Temperature Dependence of Poloxamer Insertion Into and Squeeze-Out from Lipid Monolayers

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F68, a triblock copolymer of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), is found to effectively seal damaged cell membranes. To better understand the molecular interaction between F68 and cells, we have modeled the outer leaflet of a cell membrane with a dipalmitoylphosphatidylcholine (DPPC) monolayer spread at the air−water interface and introduced poloxamer into the subphase. Subsequent interactions of the polymer with the monolayer either upon expansion or compression were monitored using concurrent Langmuir isotherm and fluorescence microscopy measurements. To alter the activity of the poloxamer, a range of subphase temperatures from 5 to 37 °C was used. Lower temperatures increase the solubility of the poloxamer in the subphase and therefore lessen the amount of material at the interface, resulting in a lower equilibrium spreading pressure. Additionally, changes in temperature affect the phase behavior of DPPC. Below the triple point, the monolayer is condensed at pertinent polymer insertion pressures; for temperatures immediately above the triple point, the monolayer is a heterogeneous mix of liquid expanded and condensed phase; for the highest temperature measured, the DPPC monolayer remains completely fluid. At all temperatures, F68 inserts into DPPC monolayers at its equilibrium spreading pressure. Upon compression of the monolayer, polymers are squeezed-out at surface pressures notably higher than those for insertion, with higher temperatures leading to a higher squeeze-out pressure. An increase in temperature decreases the solvent quality of water for the poloxamer, lowering solubility of the polymer in the subphase and thus increasing its propensity to be maintained within the monolayer to higher pressures.

Introduction

Triblock copolymers of the form poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO)-poly(ethylene oxide) (PEO) are a class of surface-active, amphiphilic molecules.[1] These commercially available noncytotoxic nonionic agents are commonly referred to as pluronics or poloxamers. Poloxamers have an extensive range of applications in the medical field such as drug solubilization, controlled release, and protection of microorganisms against mechanical damage.[2−4] Of particular interest is F68, an approximately 8.4 kDa poloxamer of the form PEO_{76}-PPO_{29}-PEO_{76} that has been proven as a surfactant sealing agent for permeabilized lipid bilayers. Increased cell permeability accounts for the majority of tissue damage in common clinical conditions after physicochemical insults such as electrical shock, significant radiation damage, thermal burns, and frostbite.[5−8] The efficacy of F68 as a cell membrane sealant was first shown when the polymer reduced leakage of carboxyfluorescein dye from loaded cells after electroporation.[9] Further studies have shown that F68 also effectively seals damaged membranes of fibroblasts after heat shock and skeletal muscle cells exposed to high-dose radiation, arresting the leakage of intracellular components.[10,11] In more recent studies, the use of F68 restored the ability of heart cells to stretch without damage in patients with Duchenne muscular dystrophy, a progressive disease of striated muscle deterioration.[12]

Although these studies indicate the effectiveness of poloxamer as a pharmaceutical agent, little is known about the molecular mechanism that mediates polymers' interaction with, and subsequent expulsion from, cells. To take a more controlled approach in the development of poloxamers as healing agents, one must understand fundamental interactions between the lipids of the cell membrane bilayer and the poloxamer molecules. While a lipid monolayer approximates only the outer leaflet of a cell membrane, this model system provides valuable insight toward the interaction between poloxamer and lipid bilayers.[13−21] Monolayer compression experiments using lipid and lipid-like molecules with a variety of poloxamers showed that polymer molecular weight and hydrophilic/hydrophobic ratio affect

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squeeze-out behavior with the more hydrophobic molecules penetrating further into the lipid layer and being maintained within the layer to a higher pressure.[17,13] Using small-angle X-ray scattering and calorimetric methods, Ishøy et al. demonstrated that P85 (PEO_{27}-PPO_{39}-PPO_{27}) inserted into the hydrophobic region of DMPC bilayers, inducing a lamellar-to-cubic phase change.[21] Homopolymers of PEO mixed with DMPC bilayers did not give analogous structure changes,[21] implicating the amphipathic nature of poloxamers as a driving force for insertion into lipid bilayers.

One of the characteristic physical properties of PEO-PPO-PEO block copolymers is their inverse temperature dependence in solubility and micellization. Contrary to most common solute−solvent interactions, an increase in temperature decreases the solubility of poloxamers.[22−25] Although a molecular level understanding of how temperature activates these solubility changes is available,[26,27] little is known about how the poloxamer structure and function induced by temperature change affect its interaction with biological membranes.

The focus of this work is to determine how the interaction of poloxamer with a lipid monolayer varies with temperature. Not only does changing the subphase temperature affect the phase behavior of the lipid membrane, but it also alters the solubility of the poloxamer and hence its partitioning to the membrane surface. We have tested how changes in this partitioning of poloxamer affect its insertion into a low-density lipid monolayer, and upon subsequent compression, its squeeze-out from the monolayer. Using a model lipid system, we have carried out isotherm measurements and fluorescence microscopy imaging to examine the interactions of F68 with zwitterionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) monolayers at an air−water interface at various temperatures ranging from 5 to 37 °C.

Changing the temperature of the system is found to affect the surface activity of the poloxamer and the phase behavior of the DPPC monolayer. The equilibrium spreading pressure (ESP) of F68 scales with temperature, and decreased solubility with increasing temperature results in a higher concentration of polymer at the interface. At the lowest temperatures tested, DPPC is below its triple point, while at the highest temperature tested, the monolayer remains fluid at all pertinent pressures. While F68 inserts into DPPC monolayers at its ESP regardless of the phase state of the monolayer, squeeze-out pressures scale with temperature; the poloxamer can indeed be maintained in the lipid monolayer to higher pressures at higher temperatures.

Materials and Methods

Lipids and Subphase. DPPC was obtained in powder form from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. The fluorescent probe used for visualization with fluorescence microscopy was Texas Red, 1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine (TR-DHPE) (Molecular Probes, Eugene, OR). Monolayer spreading solutions were prepared by dissolving DPPC in chloroform (high-performance liquid chromatography grade, Fisher Scientific, Pittsburgh, PA) at a concentration of 0.2 mg/mL and adding 0.5 mol % of TR-DHPE. Lipid solutions were stored at −20 °C in glass vials. For all Langmuir trough experiments, the subphase was ultrapure water (resistivity ≥18 MΩ·cm) processed by a Milli-Q ultrapurification setup (A-10 gradient, Millipore, Bedford, MA).

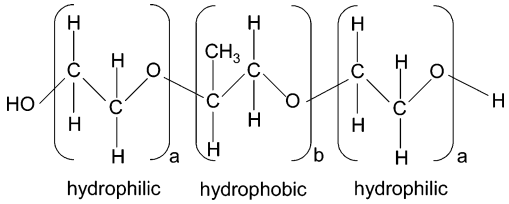

Figure 1. General chemical structure of poloxamers. F68 is of the form $(PEO)_{76}$-$(PPO)_{29}$-$(PEO)_{76}$.

F68 Solution. Solutions of the poloxamer, F68 (Figure 1) (BASF, Parsippany, NJ), with a number average molecular weight of 8370 g/mol, were made by mixing 200 mg of the poloxamer per milliliter of water in a vial with a magnetic stir bar for up to 60 min to ensure complete dissolution. Poloxamer solutions were stored at 4 °C and made fresh weekly.

Equipment. Surface pressure−area isotherms were obtained using a custom-made Teflon Langmuir trough (27.5 cm × 6.25 cm × 0.63 cm) equipped with two identical mobile Teflon barriers (l = 6.25 cm). The dual barrier setup enables symmetric compression or expansion of monolayers spread at the air−water interface, thereby increasing or reducing the surface pressure, respectively. All isotherms were run with a linear compression speed of 0.1 mm/s. A Wilhelmy balance (Reigler and Kirstein, Berlin, Germany) was used to measure surface pressure with a sensitivity of ±0.2 mN/m. The maximal working surface area of the trough was 145 cm^2, and the water subphase volume used was 95 mL. Subphase temperature was maintained within 0.5 °C of the desired temperature (range from 5−37 °C) with a home-built control station comprised of thermoelectric units (Marlow Industries, Dallas, TX) joined to a heat sink held at 20 °C by a Neslab RTE-100 water circulator (Portsmouth, NH). The subphase temperature was monitored by a submerged Teflon-coated thermistor (Omega Engineering Inc, Stamford, CT). A piece of indium tin oxide coated glass (Delta Technologies, Dallas, TX), which can be resistively heated, was placed over the trough and held at a temperature to suppress evaporative losses, minimize convective currents, and prevent condensation of water on the microscope objective.

The Langmuir trough was mounted on *x*, *y*, and *z* translation stages (Newport, Irvine, CA) that permit scanning along the air−water interface. The trough assembly was fixed to a custom-built microscope stage to allow simultaneous fluorescence microscopy with a 50X extra-long working distance objective (Nikon Y-FL, Fryer Company, Huntley, IL). A high-pressure mercury lamp was used for fluorescence excitation with excitation wavelengths between 530 and 590 nm and emission wavelengths between 610 and 690 nm, corresponding to those for TR-DHPE. The emitted light was gathered with a dichroic mirror/filter cube (Nikon HYQ Texas Red, Fryer Company, Huntley, IL). Images from the fluorescence microscope were collected at a rate of 30 frames/s using a charge-coupled device camera (Stanford Photonics Inc., Palo Alto, CA) and recorded on a Sony digital video cassette with a recorder (Sony, Tokyo, Japan). This assembly permits monolayer morphology to be observed over a large lateral area while isotherm data are obtained. The Langmuir trough and fluorescence microscope apparatus are set on a vibration isolation table (Newport, Irvine, CA) and controlled using a custom software interface designed using LabView 6.1 (National Instruments, Dallas, TX).

Critical Micelle Concentration Experiments. Using a method previously described, the critical micelle concentration (cmc) of F68 was determined by measuring its surface activity at the air−water interface as a function of concentration.[15] Aliquots of 200 mg/mL of poloxamer solution were added incrementally to the water subphase and allowed 30 min of equilibration time, and the surface pressure was then measured. The poloxamer concentration above which there is no further increase in the surface pressure indicates that an equilibrium was established between the poloxamer population adsorbed to the air−water interface and the monomer population in

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the subphase; above this concentration, the addition of poloxamer would result in the formation of micelles in the subphase. Such a concentration is therefore referred to as the cmc.

Lateral Compression Experiments. All experiments were performed on pure water at temperatures of 5, 10, 20, 30, or 37 °C. In pure lipid compression experiments, the lipid monolayer was spread by dropwise addition of the spreading solution on the water surface, and the organic solvent was allowed to evaporate for 15 min. The barriers were then compressed, and isotherm measurements in the form of surface pressure (mN/m) versus area per lipid molecule (Å^2/molecule) were taken at 1 s intervals until the system reached its compression limit. The isotherm provides information about the phases of the monolayer as a function of lipid packing density. Two different types of lateral compression experiments were performed. The sudden increase in membrane permeability due to injury has been linked to a reduction in lipid packing density and/or the formation of pores within the membrane and subsequent leakage of cellular contents.[9] The first condition is modeled with insertion stepdown experiments where the poloxamer was added to the subphase of a lipid monolayer at the bilayer equivalent pressure and the pressure was systematically lowered, decreasing lipid density at the interface until insertion occured. The latter condition was tested with pretreatment experiments, where the poloxamer was added to the subphase of a low-density lipid monolayer ($\pi = 0$) in its liquid expanded/gas coexistence.

Step-Down Experiments. Step-down experiments were carried out to identify under what lipid density conditions F68 would insert into the lipid monolayer. The lipid monolayer was compressed to a desired surface pressure of 30 mN/m to mimic the lipid packing density of a normal bilayer. When this surface pressure was attained, the barriers were switched from a compression mode to a feedback mode that maintained constant pressure by adjusting the surface area of the monolayer. F68 was then injected into the subphase underneath the monolayer. If there were no changes in the area (signifying no insertion) at a given pressure after 10 min, the surface pressure was lowered by 2 mN/m. This procedure was repeated until insertion was noted. At this point, the barriers usually expanded to the fully open position during the insertion process.

Pretreatment Experiments. In pretreatment experiments, the monolayer was first spread at a large area per molecule, and the polymer was subsequently introduced into the subphase prior to film compression to observe at what pressure F68 would be expelled from the monolayer as the tighter packing density of the membrane was restored. As in previous experiments, DPPC was spread at the air−water interface at a low surface density ($\pi \sim 0$ mN/m) and allowed to equilibrate for 15 min while the solvent evaporated. Poloxamer was then injected into the subphase and left to equilibrate for 5 min. The film was then compressed to collapse, and the observed isotherm of the system was compared to that obtained in the absence of any polymer.

Fluorescence Microscopy. During the course of all compression experiments, fluorescence microscopy images of the surface morphology were recorded on digital videotape. Because of steric reasons, the fluorescent molecule, TR-DHPE, partitions into the fluid region, rendering it bright and the condensed phase dark, thus allowing phase information to be extracted.[28]

Results and Discussion

Critical Micelle Concentration. At low temperatures and/or polymer concentrations, tri-block copolymers exist in solution as unimers. Thermodynamically stable micelles are formed with increasing copolymer concentration and/or temperature, as revealed by our surface tension measurements and the reported dye solubilization experiments by Alexandridis et al.[23] The micellization process of poloxamers is endothermic and driven by a decrease in the polarity of ethylene oxide (EO) and propylene oxide (PO) segments as the temperature increases from 5 to 37 °C, though it is likely that changes in polarity of the former contribute more to the effect.[29,30] This process is further driven by the entropic gain in water when unimers aggregate due to the hydrophobic effect.[30] Therefore, the higher the temperature, the lower the critical micelle concentration for a specific poloxamer.[30]

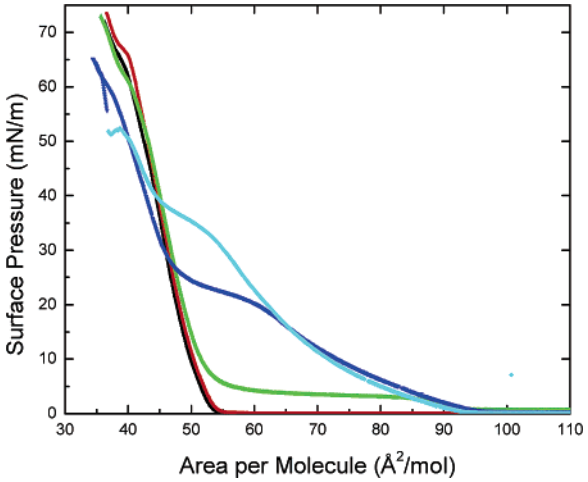

Figure 2. Isotherms of a DPPC monolayer at temperatures ranging from 5 to 37 °C: 5 °C (black), 10 °C (red), 20 °C (green), 30 °C (blue), and 37 °C (aqua). Below the triple point, at 5 and 10 °C, the monolayer proceeds directly from a gas (G)/condensed (C) coexistence to the C phase at lift-off. At the higher temperatures of 20, 30, and 37 °C, all above the triple point of the DPPC monolayer, the first-order phase transition is indicated by the plateau that correlates to a liquid expanded (LE)/C coexistence region.

To determine the cmc for the poloxamer, F68, experiments were performed as previously described;[15] the cmc of F68 at 30 °C is 1.25×10^{-4} M. It is of importance to note that literature cmc values for F68 encompass 3 orders of magnitude, ranging from 440 μM to >10 mM at room temperature. These values were obtained from a variety of techniques, including surface tension, dye solubilization, and rheometer viscosity measurements.[31−34] For each poloxamer pretreatment experiment, 200 μL of the 200 mg/mL polymer solution was added to the subphase to reach a bulk concentration of 50 μM. The exact value of the cmc is not necessary in the context of this work, as the range of reported values is well above the bulk concentration of F68 beneath the monolayer used in all of our experiments.

Previous studies have correlated the critical micellization temperature (cmt) as a function of copolymer concentration for poloxamer aqueous solutions. The cmt is defined as the copolymer solution temperature at which micelles form at a given concentration. At a bulk concentration of 0.595 mM, the cmt of F68 was reported as 52.5 °C.[23] The concentration of poloxamer (50 μM) used for each of the isotherm experiments was therefore well below the cmc at all temperatures examined. This ensured that poloxamer monomers added were not lost in the subphase in the form of micelles.

DPPC Isotherms. Surface pressure versus molecular area isotherms were measured for DPPC while concurrently imaging with fluorescence microscopy at 5, 10, 20, 30, and 37 °C. Figure 2 shows the overlay of the DPPC isotherms measured at the various temperatures. The isotherms are in agreement with published data,[35,36] and the phase transitions have been discussed extensively elsewhere.[37,38] The triple point of the system is

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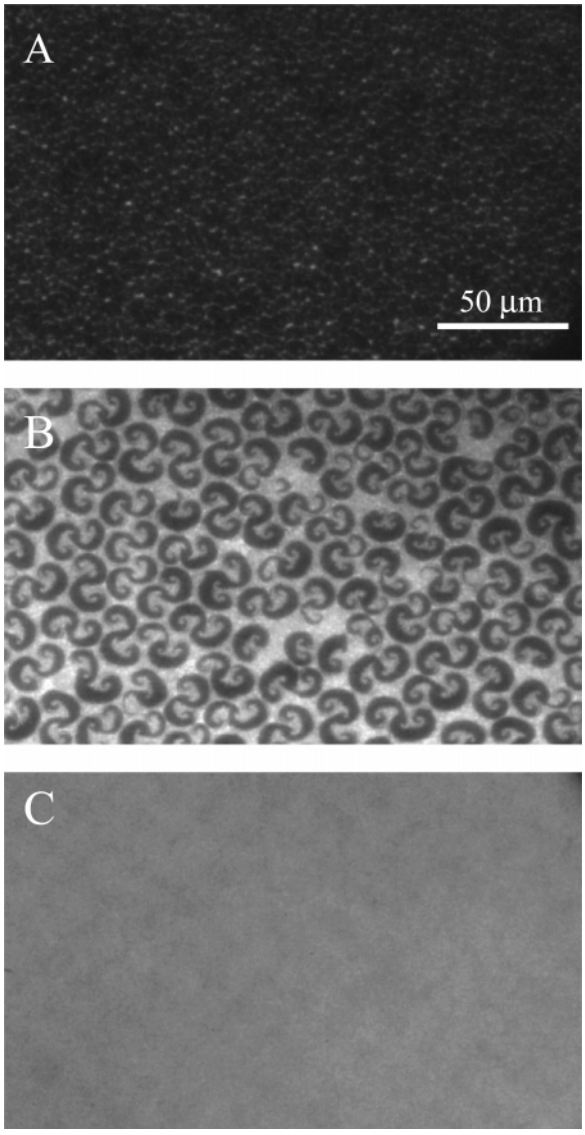

Figure 3. Fluorescence images of DPPC monolayer at 5 mN/m at different temperatures. (A) DPPC at 10 °C and a nominal area of $A_{DPPC} = 55.3$ Å^2; film entirely in C phase. (B) DPPC at 20 °C and a nominal area of $A_{DPPC} = 59.2$ Å^2; film in LE/C coexistence. (C) DPPC at 30 °C and a nominal area of $A_{DPPC} = 86.3$ Å^2; film in LE phase. The width of each micrograph is 220 μm.

defined as the temperature and pressure at which condensed (C), liquid expanded (LE), and gas (G) phases of the lipid monolayer coexist in equilibrium; below the triple point, the film goes from G to C without passing through any LE phase. At 5 and 10 °C (temperatures below the triple point), the area per molecule at lift-off is 55 Å^2/mol, and no plateau region is observed after lift-off in the isotherm. At these low temperatures, the film is in the G/C coexistence prior to lift-off and becomes a homogeneous C phase upon lift-off. At 20 °C, the system is in the vicinity of the triple point, and the C phase is found to nucleate in the presence of both the G and the LE phases. The plateau for DPPC at 20 °C occurs at the low pressure of ~3.5 mN/m, covering a wide molecular area, ranging from 80 down to 55 Å^2. All isotherms taken at temperatures above 20 °C lift-off at a molecular area of 95 Å^2, with the emergence of a uniform LE phase from the G/LE coexistence. The plateau occurs at higher surface pressures over a smaller molecular area range with increasing temperatures. It should be noted that the plateau of the 37 °C monolayer is less discernible (and at higher pressures than that at 30 °C) as it is approaching the critical temperature of ~41 °C.[39,40]

Table 1. Measured Properties of F68 at Various Temperatures: Equilibrium Spreading Pressure, Squeeze-Out Pressure from a DPPC Monolayer, and Area per Molecule at Squeeze-Out

T (°C)	ESP[a]	squeeze-out Π[b]	ΔΠ[c]	area[d] (at squeeze-out)
5	14.5	24	9.5	48
10	15.5	25	9.5	48
20	20	26	6	48
30	23	29.5	6.5	47
37	24	32	8	54

[a] ESP: equilibrium spreading pressure [b] Π is measured in mN/m. [c] ΔΠ: difference between ESP and squeeze-out pressure for each temperature. [d] Area: area per molecule (Å^2/molecule).

The FM images of the monolayer morphology show the phase state of the monolayer varies with temperature corresponding to the previous discussion. When the DPPC monolayer is at a large nominal area per molecule ($A_{DPPC} = 124$ Å^2), it is in a G/LE phase coexistence regime (or G/C in the case of DPPC at 5 and 10 °C) with the gas phase appearing dark and the LE phase bright as seen by FM. Below the triple point, the film is condensed at lift-off (Figure 3A) with subsequent dye quenching. At temperatures above the triple point, the plateau region in the isotherm correlates with the appearance of C domains that grow in size. The spiral shape of the domains has been attributed to the chirality of the DPPC molecules.[37,41,42] Simulations taking into account the chirality of the DPPC molecule as well as its large headgroup in relation to the cross-section of its aliphatic chains that leads to a spontaneous curvature of the domain within the plane of the monolayer have been used to model the spiral shape.[43] Upon further compression, the interstitial region between domain morphology becomes gray as seen with FM. This intermediate gray phase is due to a roughening at the boundary between LE and C phases, arising from an edge instability caused by differing elastic properties of the two phases.[44] FM images for a film held at a constant pressure of 5 mN/m but at 10, 20, and 30 °C show a film with a single C phase, in a LE/C coexistence, and in a fluid LE phase, respectively (Figure 3).

Equilibrium Spreading Pressure. When injected into the aqueous subphase of the Langmuir trough, amphiphilic poloxamer molecules spontaneously adsorb to the air−water interface until a certain surface pressure, the equilibrium spreading pressure, is attained. The addition of F68 to the subphase gives rise to an instantaneous increase in surface pressure from 0 to ~20 mN/m. Because the solubility of poloxamer molecules is temperature dependent, the equilibrium spreading pressure also varies with temperature. F68 follows this general trend, with equilibrium spreading pressures ranging from 14.5 to 24 mN/m in the temperature range of 5−37 °C (Table 1).

Unlike common solvent/solute interactions, polymers of the form $(PEO)_x$-$(PPO)_y$-$(PEO)_x$ are generally more soluble at lower temperatures. This can be understood in terms of an entropic

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argument, where polymer molecules with a larger radius of gyration take up much more space at higher temperatures due to a higher degree of thermal fluctuations. Therefore, at elevated temperatures, less poloxamer is soluble in the subphase and more is present at the air−water interface, raising the equilibrium spreading pressure. It has been demonstrated both experimentally and theoretically that at higher temperatures, the solubility of the poly(ethylene oxide) portion of the chain is dramatically reduced. At ambient temperatures, the structure of fluid water and the stabilizing hydrogen bond network mesh well with the polymer chains, improving polymer solubility.[45] As the temperature is increased, the water molecules are less structured due to thermal energy, breaking the hydrogen bonds, and rendering the chains less soluble. Through a quantum chemical analysis of the O−C−C−O bond structure of the poloxamer, Karlström and Lindman have suggested that differences in solubility with temperature are based on the polarity of the PEO chain groups.[27,45,46] Due to rotations around the C−C bond, the PEO block has a number of different possible conformations. Although the majority of these structures are nonpolar trans conformers with no dipole component, there are two gauche positions that are the lowest in energy and strongly polar, therefore interacting more favorably with water. At higher temperatures, the numerous nonpolar conformers are favored, and interaction with water is less energetically favorable, rendering the polymer less soluble. This theory was supported by FT Raman experiments focusing on C−H stretching modes as indicators of structure within the poloxamers as a function of temperature.[26] In aqueous solutions, the PEO block was determined to be an open coil surrounded by a zone of more structured water. Upon heating, structural features from Raman experiments revealed conformational changes of the PEO block to more disordered (due to more nonpolar trans conformers) structures that weaken the solute−solvent interactions as well as PEO's ability to screen and stabilize hydrophobic PPO blocks from water. Additionally, at low temperatures, the hydrophobic PPO subunits are disordered, and an increase in temperature increases the number of stretched trans conformers, so the PPO block progressively loses its hydration sphere and increases its hydrophobicity, driving it to the air−water interface.

Insertion Pressure. For step-down injection at a constant surface pressure, a DPPC monolayer at 10 °C was compressed to 30 mN/m and held at this surface pressure while F68 was injected into the subphase beneath the monolayer. No immediate change in area per molecule or domain morphology was observed at this elevated pressure for a period of 10 min. The pressure was lowered by 2 mN/m increments until a small amount of F68 insertion was observed at 16 mN/m (Figure 4). Since the change in area per molecule was only 2 Å^2/mol in 10 min, the pressure was further lowered to 14 mN/m where F68 rapidly inserted into the monolayer until the barriers were at their fully expanded original positions. As can be seen from the isotherm in Figure 2 and the fluorescence micrograph in Figure 3A, the DPPC monolayer at 10 °C is completely condensed at 14 mN/m, and insertion of F68 did not visibly alter the morphology of the condensed film as seen by FM; there was no appearance of a disordered lipid phase, but the dark polymer phase seen in other systems would not be visible due to lack of contrast with the condensed lipid phase.

When a similar experiment was performed at 30 °C, insertion of F68 and full expansion of the barriers occurred at 22 mN/m, a pressure at which the DPPC film was in the LE/C coexistence.

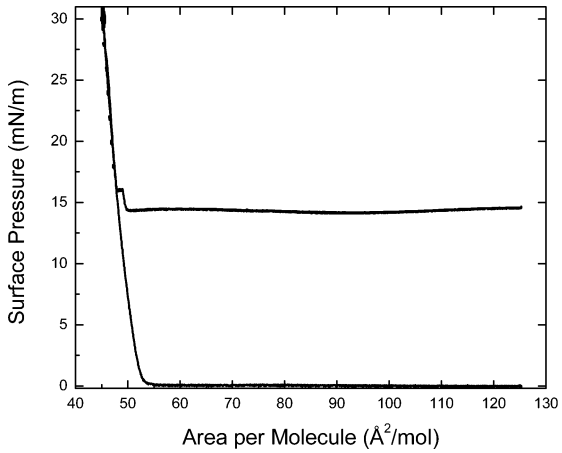

Figure 4. Injection of F68 into the water subphase of a DPPC monolayer and subsequent insertion at 10 °C. Monolayer film was compressed to 30 mN/m after which F68 was injected into the subphase. The change in area per molecule was monitored to check for insertion of the poloxamer into the monolayer. If there were no changes in area per molecule (or no insertion) at the maintained pressure after 10 min, the surface pressure was incrementally lowered by 2 mN/m until a small, but substantial, insertion was noted at 16 mN/m. When the pressure was further lowered to 14 mN/m, the barriers expanded to their fully open positions during the insertion process.

FM showed that insertion of F68 at this pressure decreased the amount of condensed phase, giving rise to a more fluid phase of intermediate brightness.

The equilibrium spreading pressure of the polymer was found to be 14.5 mN/m at 10 °C and 23 mN/m at 30 °C. Using scanning angle reflectometry, Charron et al. saw insertion of polystyrene-PEO diblock copolymers into LE and LE/C phases of DPPC lipid monolayers but not the condensed regime.[47] This led to the conclusion that polymer insertion only occurs in fluid regions. Conversely, our results for the 10 °C case show insertion of F68 at its equilibrium spreading pressure into a condensed monolayer, in contradiction to Charron's fluidity assumption. Thus, our results indicate that the poloxamer inserts into the lipid film at a pressure approximately equivalent to the equilibrium spreading pressure, regardless of how the phase behavior of the DPPC monolayer is altered by the temperature change.

Squeeze-Out Pressure. Pretreatment experiments are a measure of how well the poloxamer is maintained within the monolayer upon compression to high surface pressures. For a pretreatment experiment, DPPC was spread at a high area per molecule ($\pi = 0$ mN/m). Without compressing the lipid film, F68 was injected into the subphase to allow for maximal insertion and adsorption at the air−water interface. The presence of the polymer at the interface gave rise to an instantaneous increase in surface pressure to the ESP of the polymer at a given temperature. The lipid−poloxamer system was then compressed to collapse. Figure 5 shows results obtained from the F68-pretreated DPPC monolayer at 5 °C. Beyond a surface pressure of 24 mN/m, the isotherm of the F68-pretreated monolayer reverts to that of a pure DPPC monolayer, suggesting that all F68 has been eliminated from the film.

Similar pretreatment experiments were performed at various temperatures, providing squeeze-out pressures of 25 mN/m for 10 °C, 26 mN/m for 20 °C, 29.5 mN/m at 30 °C, and 32 mN/m at 37 °C (Figure 6 and Table 1). At successively higher temperatures, the squeeze-out pressure of F68 is higher. This is due to the interplay of two effects that change the properties of

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(47) Charron, J. R.; Tilton, R. D. *Langmuir* **1997**, *13*, 5524.

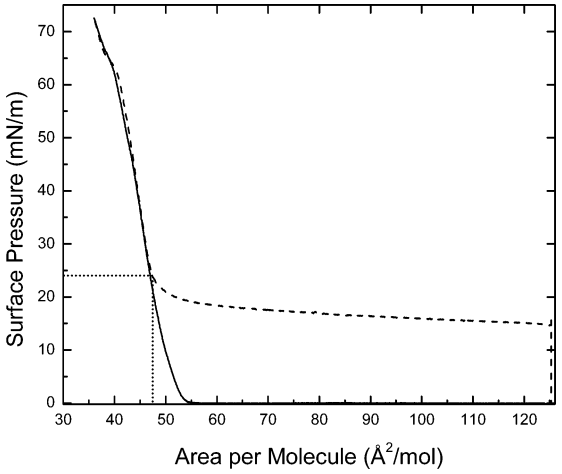

Figure 5. Lateral compression isotherms of DPPC (line) and DPPC pretreated with F68 (dashed) on water at 5 °C. At $\pi \geq 24$ mN/m and an area per molecule of 48 Å, the isotherm of the poloxamer-pretreated system overlaps with that of the pure lipid, indicating squeeze-out of poloxamer from the lipid monolayer. 5 °C is below the triple point of DPPC, so the film is condensed at pressures beyond lift-off. Surface pressure and area per molecule at squeeze-out are marked.

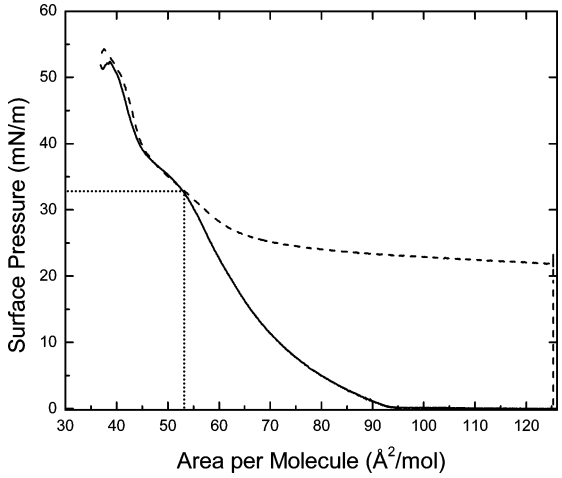

Figure 6. Lateral compression isotherms of DPPC (line) and DPPC pretreated with F68 (dashed) on water at 37 °C. At $\pi \geq 32$ mN/m, the isotherm of the pretreated system overlaps with that of the pure lipid, indicating squeeze-out of poloxamer from the lipid monolayer. This occurs at an area per molecule of 54 Å^2, prior to the phase transition to LE/C coexistence. Surface pressure and area per molecule at squeeze-out are marked.

the lipid−poloxamer system at different temperatures, namely, the phase behavior of the lipid monolayer and the structure and physical properties of the polymer. Using small-angle X-ray scattering, Firestone et al. demonstrated how poloxamer architecture affected its propensity to be incorporated into a lipid bilayer; commensuration of the PPO chain with the dimension of the lipid acyl chain is necessary.[48] In the case where poloxamer insertion into lipid bilayers is observed (for F68 and F88 (PEO_{117}-PPO_{47}-PEO_{117})), all scattering features revert back to those characteristic of a pure lipid bilayer if the temperature is sufficiently reduced. They inferred this observation as the result of a lesser amount of polymer insertion due to enhanced solubility of the poloxamer at lower temperatures. Our results of the poloxamer's decreased ability to be maintained within the monolayer at reduced temperatures supports their assertion.

A change in temperature can alter the solvent condition for one or more blocks in the copolymer. However, the various factors responsible for the solvent quality, such as temperature, pressure, and molecular architecture, are often complex and cannot be treated as independent of one another. As discussed previously, conformational changes and dehydration of both PEO and PPO blocks decrease the solubility of the poloxamer at elevated temperatures. At a lower temperature, on the other hand, the poloxamer is more soluble in the subphase and has a lower stable equilibrium pressure. Thus, our results indicate a change in the solubility of the poloxamer with temperature, which in turn affects the ESP of the polymer. It therefore follows directly that the squeeze-out pressure, the pressure up to which the poloxamer can be maintained within the monolayer, scales with temperature in a similar fashion.

Thus far, the discussion on the squeeze-out pressure has focused on the temperature effects on the copolymer. However, since this is a binary system of poloxamer and DPPC, the effect of temperature on the lipid monolayer should also be examined. As the temperature is increased, so is the fluidity of the monolayer. At a surface pressure of ∼20 mN/m, the DPPC monolayer is completely condensed at 5 and 10 °C but exists as a heterogeneous film above the triple point at 30 °C and becomes increasingly fluid at an even higher temperature of 37 °C. While the phase state of the monolayer at which F68 inserts varies at different temperatures (from the step-down assay), the area per molecule at squeeze-out (from the pretreatment assay), 48 Å^2/mol, is the same for all temperatures except 37 °C when the nominal squeeze-out area per molecule is 54 Å^2/mol. At lower temperatures, the poloxamer squeeze-out occurs from a condensed film (at 5, 10, and 20 °C) or a film in the LE/C coexistence (at 30 °C) and at a pressure higher than the pressure observed for any phase transition plateau. At 37 °C, however, F68 is expelled from a fluid film at a surface pressure lower than that for the first-order LE/C phase transition, thus accounting for the difference in area per molecule upon squeeze-out as DPPC molecules in a fluid state have a larger nominal area than condensed ones.

Under all experimental temperatures examined, the poloxamers are not squeezed-out of the monolayer until reaching a surface pressure much higher than the poloxamer's equilibrium spreading pressure (see Table 1). This indicates that once the poloxamer is incorporated into the lipid monolayer, there is a favorable interaction between the polymer and the lipid that maintains the poloxamer's position at the interface to a much higher pressure. The pressure difference between equilibrium spreading and squeeze-out varies from 6 to 9.5 mN/m with no strong trend linking the lipid phase state differences as was discerned from isotherm and fluorescence microscopy measurements. For the condensed films (5, 10, and 20 °C), the differences are 9.5, 9.5, and 6 mN/m, respectively, while the heterogeneous DPPC film (30 °C) had a pressure difference of 6.5 mN/m. At 37 °C where the monolayer is in the LE phase at squeeze-out, the pressure difference increases to 8 mN/m.

During a pretreatment experiment, F68 molecules at higher temperatures were maintained within the DPPC monolayer to a much higher surface pressure. In these experiments, the temperature differences are shown to have their greatest effect on the structure and solubility of the poloxamers. Elevated temperatures favor dehydrated extended nonpolar conformations of the polymer that are energetically more favored to exist at the air−water interface. The extended conformation of the central PPO blocks may enable the polymer to have more favorable contacts with neighboring hydrophobic lipid tails. This increase in PPO block volume along with thermal disordering of the

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hydrophillic PEO tails may create more steric hindrance for the molecule upon expulsion from the monolayer. These effects combined with a decrease in solubility contribute to elevated squeeze-out pressures at higher temperatures.

Conclusion

We have examined the effects of temperature on the interaction between a DPPC monolayer and poloxamers injected into the subphase beneath the film. Changes in the interaction are caused by the interplay of altering the fluidity of the lipid monolayer with changing the solubility of the poloxamer, hence the driving force to propel the poloxamer to the surface.

The equilibrium spreading pressure of the F68 polymer varies with temperature. The higher the temperature, the higher the ESP, spanning a range of 14.5 mN/m at 5 °C up to 24 mN/m at 37 °C. This can be understood in terms of the decreased solubility of the poloxamer at higher temperatures due to conformational changes and increased thermal fluctuations, coupled with a decreased polarity and therefore hydrophilicity of the PEO block. As the amphiphilic poloxamer must partition between the subphase and the interface, a decrease in solubility results in an increase in materials adsorbed at the interface.

Injection or stepdown experiments show that F68 inserts into the monolayer at its equilibrium spreading pressure. It follows that at 37 °C, F68 would insert into DPPC monolayers at pressures of 24 mN/m or below. Pretreatment or squeeze-out experiments show that the higher the temperature, the higher the squeeze-out pressure of the polymer from the monolayer. This general trend arises from the change in solubility of the polymer with temperature, which creates a different driving force for the amphiphillic molecule to be at the interface. It is of interest to note that at physiological temperatures, the insertion pressure of F68 is lower than the bilayer equivalent pressure, while squeeze-out occurs at pressures correlating to a healthy cell membrane.[49]

Our results indicate that altering the poloxamer's solubility by modulating the temperature can affect its interactions with and insertion into a lipid monolayer. These lipid monolayer studies thus provide insight into the fundamental interactions of F68 with the outer leaflet. As the cell membrane is a bilayer with a heterogeneous mix of lipids and proteins, similar studies with more biologically relevant, multicomponent systems are currently underway.

During electroporation, radiation injury, electrical shock, and thermal burns, there are not only physical disruptions to the integrity of the cell membrane but also a local heating effect. The higher temperature would no doubt increase the fluidity of the cell membranes and decrease the solubility of the poloxamer in surrounding tissues. The question arises as to whether such a local heating effect will result in a higher degree of poloxamer insertion into the membrane as indicated by our monolayer studies. The change in solubility suggests a possible targeting feature in that localized heating could be used to change the fluidity of a specific set of cells so as to promote the insertion of a polymer. Alternately, a specific poloxamer could be designed to have a certain equilibrium spreading/insertion pressures at 37 °C, depending on its role in healing, drug delivery, or permanent incorporation into a cell.

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