Interaction of Poloxamers with Liposomes: An Isothermal Titration Calorimetry Study

Guohui Wu and Ka Yee C. Lee*

Department of Chemistry, Institute for Biophysical Dynamics & James Franck Institute, The University of Chicago, Chicago, Illinois 60637

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The interaction between lipid bilayers and poloxamers has recently attracted much attention, and contradicting effects of poloxamers have been revealed on the integrity of lipid membranes; poloxamers are reported to be either effective sealants or permeabilizers of cell membranes depending on the cell type. To elucidate poloxamer−membrane interactions, we study the roles played by the lipid bilayer phase structure, poloxamer concentration, and temperature using isothermal titration calorimetry (ITC). Our results indicate that the lipid bilayer phase structure plays a critical role in its interaction with poloxamers. Poloxamers are found to partition only into fluid-phase, not gel-phase, membranes. Moreover, the partitioning of poloxamers into fluid-phase liposomes increases with temperature, owing to the enhancement in both the membrane fluidity and the hydrophobicity of the poloxamer at elevated temperatures. Our ITC results also point to a saturation concentration of poloxamers, below which poloxamers can partition into a lipid bilayer without disrupting liposomes and above which they instead disintegrate liposomes into micelles. To address the long-standing question as to whether poloxamers migrate through the lipid bilayer after their adsorption onto preformed liposomes, two ITC protocols are used to cross-validate the distribution of poloxamers over the two leaflets in the bilayer. We find that on a short timescale (∼200 min) poloxamers partition into the outer leaflet of the fluid-phase lipid bilayer without much interactions with the inner leaflet. However, on a longer timescale (38 h), they are found to migrate through the bilayer and eventually establish an even distribution in both the inner and outer leaflets of the membrane.

Introduction

Poloxamers, a family of nonionic triblock copolymers with a structure of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), have gained increasing attention for their capability to repair biological membranes damaged by electrical shock, thermal burns, intense ionizing radiation exposure and sickle cell disease.[1−11] Recent studies have shown that poloxamers have the potential to represent a new therapeutic approach for cardiomyopathy and heart failure in muscular dystrophy where the membrane integrity can be restored.[12] Besides acting as membrane sealants, poloxamers can interact with membranes to inhibit drug efflux so as to serve as potent sensitizers for drug-resistant cancer cells to improve drug transport.[13] The level of interaction between poloxamers and lipid membranes can also be tuned by varying the relative size of the hydrophobic block poly(propylene oxide) (PPO) and the hydrophilic block poly(ethylene oxide) (PEO) segments of the polymer.[13] However, the development of poloxamers into viable therapies has been hampered by the lack of a clear understanding of the underlying membrane-interacting mechanisms. For example, the optimal dosing and long-term effects of poloxamers on humans remain unexplored and are crucial steps in clinical trials, which are affected by the extent of interaction between poloxamers and damaged or intact membranes.

Previous studies have revealed that the lipid packing density in Langmuir monolayers influences the incorporation of poloxamers into membranes by allowing of poloxamer insertion only at low lipid packing densities.[14−17] This suggests that poloxamers preferentially interact with damaged membranes over intact ones. The once-inserted poloxamer can also be eliminated from the membrane when the lipid packing density increases beyond a threshold "squeeze-out" pressure. This selective insertion has been observed in lipid bilayers, which are more relevant models for mimicking cell membranes, and the subsequent membrane morphologies are drastically different as revealed by atomic force microscopy and cryo-electron microscopy.[14,18] However, the incorporation of poloxamer into the lipid membrane has not been addressed in any quantitative fashion. In this article, we present data from isothermal titration calorimetry (ITC) to study interactions between poloxamers and liposomes quantitatively. The insertion of poloxamers into lipid bilayers (or the partitioning of poloxamers from water into the lipid bilayer) is investigated at temperatures above and below the main transition temperature, T_m, of the liposomes. By examining both the ordered gel phase and the disordered fluid phase of the lipid bilayer, we confirm that the lipid packing density indeed regulates the manner in which poloxamers associate with membranes. Furthermore, we are successful in obtaining the distribution of poloxamers across the two leaflets of the lipid membrane after their initial adsorption onto preformed liposomes. Our results are independently obtained by two different methods.

Despite the widely accepted notion that poloxamers can stabilize and seal damaged membranes, the diametrically opposite effect of bilayer permeabilization by poloxamers has also been reported. For the same poloxamer, countering observations have been reported: during the first hour of exposure to 0.1 mg/mL poloxamer 338 (P338), the entrapped fluorescent dye inside egg-PC liposomes diminishes by 30–70%,[20] whereas P338 has no membrane permeabilization effects on cancer cells because intracellular drug retention is

* Corresponding author. Tel: 773-702-7068. Fax: 773-702-0805. E-mail: kayeelee@uchicago.edu.

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actually enhanced in the presence of P338.[13] Evidence of membrane permeabilization by poloxamers has come from several groups. Hui et al. have invented a method for controlled release from large unilamellar vesicles (LUV) based on the incorporation of poloxamers into lipid membranes at a temperature above their critical micelle temperature, which subsequently causes the leakage of LUVs.[19] These seemingly conflicting behaviors of poloxamer−membrane interaction have never been systematically studied. Our results here reveal that both the poloxamer concentration and the phase state of the membrane play decisive roles in determining the poloxamer's optimal performance. Using ITC and poloxamer 338 (P338), whose molecular weight is one of the largest among all those commercially available, as a model poloxamer, we have systematically examined the effects of poloxamer concentration on poloxamer−liposome interactions. Our results clearly show the existence of a saturation limit at which the lipid membrane can accommodate the maximum number of poloxamers but beyond which the liposome is disrupted or disintegrated. Our findings agree well with the practical observation that poloxamers at low concentrations inhibit drug efflux from drug-resistant cancer cells while inducing drug removal from the cells at concentrations above the critical micelle concentration (cmc).[13] In real cell membranes, various components regulate lipid packing or lipid composition to alter the membrane fluidity and membrane microviscosity, which in turn can modulate poloxamer−membrane interactions and can help stabilize the membrane structure. For example, it has been shown that the addition of cholesterol to eggPC decreases the fluidity of the membrane and can also considerably decrease the dye efflux induced by poloxamers (e.g., 0.1 mg/mL P338).[20] Furthermore, the determination of the saturation limit for poloxamers can provide a rational basis for optimizing poloxamer formulation for a variety of existing and novel therapeutic applications.

Materials and Methods

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Ultrapure water (resistivity ≥18MΩ cm) was obtained from a Milli-Q UV Plus system (Millipore Inc., Bedford, MA). A 200 mg/mL P338 (MW = 16 250, 80 wt % PEO) stock solution was prepared by adding P338 (BASF, Parisippany, NJ) and ultrapure water to a vial containing a magnetic stirring bar, which was then left to mix on a stirring plate for 0.5 h to ensure complete dissolution. The P338 solution was diluted further to the desired concentrations.

Liposome Preparation. Liposomes were prepared via the freeze−thaw extrusion method. First, the dry lipid (as a film dried from a chloroform solution under a stream of nitrogen then vacuum) was dispersed in water and vortex mixed for 1 h at a temperature of about 10 °C above the main transition temperature T_m[21−23] to ensure complete hydration. Next, the lipid suspension was put through freeze−thaw cycles by freezing with liquid nitrogen and then heating in a water bath (10 °C higher than T_m) for at least 10 repetitions. The solution was then extruded 17 times through a polycarbonate filter having a pore size of 100 nm with the aid of a lipid miniextruder (Avanti Polar Lipids Inc., Alabaster, AL) to form unilamellar liposomes.[24] During extrusion, the temperature of the lipid solution was always kept higher than T_m. The size distribution of the resulting liposomes was determined by dynamic light scattering (PD2000DLS, Precision Detectors, Franklin, MA) and was typically found to be centered at a diameter of about 140 nm with a standard deviation of 30 nm. The loss of lipid concentration after similar extrusion procedures was reported to be less than 5% by high-performance liquid chromatography (HPLC).[25] Liposomes were stored for at least 4 h at the desired temperature for a given experiment before use.

Two methods were used to incorporate poloxamers into liposomes. In the first method (I), the poloxamers actively participated in the liposome bilayer formation and are expected to distribute evenly across the bilayers. To achieve this, the liposomes were prepared using the hydration and extrusion steps in the presence of poloxamers at the desired concentration. In the second method (A), the poloxamers were added to preformed liposomes and adsorbed onto their surfaces. Here, liposomes were first formed and then mixed and diluted with the poloxamer solution to the desired final concentration at a temperature higher than T_m right before the titration experiments. Hence, we use method I to denote liposomes prepared with poloxamers added initially and method A for liposomes prepared with poloxamers added after the liposomes were already formed. Methods I and A for the formation of mixed lipid/poloxamer liposomes were used in the release and pseudorelease ITC protocols described below, respectively. Liposomes were freshly made every 2 weeks.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was carried out using the VP-type instrument produced by MicroCal Inc. (Northampton, MA). ITC experiments were performed at different temperatures so that liposomes were either in the ordered gel phase or the disordered fluid phase. The cell (volume 1.4045 mL) for the ITC measurement was filled with a poloxamer solution at a concentration below the cmc.[26] A second cell, the reference cell, contained only the buffer (H_2O) and provided a reference point for the actual experiment. The injection syringe was filled with 298.67 μL of a liposome dispersion at a concentration of 15 mg/mL, and a series of injections (typically 10 μL each) were made. As recommended by the manufacturer, a prior 1 μL injection was carried out without taking into account the corresponding observed heat because the first injection was subject to large errors as a result of the diffusion of solutions across the syringe tip during the pretitration equilibration period. At each injection, the injected lipid solution mixed with the polymer solution in the cell; interactions between poloxamers and the lipid membrane could subsequently lead to a characteristic heat signal. Integration of the individual calorimeter traces therefore yielded the heat of binding, h_i, of each injection step.

Three protocols for the ITC experiments were used in this work: (1) uptake, in which liposomes were injected with a syringe into a poloxamer solution in the cell; (2) release, in which a liposome solution of mixed lipid/poloxamer prepared by method I with poloxamer participating in the liposome formation was injected with a syringe into water; and (3) pseudorelease, in which a liposome solution of mixed lipid/poloxamer, prepared by method A with poloxamer adsorbed to preformed liposomes, was injected with a syringe into the cell 15 min after the poloxamers and preformed lipsomes were mixed. The purpose of the simultaneous application of the uptake and release protocols was to obtain thermodynamic parameters for the reaction. These parameters include the partition coefficient, K, and the molar enthalpy of partitioning, ΔH, for the poloxamer partitioning between the aqueous phase and the lipid bilayer. Furthermore, this combination of protocols also allows us to determine the poloxamer's distribution across the membrane by fitting the parameters of the fraction of

materials in the partitioning reactions and to identify whether the poloxamer can permeate through the lipid bilayers to access the liposome interior in the course of the mixing experiment.[27]

Another direct way to check for the transmembrane distribution of poloxamers on the timescale of the ITC experiment can simply be done by comparing the data from the release versus the pseudorelease protocols. Here, the idea is to perform two ITC experiments using mixed lipid/poloxamer liposomes prepared by methods I and A, each with the same concentrations for both components. If the permeable model is appropriate, then the poloxamer should have diffused through and distributed evenly across the lipid bilayers within 15 min after the mixing of poloxamers and preformed liposomes in method A. Consequently, the pseudorelease protocol would have exactly the same starting sample condition as the release protocol where poloxamers participated in the liposome formation via method I, and hence identical ITC data should be generated in these two protocols. However, if the impermeable model is valid, then poloxamers in the pseudorelease protocol would be associated only with the outer leaflet of the lipid bilayer and therefore would be distributed unevenly across the membrane. As a result, the starting sample conditions for the ITC experiments would be different for the release and the pseudorelease protocols, with the former generating less heat than the latter because part of the poloxamer is trapped in the inner leaflet of the bilayer and not available for transfer to the aqueous phase in the cell. In contrast, almost all associated poloxamers are available for partitioning into the aqueous phase in the pseudorelease case.

Partitioning Model. For our data analysis, we applied nonlinear least-squares curve fitting using the model suggested by Heerklotz for the partitioning of surfactants into lipid membranes for both the uptake and release protocols.[27] A different model describing uptake protocol by Hoyrup[25] was also tested, and very similar results were obtained but are not reported here.

Briefly, in Heerklotz's model the partition coefficient K is defined in terms of mole fractions[27,28]

$$K = \frac{P_b W}{(P_b + L)(P_t - P_b)} \tag{1}$$

where $W = 55.5$ M is the molarity of water and L and P are the lipid and poloxamer (effectively playing the role of the detergent) concentrations. Subscripts t and b represent the total concentration of the poloxamer and the concentration of the poloxamer in the bilayers.

The normalized heat q_{obs} resulting from the total amounts of lipid and poloxamer in the cell introduced upon injections is expressed as

$$q_{obs} = \Delta H\left[X_P^{syr}\frac{\partial P_b}{\partial P_t} + (1 - X_P^{syr})\frac{\partial P_b}{\partial L} - \frac{P_b^{syr}}{P_t^{syr} + L^{syr}}\right] + q_{dil} \tag{2}$$

where

$$\frac{\partial P_b}{\partial L} = -\frac{1}{2} + \frac{K(P_t + L) + W}{2\sqrt{K^2(P_t + L)^2 + 2KW(L - P_t) + W^2}}$$

$$\frac{\partial P_b}{\partial P_t} = \frac{1}{2} + \frac{K(P_t + L) - W}{2\sqrt{K^2(P_t + L)^2 + 2KW(L - P_t) + W^2}}$$

ΔH is the molar enthalpy of partitioning, which is the molar heat resulting from the transfer of the poloxamer from water to the bilayer: $\Delta H = h_P^b - h_P^w$, X_P^{syr} denotes the total poloxamer mole fraction in the syringe. The term q_{dil} is the molar heat of dilution of the injectant, measured separately by a control experiment.

In Heerklotz's model, the transmembrane distribution of poloxamer is accessed using a fitting procedure. To consider the possibility that not all of the molecules are able to redistribute across the bilayers on the ITC experimental timescale, the total lipid and poloxamer concentrations are replaced by effective concentrations that do not include molecules trapped inside the liposomes,

$$P_t \rightarrow \gamma_P P_t \tag{3}$$

$$L \rightarrow \gamma_L L \tag{4}$$

where γ_P and γ_L are the fractions of poloxamers and lipids involved in the partitioning, respectively. γ_P and γ_L have the same value of 1 in the permeable model but different values in the impermeable model. In the impermeable model, when the poloxamer cannot cross the lipid bilayer, injecting liposomes into a poloxamer solution (uptake protocol) results in a scenario where all of the poloxamers are free to partition ($\gamma_P = 1$) but only lipids in the outer leaflet of liposomes are accessible ($\gamma_L = 0.5$). For a release experiment, however, both the lipid and the poloxamer species may be partially trapped inside the inner leaflet of the bilayer ($\gamma_P = 0.5$ and $\gamma_L = 0.5$ if the poloxamer distributes evenly in the first place). By substituting eqs 3 and 4 into the fitting models and simultaneously fitting both the uptake and release data, γ_P and γ_L can be obtained, thus allowing one to discern whether the membranes are permeable to the poloxamer on the timescale of the titration experiment.

Results

Heat of Dilution. All ITC experiments involve the addition of liposomes (made of either pure lipid or mixed lipid/poloxamer) to a pure water or a poloxamer solution bath. Such mixing can result in heat generated from three possible sources: the dilution of liposomes, the dilution of poloxamers, and the partitioning of poloxamer from water into lipid bilayers. It is therefore necessary to measure the heat of dilution for both the liposome and the poloxamer to provide a baseline for subsequent measurements. The heat of dilution of liposomes was measured by injecting DMPC or POPC liposomes into pure water. A typical plot of the calorimetric data obtained from diluting DMPC liposomes at 5 °C is shown in Figure 1. Each injection of the liposome solution produces a small exothermic heat flow, which remains constant throughout the entire titration process. The heat per injection was integrated and normalized with respect to the number of moles of the lipids injected, giving rise to the molar heat of dilution for DMPC liposomes q_{dil} (DMPC) = −0.008 kcal/mol. The heat of dilution for DMPC liposomes at 5, 30, and 37 °C was obtained as listed in Table 1; only small changes in q_{dil} as a function of temperature were observed. Similarly, at 30 °C the heat of dilution for POPC liposome, q_{dil} (POPC) = −0.018 kcal/mol, was determined.

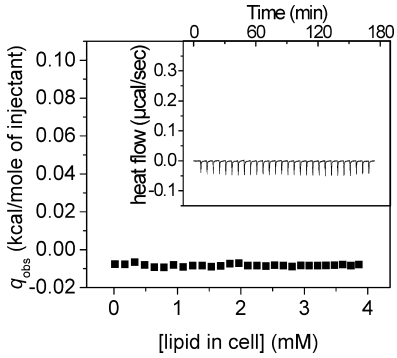

Figure 1. Isothermal titration calorimetry of introducing a series of injections (10 μL each) of a 15 mg/mL DMPC liposome solution into a pure water cell at 5 °C. The data are integrated heat per injection normalized with respect to the number of moles of DMPC injected. The absolute value of the dilution heat is small. (See Table 1.) The inset shows the raw data of the sequential injections.

TABLE 1: Heat of Dilution of 15 mg/mL DMPC Liposomes in Pure Water at Various Temperatures

temperature (°C)	q_{dil} (kcal/mol)
5	−0.008[a]
30	−0.011[a]
37	−0.010[a]

[a] Negative sign indicates that the heat is exothermic.

To obtain the heat of dilution for the poloxamer in the cell when the titrant was added, we have carried out measurements in which pure water was injected into the P338 solution. Because both the heat of dilution of the liposomes and the partitioning heat are normalized by the amount of lipid added, we also normalize the measured heat flow in diluting poloxamers by the corresponding amount of lipid in the partitioning experiments (typically 15 mg/mL DMPC). In this way, we can effectively compare the heat due to dilution effects with that from partitioning effects. At a concentration of 0.6 mg/mL P338, q_{dil} (P338) = −0.004 kcal/mol was obtained. The molar heat of dilution q_{dil} is very small and can be used in the equilibrium partitioning model described by eq 2.

Partitioning of Poloxamers into Fluid-Phase DMPC Bilayers. At temperatures higher than 24 °C, the DMPC liposome is in the fluid phase. The calorimetric data of injecting 15 mg/mL of fluid-phase DMPC liposomes into 0.05 mg/mL P338 solution at 30 °C are shown in Figure 2A. The results are different from the diluting process discussed above. Here, each injection produces an endothermic heat flow that decreases with each injection as less and less free P338 is available in the aqueous phase to partition into the lipid bilayer. Eventually, the heat flow reverts to that of dilution because all poloxamers in the cell have been used for their incorporation into the liposomes, leaving no free polymers for further reaction. This clearly shows that P338 is incorporated into the fluid-phase DMPC bilayer. The solid line in Figure 2A is a nonlinear least-squares fit of the measured titration data to the equilibrium partitioning model described by Heerklotz,[27] assuming that the bilayer is impermeable to P338. The fitted parameters are ΔH = 12.8 kcal/mol and $K = 6.17 \times 10^4$. The permeable model has also been thoroughly tested, but no consistent results can be obtained from fitting data from both the uptake and the release protocols, thus confirming the invalidity of the permeable model for our system. (See Distribution across the Bilayer.)

The effect of temperature on the partitioning of the poloxamer into fluid bilayers was evaluated at the elevated temperature of 37 °C while maintaining all other experimental conditions the same as at 30 °C; the resulting calorimetric data are shown in Figures 2B. Detailed fitting of parameters to the data show that the partition coefficient K at 37 °C is 4.6 times that of 30 °C and the molar enthalpies of partitioning ΔH at 30 and 37 °C are very close, with values of 12.8 and 12.0 kcal/mol, respectively. The higher K value clearly indicates that the interaction between the poloxamer and the fluid-phase bilayer becomes stronger (more spontaneous) with increasing temperature.

Gel-Phase DMPC Liposome Titrated into Poloxamer Solution. Figure 3 shows the calorimetric data from the isothermal titrations of a 15 mg/mL gel-phase DMPC liposome solution into a 0.1 mg/mL P338 solution maintained at 5 °C, in which the DMPC liposomes were in the gel phase. Unlike the case of the fluid-phase membrane, results for the gel-phase liposomes are almost identical to the measured heat of dilution in the absence of poloxamers (Figure 1). The heat flow is small, exothermic, and independent of the amount of lipid injected. The integrated heat normalized by the injected amount of DMPC gives an average value of −0.013 kcal/mol, which is close to the sum of the heat of dilution from DMPC liposomes (−0.008 kcal/mol) and the P338 (−0.004 kcal/mol) solution. These small, constant heat flows point to the fact that no poloxamers were incorporated into the gel-phase lipid bilayer.

To confirm that the phase state of the membrane truly dictates the ability of poloxamer to interact, we have carried out experiments at much higher lipid and poloxamer concentrations. Similarly small and exothermic heats of dilution were also measured for DMPC liposomes (75 mg/mL) or P338 solutions (50 mg/mL) at 5 °C (data not shown).

At low temperatures, besides the relatively tight lipid packing in the gel-phase liposomes, the increase in poloxamer solubility in water[26] could also play a role in inhibiting the poloxamer from partitioning into the lipid bilayer as a result of the lower affinity of the PPO block for the hydrophobic environment in the core of the lipid bilayer. To delineate the membrane fluidity effect from the poloxamer solubility effect, we performed a similar experiment at 5 °C but with fluid-phase POPC liposomes (T_m = −2 °C) at 15 mg/mL titrating into a 2 mg/mL P338 solution. Data similar to those in Figure 2 were obtained (data not shown) with endothermic heat flows decreasing with injections until only an exothermic heat of dilution was observed. The molar enthalpy of partitioning, ΔH, and the partition coefficient, K, were fitted to be 0.232 kcal/mol and 7.61×10^5, respectively. These results clearly demonstrate that P338 partitions into fluid-phase POPC liposomes at 5 °C, thus allowing us to conclude that it is the membrane phase structure rather than the change in poloxamer solubility that dictates the manner in which the poloxamer interacts with the lipid bilayer, with the poloxamer partitioning into disordered fluid-phase membranes but not ordered gel-phase membranes.

To elucidate the effect of temperature further, experiments were carried out at temperatures below and above T_m for DMPC (24 °C) for a systematic study of the effect of bilayer phase structure on interactions between poloxamers and liposomes. As shown in Table 2, the partitioning of poloxamers into the lipid bilayer is strongly influenced by the underlying gel- or fluid-phase structure of the DMPC liposomes. Below T_m (at T = 5, 20, and 22 °C), a constant small amount of exothermic heat is generated despite the presence of poloxamers in the cell, signifying that the observed effect is due only to dilution. These results clearly indicate that the gel-phase bilayer inhibits the incorporation of poloxamers into the liposomes as long as the liposomes are kept at temperatures below T_m irrespective of

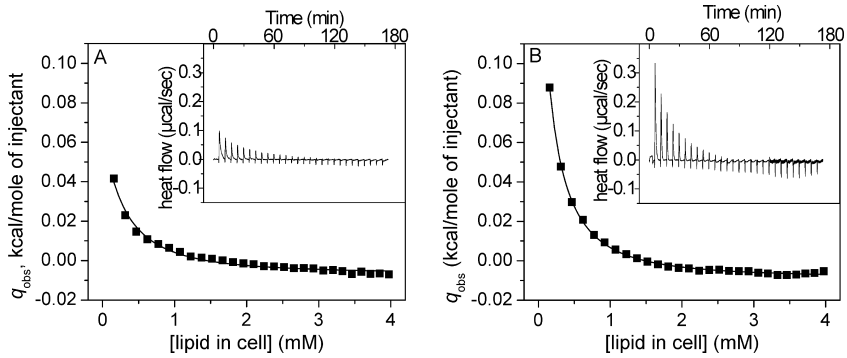

Figure 2. Isothermal titration calorimetry of 0.05 mg/mL P338 with 15 mg/mL DMPC liposomes at (A) 30 and (B) 37 °C. The data are the integrated heat per injection normalized with respect to the number of moles of DMPC injected. The solid lines are the least-squares fits of the calorimetric data with the Heerklotz's partitioning model, where P338 does not permeate through the membrane on the experimental timescale. The fitting parameters are $\Delta H = 12.8$ kcal/mol and $K = 6.17 \times 10^4$ at 30 °C and $\Delta H = 12.0$ kcal/mol and $K = 28.2 \times 10^4$ at 37 °C. The inset shows the raw data of the sequential injections.

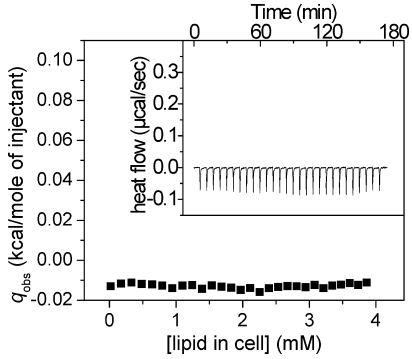

Figure 3. Isothermal titration calorimetry of 0.1 mg/mL P338 with 15 mg/mL gel-phase DMPC liposomes at 5 °C. The data are the integrated heat per injection normalized with respect to the number of moles of DMPC injected. The small, constant, exothermic heat flows are similar to the heat of dilution, indicating that there is no incorporation of poloxamers into the gel-phase lipid bilayer. The inset shows the raw data of the sequential injections.

TABLE 2: Molar Enthalpy Change, ΔH, Resulting from Titrating a 15 mg/mL DMPC Liposome Solution into a 0.1 or 0.05 mg/mL P338 Solution as a Function of Temperature

concentration (mg/mL)[a]	temperature (°C)	ΔH (kcal/mol)	K
$P^{syr} = 0$, $L^{syr} = 15$, $P_o = 0.1$, $L_o = 0$	5	−0.013[b]	
$P^{syr} = 0$, $L^{syr} = 15$, $P_o = 0.05$, $L_o = 0$	20	−0.020[b]	
	22	−0.020[b]	
	26	5.37[c]	6.91×10^4
	30	12.8[c]	6.17×10^4
	37	12.0[c]	28.2×10^4

[a] P^{syr} and L^{syr} are the poloxamer and lipid concentrations in the syringe; P_o and L_o are the initial poloxamer and lipid concentrations in the cell. [b] Small exothermic heat flow is independent of the injection, signifying that it is due to the heat of dilution. [c] At 26, 30, and 37 °C, the data were fitted with the model described by Heerklotz[27] (uptake protocol) where P338 does not permeate through the membrane on the experimental timescale.

variations in temperatures and lipid or poloxamer concentration. Above T_m, a large amount of endothermic heat is produced by the partitioning of poloxamers into lipid bilayers. The spontaneity of this partitioning process increases with temperature, as shown by the increase in K from 26 to 37 °C. Therefore, there

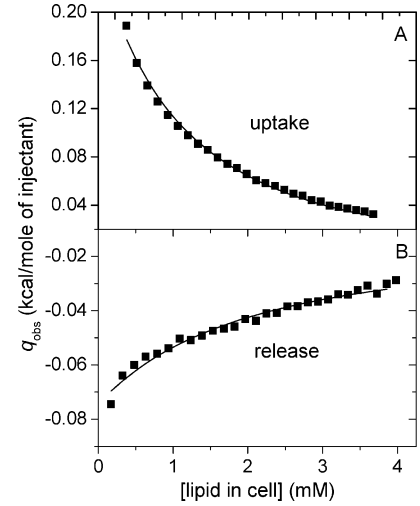

Figure 4. Data of (A) uptake (titrating 15 mg/mL DMPC liposomes into 0.5 mg/mL P338) and (B) release (dilution of DMPC liposomes preloaded with P338 in both outer and inner leaflets, with the liposome concentration at 15 mg/mL and the P338 concentration at 2 mg/mL) experiments investigating the partitioning of poloxamers between water and DMPC liposomes at 30 °C. The experimental setup and the model parameters for the solid fit lines are in Table 3. (Here only the fit to the impermeable model is shown.)

is no doubt that the gel- or fluid-phase structure of the liposome plays a crucial role in the partitioning of poloxamers into lipid membranes.

Our data further indicate that the poloxamer inserts into the fluid-phase lipid bilayer instead of adsorbing onto the surface of the liposomes; otherwise, there should be no dramatic differences in the reaction heat between gel- and fluid-phase liposomes.

Distribution across the Bilayer. ***Uptake/Release Method.*** The determination of the distribution of the poloxamer across the lipid bilayer has been a long-standing question.[29] By simultaneously fitting the uptake and release titration data, one can determine whether the poloxamer accesses the liposome interior[27] on the experimental timescale (typically 200 min). Figure 4 shows one set of data with both uptake and release protocols for the DMPC liposome/P338 system at 30 °C. Table 3 shows the parameters fitted for the uptake and release protocols using Heerklotz's model;[27] here the total lipid and poloxamer concentrations were corrected by their effective concentrations using γ_D and γ_L (Table 3).

From Table 3, we can see that the permeable and impermeable models can both be fit with the data, giving similar χ^2 values.

TABLE 3: Type of Experiment Performed and the Fitting Parameters Obtained for the Uptake, Release, and Pseudorelease Experiments at 30 °C

		permeable model				impermeable model			
type of experiment	concentration (mg/mL)	γ	ΔH (kcal/mol)	$K/10^4$	$\chi^2/10^{-6}$	γ	ΔH (kcal/mol)	$K/10^4$	$\chi^2/10^{-6}$
uptake	$P^{syr} = 0$ $L^{syr} = 15$ $P_o = 0.5$ $L_o = 0$	$\gamma_P = 1, \gamma_L = 1$[a]	21.8	1.72	17.3	$\gamma_P = 1, \gamma_L = 0.5$[a]	10.9	3.40	17.3
release	$P^{syr} = 2$ $L^{syr} = 15$ $P_o = 0$ $L_o = 0$	$\gamma_P = 1, \gamma_L = 1$[a]	10.5	1.25	3.27	$\gamma_P = 0.5, \gamma_L = 0.5$[a]	10.5	2.50	3.27
pseudorelease 2 (38 h after mixing)	$P^{syr} = 2$ $L^{syr} = 15$ $P_o = 0$ $L_o = 0$	$\gamma_P = 1, \gamma_L = 1$[a]	9.51	1.84	1.25	$\gamma_P = 0.5, \gamma_L = 0.5$[a]	9.51	3.68	1.25

[a] γ_P and γ_L are the fractions of poloxamers and lipids in the partitioning reaction, respectively. To compare whether permeable or impermeable models give consistent fitting parameters, γ_P and γ_L are fixed during data fitting.

TABLE 4: Simultaneous Fitting for Uptake and Release Experiments at 30 °C to Determine the Distribution of F108 across the Bilayer

experiment setup	concentration (mg/mL)	γ	ΔH (kcal/mol)	$K/10^4$	$\chi^2/10^{-6}$
uptake	$P^{syr} = 0$ $L^{syr} = 15$ $P_o = 0.5$ $L_o = 0$	$\gamma_P = 0.997$, $\gamma_L = 0.586$	10.8	3.37	7.30
release	$P^{syr} = 2$ $L^{syr} = 15$ $P_o = 0$ $L_o = 0$	$\gamma_P = 0.336$, $\gamma_L = 0.475$			

However, only the impermeable model gives rise to the comparable fitted parameters for both the uptake and release experiments. (ΔH is 10.5 and 10.9 kcal/mol and K is 2.50×10^4 and 3.40×10^4 in release and uptake experiments, respectively.) Although the uptake and release protocols lead to different kinetically entrapped nonequilibrium states, a consistent fit of both the uptake and the release data is possible only when a valid assumption is made regarding the membrane permeability. On the contrary, models assuming permeable membranes give rise to quite inconsistent ΔH values between the two protocols (ΔH is 10.5 and 21.8 kcal/mol in the release and uptake experiments, respectively). Thus, the assumption that the poloxamer is permeable to the lipid bilayer is ruled out, whereas the assumption that the poloxamer does not access the liposome interior during the experimental time is established on the basis of the fitted parameters.

Another way to determine whether the permeable or the impermeable model is correct is to fit the uptake and release data simultaneously to obtain γ_P and γ_L (shown in Table 4). In the uptake protocol when poloxamers adsorb to preformed liposome surfaces, the fitted results show that $\gamma_P = 0.997$ and $\gamma_L = 0.586$, indicating that 99.7% of poloxamers are available to partition from water into the lipid bilayer whereas only 58.6% of lipids are accessible to the poloxamers. Meanwhile, in the release protocol the fitted parameters of $\gamma_P = 0.336$ and $\gamma_L = 0.475$ show that 33.6% of poloxamers and 47.5% of lipids participate in the partitioning reaction. These results confirm that on the timescale of ITC experiments (200 min) the impermeable model is correct. The fitted molar enthalpy of partitioning ΔH and partition coefficient K in Table 4 also agree very well with the values in Table 3.

Release/Pseudorelease Method. The asymmetric distribution of poloxamers after partitioning into the lipid bilayer was also unambiguously corroborated by the release/pseudorelease method. The hypothesis used here is that for the same lipid and poloxamer concentrations used in both the release and the pseudorelease protocols, if the membrane is impermeable then the different kinetically trapped starting states between the release (diluting a liposome solution preloaded with poloxamers in both the outer and inner leaflets) and the pseudorelease (diluting a liposome solution with poloxamers only incorporated in the outer leaflet) protocols should lead to different results. Otherwise, if the permeable model is correct, then the poloxamer should diffuse across the lipid bilayer when prepared by method A and hence the release and pseudorelease experiments should generate very similar, if not identical, ITC data.

Figure 5 shows the results from experiments testing this hypothesis in which two kinds of pseudorelease experiments were performed:

(1) In pseudorelease experiment P-R1, P338 was added to preformed DMPC liposomes and the titration commenced 15 min after mixing; the mixing time is short and P338 should stay in the outer leaflet of the liposomes only if the impermeable model is correct. (2) In pseudorelease experiment P-R2, P338 was added to preformed DMPC liposomes and titration commenced 38 h later. The purpose here was to test whether 38 h was a long enough duration for the poloxamers to migrate across the lipid bilayer to reach and to interact with the inner leaflet of the lipid bilayer.

Results from both P-R1 and P-R2 experiments were compared with those obtained from release experiment R in which P338 was preloaded into DMPC liposomes during liposome formation so that the poloxamers were in both the inner and outer leaflets of the lipid bilayer. As can be clearly discerned from Figure 5, the curves from the P-R1 and R data are rather different in shape. In addition, the P-R1 experiment generates more exothermic heat than release experiment R, and the observed differences are in line with the impermeable model. The lipid bilayer thus appears to be impermeable to the poloxamer on the short timescale of the P-R1 experiment (~200 min). The total lipid and poloxamer concentrations in the P-R1 and R experiments are the same, yet the P-R1 experiment exhibits a larger

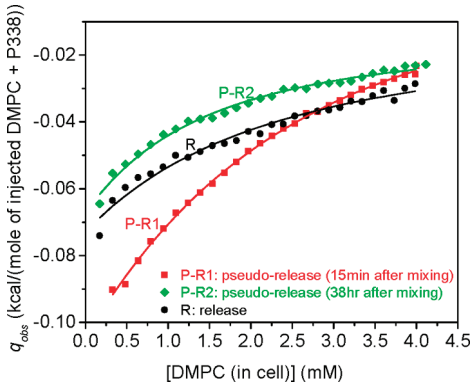

Figure 5. Data from the release experiment, R, and the two pseudorelease experiments, P-R1 and P-R2, at 30 °C, investigating the partitioning of P338 between water and DMPC liposomes. In all three experiments, the DMPC liposome/P338 mixture (15 mg/mL DMPC and 2 mg/mL P338) was injected into the cell containing water. In release experiment R (black circles), P338 participated in the liposome bilayer formation and hence stayed in both the outer and inner leaflets of the membrane; in the pseudorelease experiment P-R1 (red squares), P338 was added to preformed DMPC liposomes and the titration occurred 15 min after mixing; in pseudorelease experiment PR-2 (green diamonds), P338 was added to preformed DMPC liposomes and the titration occurred 38 h after mixing. The solid lines are fits to the data with an impermeable model where poloxamers do not migrate through the lipid bilayer on the timescale of ITC experiments (200 min). The experimental protocols and model parameters are shown in Tables 3−5.

TABLE 5: Parameters for Fitting Release and Pseudorelease Experiments Simultaneously at 30 °C for Both the PR-1 and PR-2 Experimental Protocols

		impermeable model			
experimental protocol	concentration (mg/mL)	γ	ΔH (kcal/mol)	$K/10^4$	$\chi^2/10^{-6}$
pseudorelease 1 (15 min after mixing)	$P^{syr} = 2$ $L^{syr} = 15$ $P_o = 0$ $L_o = 0$	$\gamma_P = 1$, $\gamma_L = 0.21$	10.0	3.54	0.89
pseudorelease 2 (38 h after mixing)	$P^{syr} = 2$ $L^{syr} = 15$ $P_o = 0$ $L_o = 0$	$\gamma_P = 0.45$, $\gamma_L = 0.61$			

exothermic heat of reaction. This is due to the fact that in P-R1 all of the poloxamers are associated only with the outer leaflet of the bilayer and are therefore all available to participate in partitioning whereas in R half of the poloxamer population is not available for partitioning. The difference in the actual assessable concentration of poloxamers partitioning from the lipid bilayer to water can therefore account for the difference in the exothermic heats observed.

In Figure 5, it is interesting to note that if enough time is allowed after the addition of poloxamers to preformed liposomes in the pseudorelease protocol (here 38 h elapse before the start of the P-R2 experiment) then similar data between release experiment R and pseudorelease experiment P-R2 are found except for an almost constant vertical offset between the two sets of data points.[30] The observed offset is ~0.008 kcal/mol, less than the heat of dilution. The P-R2 results were fitted by the equilibrium partitioning model of Heerklotz,[27] and the fitting parameters are listed in Table 3. The parameters agree very well with those from release experiment R, indicating that P-R2 and R have similar starting sample conditions. Together these results suggest that when the poloxamers and the liposomes are mixed, kinetically trapped poloxamers in the outer leaflet of the membrane would diffuse slowly through the bilayer, eventually reaching an even distribution between both leaflets.

It should be noted that it is possible to obtain information on the effective poloxamer and lipid concentrations that actually participate in the partitioning reaction. This can be achieved by fitting the P-R1 and P-R2 data simultaneously with γ_P and γ_L as open parameters. The parameters from such simultaneous fittings are listed in Table 5. The fitted molar enthalpy of partitioning, ΔH, and the partition coefficient, K, shared by both P-R1 and P-R2 agree well with those obtained from uptake and release experiments (Table 3 and 4). The fit gives the effective poloxamer and lipid fractions participating in the partitioning: $\gamma_P = 1$ and $\gamma_L = 0.21$ for P-R1 and $\gamma_P = 0.45$ and $\gamma_L = 0.61$ for P-R2. The fitted values of γ_P and γ_L confirm that in P-R1 poloxamers associate only with the outer leaflet of the bilayer (only 21% of the lipids are involved) and are all involved in the partitioning whereas in P-R2 almost half ($(1 - 0.45) \times 100\% = 55\%$) of the poloxamer is trapped in the inner leaflet of the membranes.

ITC with Considerably High Poloxamer Concentrations. It has been suggested that when a surfactant solution of concentration higher than the cmc is titrated into a liposome solution, the surfactant−lipid phase diagram crosses three stages with increasing surfactant concentration:[33,34] (1) There is a distribution of surfactant between the bilayer and the aqueous phase, forming a mixed lipid/surfactant bilayer. With each injection, the concentration of surfactants in the membrane increases until the bilayer is saturated with surfactants, at which point the bilayer can no longer tolerate further addition of surfactants and starts to disintegrate. (2) Further addition of surfactants induces the formation of surfactant−lipid micelles, hence the system is in a lipid−surfactant bilayer and lipid−surfactant micelle coexistence state. (3) As the surfactant concentration is increased further, all bilayers are dissolved and the system enters the single micelle-phase region. Therefore, the injected liposome is expected to disintegrate when the P338 concentration reaches values above its cmc (8 mg/mL).[26] Uptake experiments with fixed DMPC−liposome concentration at 15 mg/mL but varying P338 concentrations were performed at 30 °C. Similar titration data to those in Figure 2 were obtained when the P338 concentration was in the range from 0.05 to 10 mg/mL. However, when DMPC (15 mg/mL) liposomes were titrated into P338 (20 mg/mL) (Figure 6), large exothermic heat flow was produced with the first few injections. Afterwards, the heat flow returned to being endothermic as observed in the uptake experiments (Figure 2).

Because the concentration of P338 at 20 mg/mL is higher than the cmc (8 mg/mL)[26] of P338 at 30 °C, we conjecture that the liposomes are disintegrated and solubilized to micelles during the first few injections,[35,36] after which further injections led to the coexistence of DMPC/P338 liposomes and micelles. This is indeed corroborated by our recent results where cryo-electron microscopy was used to reveal the morphology of particles in the mixed solutions between P338 and DMPC liposomes.[18]

The ability of P338 to disintegrate liposomes also depends on the stability of the liposomes. It is known that liposomes are metastable but reasonably long-lived on experimental timescales. Our experience indicates that the age of the liposome undoubtedly affects its stability. Properties of the bilayer, such as its fluidity, composition, and structural changes with storage time, all play a role in determining the stability.[37,38]

Our work shows that the poloxamer can incorporate into fluid lipid bilayers as long as the concentration of the poloxamer is

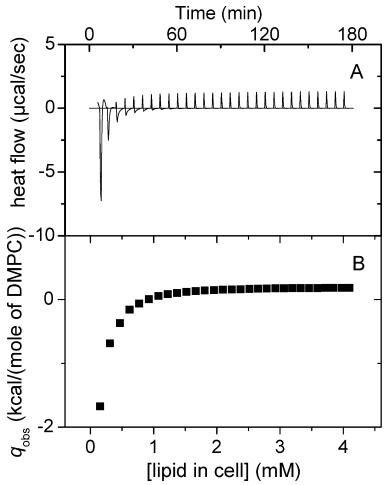

Figure 6. Isothermal titration calorimetry of 20 mg/mL P338 with 15 mg/mL DMPC liposomes at 30 °C. (A) Heat flow vs time. (B) Integrated heat per injection, normalized with respect to the number of moles of DMPC injected. The heat of the first few injections is exothermic and large (the largest heat flow is about a decade larger in amplitude than that in normal uptake experiments, see Figure 2), suggesting that the liposome is disintegrated by P338 at a concentration above the cmc to form mixed DMPC/P338 micelles.

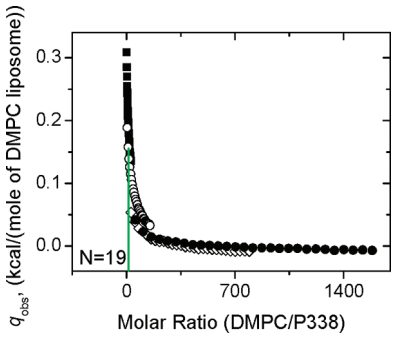

Figure 7. Isothermal titration calorimetry data of titrating 15 mg/mL DMPC liposomes at 30 °C into P338 solution at different concentrations: (□) 3, (○) 0.5, (◇) 0.1, and (●) 0.05 mg/mL. The titration data cover the entire range from ratios that yield large heats of reaction to those that give rise to the flat baseline owing to dilution effects. From the half-maximum of the observed heat, the binding stoichiometry was determined to be around $N = 19$ DMPC molecules per P338.

below the saturation limit. Under these conditions, the poloxamer can act to stabilize and help repair membranes.[1−11] When administered at a concentration above the saturation limit, the poloxamer changes its role drastically from a stabilizing agent to a disintegrating agent. Therefore, whether the poloxamer acts as a sealant or a permeabilizing agent depends on the membrane and the amount of poloxamer present. Our data act to clarify the seemingly conflicting reports in the literature, where the permeability of eggPC liposomes was shown to increase with the loss of encapsulated marker in the presence of poloxamers on the one hand,[20] whereas the same poloxamer was reported widely as an effective membrane sealant on the other hand.[7,8,10,11,39] The seemingly contradictory effects of poloxamers are likely due to the use of different membranes with varying lipid compositions as well as the different concentrations and/or ratios of lipids and poloxamers employed. As a matter of fact, the saturation limit could vary among different lipid membranes. For example, Jamshaid and et al. have shown that at 0.1 mg/mL and 37 °C (the reported cmc for the given experimental condition), P338 induces considerably higher content release from liposomes prepared by egg PC alone than those prepared from egg PC and cholesterol (1:1 molar ratio). Our work thus points to the importance of identifying the saturation limits of poloxamers in different membranes, which could help to formulate the appropriate poloxamer concentration as well as poloxamer/lipid ratio for the purposes of either stabilizing or destabilizing lipid membranes for a variety of applications.

Stoichiometry of Binding. The association of poloxamers to preformed liposomes can be treated as a binding reaction, with the stoichiometry N being the lipid/poloxamer ratio that can be reached before the liposome is disintegrated into micelles. The stoichiometry of this reaction can be determined from the titration data, and here we present results obtained for such a determination. To ensure that a wide fraction of the binding isotherm could be observed, 15 mg/mL DMPC liposomes was titrated into P338 solutions of varying concentrations, and the results are shown in Figure 7. Four ITC binding curves acquired at different poloxamer concentrations in the cell give the entire transition range from a lipid/poloxamer molar ratio that gives a large heat of reaction to that providing a plate-flat baseline. The binding isotherm allows for the estimation of the stoichiometry of partitioning, and a value of $N = 19$ DMPC molecules per P338 is found from these experiments. This partitioning stoichiometry was obtained by identifying the molar ratio DMPC/P338 corresponding to the half-maximum of the observed heat.[40]

Discussion and Conclusions

The membrane phase structure plays a critical role in the interaction between poloxamers and membranes. Although poloxamers do not partition into ordered gel-phase bilayers, they do so with ease into the disordered fluid-phase lipid bilayers, with the level of interaction increasing with bilayer fluidity. In fact, it is the difference in the lipid packing between fluid- and gel-phase membranes that regulates the level of poloxamer/lipid association. In the context of membrane sealing, the capability of the poloxamer to distinguish the small difference between fluid- and gel-phase lipid bilayers suggests that it is possible for the poloxamer to associate only with damaged cell membranes that exhibit enhanced permeability as a result of a reduction in the total lipid density while not interfering with healthy membranes with lipid molecules packed tightly enough for barrier functions. Our liposome findings corroborate the conclusions drawn from our earlier monolayer studies that poloxamers insert only into monolayers with the lipid packing density below a certain threshold value and that poloxamers can gracefully exit the membrane when the membrane's structural integrity is restored.[17]

In this work, single-component synthetic bilayers were used to elucidate the interaction between lipids and poloxamers. It should be kept in mind that real cell membranes are multicomponent systems consisting of unsaturated lipids and cholesterol and are fluid-phase under physiological conditions. Unlike our simple model systems, nonlipid components are present in real membranes, and they no doubt can exert an influence on poloxamer−membrane interactions. For instance, it has been shown that the addition of cholesterol considerably decreases the efflux rate of entrapped 6-carboxyfluorescein from liposomes prepared from egg PC when suspended in poloxamer P338.[20] The subtle interplay of the various components in real membranes on lipid packing may in fact alter the fluidity of the membrane enough to prevent the incorporation of poloxamer into healthy cells. It is only with the disruption of lipid packing, due to traumas or diseases, that the association of certain poloxamers with lipid membranes is promoted. To correlate our

findings of poloxamer−membrane interactions in synthetic lipid bilayers with those found in real membranes, further investigations are needed.

Our previous work has further demonstrated that poloxamers with different molecular weights and/or hydrophobicity exhibit different capabilities to associate with membranes.[41] Poloxamers appropriate for different degrees of loss of membrane integrity can therefore be designed to yield the desired membrane-interacting capability so that they would seal and repair damaged cell membranes while leaving normal and intact cells alone. In particular, the design of poloxamers catering to different cell types should also be pursued. Because our data indicate that the level of poloxamer interaction depends on the phase state of the lipid bilayer, which in turn is temperature-dependent, one can envision designing temperature-controlled therapies involving poloxamers.[18]

The transfer of poloxamers from the aqueous phase to the fluid-phase lipid membrane has been shown to be endothermic. This implies that the driving force for the partitioning of poloxamers into lipid membranes is dominated by entropy.[42] The gain in entropy is due to the release of ordered water molecules surrounding the PPO chain of the poloxamer upon partitioning from the aqueous to the lipid bilayer phase. This gain is expected to contribute significantly to the partitioning free energy. Furthermore, the favorable interaction between the hydrophobic acyl chain of the lipid and the PPO block of the poloxamer is also important and likely contributes additionally to the enthalpy−entropy balance of the free energy of partitioning.

The asymmetric bilayer distribution of poloxamers after partitioning from the aqueous phase into the liposome is also demonstrated in the data presented here. Poloxamers are found to incorporate predominantly into the outer leaflet of the liposome on the timescale of the ITC experiment (∼200 min) and only slowly migrate through the bilayer to eventually result in an even distribution after incubation for an extended period of time. Our results thus indicate that the incorporation of poloxamers into the outer leaflet of the membrane is a relatively fast process whereas the transfer across the membrane is a significantly slower one. This is reasonable because the lipid bilayer serves as a strong impediment to passive diffusion of the highly hydrophilic PEO blocks across the nonpolar acyl chain region. The transfer of the poloxamer to the inner leaflet is slow because a large energy barrier needs to be overcome and hence is not observed within the ITC experimental time frame (200 min). This is in agreement with a previous report stating that after its addition to preformed liposomes the poloxamer does not insert into the core of the fluid lecithin bilayer using hydrophobic dyes as bilayer probes,[43] though the timescale of their measurements was not noted. The asymmetric poloxamer distribution is expected to be influenced by the acyl chain length and the overall geometry of the lipid molecules (which affects the packing of the membrane) as well as the hydrophobicity of the poloxamer; poloxamers with shorter PEO blocks are likely to undergo transbilayer diffusion more readily.

The asymmetric poloxamer distribution further helps rule out the possibility that the poloxamer adopts a conformation that spans across the bilayer with its two PEO blocks dangling at opposite ends of the membrane right after its incorporation. Moreover, the fact that in a short period after mixing, poloxamers predominantly interact only with the outer leaflet or half of the membrane justifies the use of the lipid monolayer as a model system;[17] such a system is effective in providing insight into lipid−poloxamer interactions in the initial stage.

Our work also helps to elucidate the molecular mechanisms responsible for membrane disruption by poloxamers. As expected, the effect of poloxamer on lipid membranes (either aiding in their repair or their disintegration) is very concentration-dependent. For single-component synthetic membranes, a saturation limit exists below which the poloxamer partitions into and stabilizes the membrane structure but above which the poloxamer actually destroys the structural integrity of the liposome. This concentration effect is not restricted only to lipid−poloxamer interactions but can be broadly applicable to other amphiphilic detergents. Understanding the factors contributing to these almost opposite effects on membrane stability is therefore significant in the context of designing effective liposomal drug-delivery systems.[44]

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(30) One plausible explanation for the vertical offset between the P-R2 data and the R data is a slight loss of lipids adsorbed onto the miniextruder during the preparation of pure liposomes for the P-R2 experiment; On the opposite, the presence of poloxamers in the miniextruder to prepare lipid/poloxamer liposomes for the release experiment R acts to minimize the loss of lipids because it is known that the addition of poloxamer can reduce surface adsorption.[4,31,32] The different levels of lipid loss could account for the small offset observed. However, careful quantification of materials is needed in future experiments to confirm this.

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(40) It is hard to establish the baseline at a low molar ratio of DMPC/P338 for the partitioning of poloxamer into the lipid bilayer. There are two ways to decrease the DMPC/P338 molar ratio to perform titrations: (1) Maintain a reasonable liposome concentration and increase the P338 concentration in the cell. This method can cover a relatively large molar ratio range, but liposomes were disrupted at P338 concentration higher than the cmc (discussed in the previous section). (2) Keep the P338 concentration below the cmc, and decrease the DMPC liposome concentration. This method covers a very limited molar ratio range, and we find that the maximum observed heat is around 0.31 kcal/mol. If extremely careful experiments were performed to find a slightly higher maximum observed heat, then the stoichiometry N would be slightly smaller than $N = 19$ DMPC molecules per P338. Hence, $N = 19$ is the upper limit here.

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