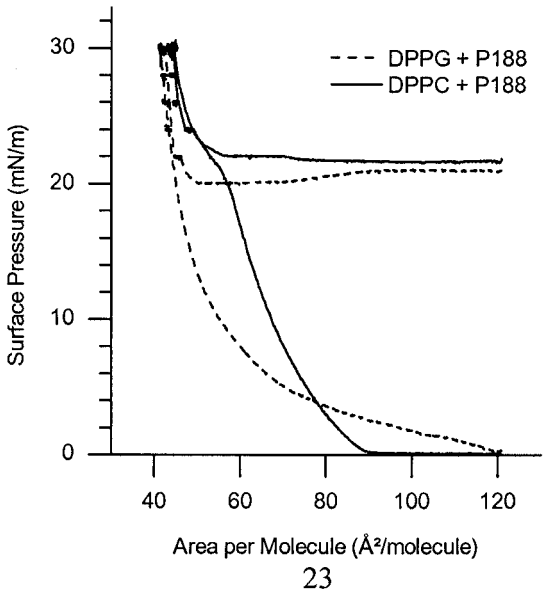

FIGURE 3 Injection of P188 into the water subphase of DPPC and DPPG monolayers at 30°C. Monolayer films were compressed to 30 mN/m before P188 was injected into the subphase. No change in area per molecule was observed at 30 mN/m, so the pressure was lowered by 2 mN/m, and the monolayer was observed for 10 min to allow for insertion. If no insertion was observed, the surface pressure lowering procedure was continued until insertion was noted, and the barriers expanded to their original precompression position.

ence (Andelman et al., 1994; Kaganer et al., 1999; Knobler and Desai, 1992; Lee et al., 1998; Lipp et al., 1997; McConnell, 1991; Möhwald, 1990, 1993; Stine, 1994; Weis, 1991). As the surface area for each molecule was reduced, the onset of the LE/LC phase transition occurred at 20 mN/for DPPC, and at 6 mN/m for DPPG, each lipid forming condensed domains in a LE background.

Injection results

For injection experiments at a constant surface pressure, DPPG monolayers were compressed until they reached 30 mN/m and held at this surface pressure while P188 was administered. No immediate change in the area per molecule or morphology was observed at this pressure for a period of 10 min (Fig. 3). Subsequently, the surface pressure was lowered to 28 mN/m, but still no observable change was detected. A pressure step-down procedure was then adopted until a low level of P188 insertion was observed at 22 mN/m. Because this change in the effective area per lipid molecule was only ~3 Å^2/molecule for DPPG after 10 min, the surface pressure was lowered again to 20 mN/m. Rapid insertion of P188 into the DPPG monolayer was detected at this pressure with an overall change in an area per molecule of 74 Å^2/molecule, or until the barriers were expanded to their original position in ~30 min (see expansion in Fig. 3). It should be noted that P188 insertion could have occurred at a faster rate and could have continued if not for the physical limitations of the apparatus.

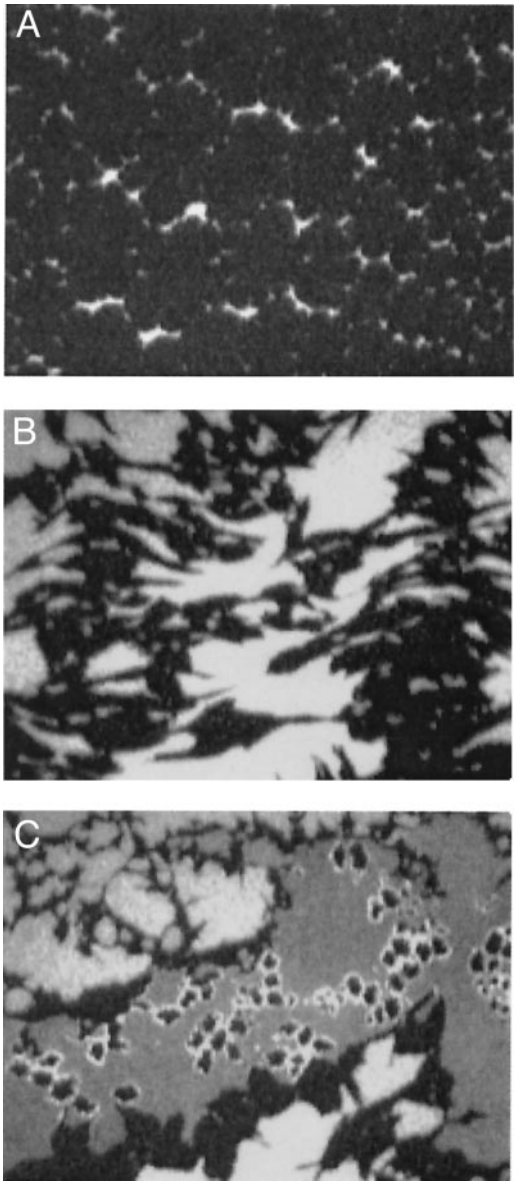

FIGURE 4 Fluorescence images during the P188 injection experiment into a DPPG monolayer at 30°C on a water subphase. (*A*) DPPG monolayer before P188 injection at 30 mN/m, which shows that the area covered by the condensed phase (*dark*) is substantially more than that of the fluid phase (*bright*). (*B*) DPPG monolayer after P188 insertion at a surface pressure of 20 mN/m and surface area of 78 Å^2/molecule. There is a decrease in the amount of condensed phase and a corresponding increase in the amount of fluid phase. P188 incorporation into the monolayer causes the condensed phase domains to become elongated, creating a network structure. (*C*) DPPG monolayer after P188 insertion (at a later time than in *B*) at a surface pressure of 21 mN/m and a surface area of 120 Å^2/molecule. There is a significant decrease in the percentage of condensed phase domains, and the formation of a phase of intermediate brightness (*gray*).

Fig. 4, *A–C*, shows the morphology of monolayers of DPPG on a water subphase at 30°C before and after P188

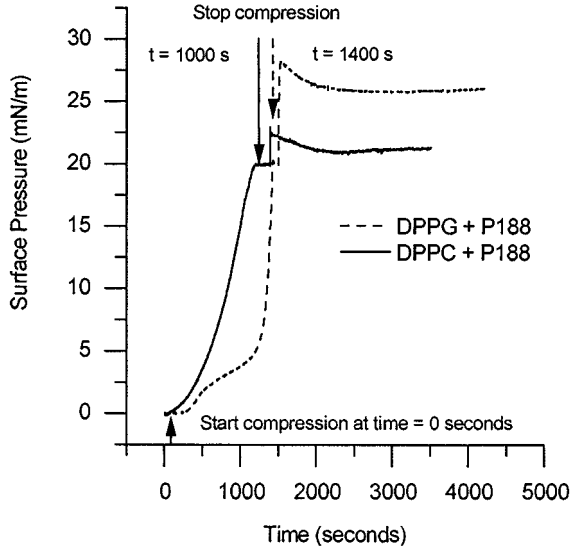

FIGURE 5 Constant area measurements of DPPC and DPPG monolayers after P188 injection at 20 mN/m at 30°C on a water subphase. The surface pressure increases maintained were 2 mN/m for DPPC and 6 mN/m for DPPG.

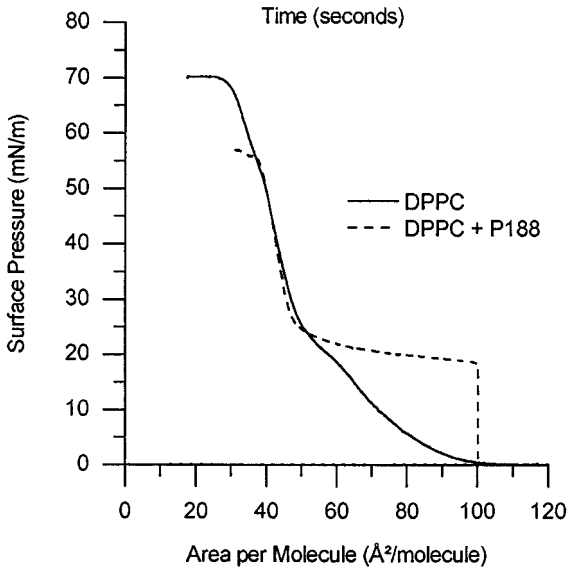

FIGURE 6 Lateral compression isotherms of DPPC and P188 pretreated DPPC on a water subphase at 30°C. At surface pressures of 25 mN/m and greater, the isotherm of the P188-treated systems overlaps that of the pure lipid, indicating that P188 is "squeezed out" of the film at higher surface pressures equal to 25 mN/m or greater.

injection. Before injection, the condensed flower-shaped domains of DPPG occupy a much higher area fraction than the LE phase at 30 mN/m (Fig. 4 *A*). Upon the insertion of P188 at 20 mN/m, the condensed domains become elongated, forming a more network-like structure with various size domains linked (Fig. 4 *B*). Additionally, there is a drastic increase in the percentage of LE or disordered phase, indicating the disordering of lipid molecules by the incorporation of P188. An additional phase of intermediate brightness is also observable (Fig. 4 *C*).

A similar constant surface pressure experiment was performed with DPPC, and isotherm data and FM images were collected. As with DPPG, no observable change in the area per molecule at 30 mN/m was noted after P188 administration. Although there was a small increase in area at 24 mN/m, substantial insertion of P188 was not observed until the surface pressure was lowered to 22 mN/m, at which point a rapid expansion in the area per molecule from 54 $Å^2$/molecule to a full expansion of the barriers to 120 $Å^2$/molecule (Fig. 3). Morphologically, the insertion of P188 at 22 mN/m caused the condensed domains of DPPC to disappear, thereby creating a homogeneous LE phase (data not shown).

For injection experiments at constant area, each pure monolayer was compressed to 20 mN/m over a period of roughly 1000 s, and the barriers are left stationary to keep the area constant. P188 was injected into the subphase at this constant area, and subsequent insertion of the polymer into the lipid film resulted in an increase in the surface pressure. In both cases, there were dramatic surface pressure increases immediately upon the administration of P188 (see sharp jumps in surface pressures in Fig. 5). The DPPC and DPPG films showed an immediate increase in surface pressure by 3 and 8 mN/m, respectively. Both systems were left to equilibrate for ~1 h after injection and were able to maintain the increased surface pressures of 22 mN/m for DPPC and 26 mN/m for DPPG.

Pretreatment results

Pretreatment experiments were performed to test the extent of involvement of the poloxamer in monolayers at high surface pressures of 30 mN/m and beyond. In these experiments, the lipid was spread at a high area per molecule ($\pi \approx 0$ mN/m), and P188 was added to the system to allow for maximal insertion. For both lipid monolayers, the addition of P188 to the subphase resulted in the partitioning of P188 to the air-water interface, which gave rise to an instantaneous increase in surface pressures from 0 to ~20 mN/m. The heterogeneous lipid-poloxamer system was then compressed fully. Figs. 6 and 7 show that as the lipid systems were compressed to high surface pressures, the isotherms of the poloxamer-pretreated monolayers reverted to those of the pure lipids, suggesting that P188 had been eliminated from the system. These observations suggest that P188 activity is localized, capable of incorporating itself into the monolayers only when the film pressure is below 25 mN/m for DPPC and 28 mN/m for DPPG. At pressures greater than 25 mN/m for DPPC and 28 mN/m for DPPG, P188 is "squeezed out," or eliminated from the film, and its involvement is no longer detectable. It should be pointed out

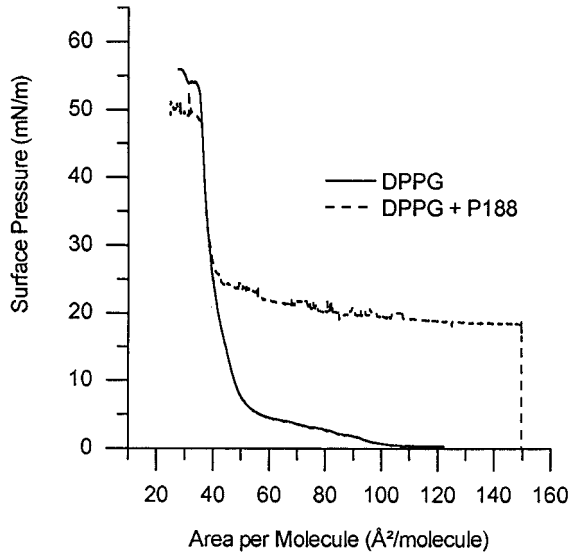

FIGURE 7 Lateral compression isotherms of DPPG and P188 pretreated DPPG on a water subphase at 30°C. At surface pressures of 28 mN/m and greater, the isotherm of the P188-treated systems overlaps that isotherm of the pure lipid, indicating that P188 is "squeezed out" of the film at higher surface pressures equal to 28 mN/m or greater.

that the pressure at which the polymer is squeezed out of the film is slightly higher that than for polymer insertion. No FM images were collected for these experiments because the addition of P188 at such a high area per molecule does not result in any discernable surface morphology.

CONCLUSION AND DISCUSSION

Our results demonstrate that P188 is a highly surface-active copolymer, attaining a maximal surface pressure of 22 mN/m at a concentration of 50 μM at 30°C in a water subphase. This high surface activity probably aids in its absorption and facilitates its insertion into lipid monolayers.

Injection experiments show that P188 does not insert into lipid monolayers at high surfaces pressures, but does insert into lipid monolayers of DPPC and DPPG at 22 mN/m, with the ease of insertion increasing drastically when the surface pressure is decreased to 20 mN/m. Therefore, it can be concluded that P188 will only adsorb onto damaged portions of electroporated cells, where the local lipid packing density is reduced, thereby localizing its effect. This observation suggests that P188 would only interact with compromised bilayers, and would not nonspecifically insert into membranes that were not affected. Moreover, similar injection results for DPPC and DPPG monolayers suggest that P188 insertion is not influenced by electrostatics because similar interaction is observed with a lipid headgroup of a different charge. Morphologically, our results show that the insertion of P188 results in the increase of the LE phase. The presence of the polymer thus disorders the packing of the film.

Results from the constant area experiments show that in a confined area, at surface pressures of 20 mN/m, P188 readily inserts into DPPC and DPPG monolayers, increasing the surface pressure of the systems from 20 mN/m to 22 mN/m for DPPC and from 20 mN/m to 26 mN/m for DPPG. These experimental results therefore indicate that P188 can effectively insert into the injured region where the local lipid packing density is reduced and help increase the local packing density.

The inserted P188, however, cannot maintain its position within the monolayer at surface pressures of 25 mN/m or higher for DPPC and 28 mN/m or higher for DPPG. At higher surface pressures, P188 is "squeezed out" of the film, leaving pure DPPC or DPPG in the pretreatment experiments. The incapability of P188 to sustain its involvement in the system at high surface pressures can be beneficial in terms of its application. After electroporation, cells may activate a self-healing process, restoring the structural integrity of the bilayer. Consequently, as the cell heals and the lipid packing of the membrane is regained, P188 can be easily removed from the cell membrane.

These results support our hypothesis that the poloxamer interacts with structurally disrupted monolayers because it is only incorporated into the lipid film where the local lipid packing density is reduced, and is effectively excluded from the film when the lipid packing density of a normal membrane is re-established. Results also indicate that the mechanism of P188 insertion is most likely dependent upon the poloxamer's interaction with the hydrophobic portions of the lipid monolayers, thereby suggesting that the hydrophobic subunit of the poloxamer is inserted into the acyl chain portion of the lipid film. Our results point to the amphipathic nature of P188 as the physical driving force for the polymer to seek out the membrane surface and the hydrophobicity of the mid-section of the polymer for the subsequent insertion. If this is indeed the case, the surface pressure at which the polymer inserts into the lipid monolayer, as well as that for its elimination from the surface film should depend on the hydrophilic:hydrophobic ratio of the triblock copolymer. Thus, future experiments aim to extend studies to poloxamer family members with the same hydrophilic chain lengths as P188, but different hydrophobic chain lengths and vice versa. In addition, poloxamers of greater molecular weight but with the same hydrophobic: hydrophilic chain ratios will be investigated, along with structurally similar diblock copolymers. Results from these studies will provide information on the influence of molecular weight, varying chain lengths and ratios, and the effectiveness of diblock versus triblock structures on the polymer's ability to seal damaged membranes.

S.A.M. and K.Y.C.L. are grateful for the support from the March of Dimes Basil O'Connor Starter Scholar Research Award (5-FY98-0728), the Pack-

ard Foundation (99-1465), and the Camille and Henry Dreyfus New Faculty Award (NF-98-048). The experimental apparatus was made possible by a NSF CRIF/Junior Faculty grant (CHE-9816513). S.A.M. would like to thank Ajaykumar Gopal and Canay Ege for valuable input and experimental help and Joseph Kurutz for help with the manuscript. J.H. and R.C.L. acknowledge support from the Electrical Power Research Institute (EPRI-2914-03) and the National Institutes of Health (R01-GM53113 and R01-GM61101). The work is also made possible by a University of Chicago Interdepartmental Collaboration Starter grant.

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