

Effects of bilayer phases on phospholipid-poloxamer interactions†

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Poloxamers are amphiphilic copolymers capable of interacting with biological membranes, while the fundamental mechanism of the interactions is not yet fully understood. Using liposomes as model membranes, we have investigated the interactions of the poloxamer with phospholipids bilayers by isothermal titration calorimetry (ITC) and electron cryomicroscopy (cryo-EM). The results suggest that the phase structure of the lipid bilayer plays a critical role in regulating poloxamer insertion into lipid bilayers. ITC shows that the poloxamer is incorporated into the liposome at temperatures (T) above the bilayer main phase transition temperature (T_m) but the incorporation is completely inhibited otherwise. This distinct effect from the phase structure of the lipid bilayer determines the concentration of poloxamer incorporated into the membrane and affects the morphology of the self-assembled structure of lipid-poloxamer mixtures. When poloxamers are introduced at concentrations above the critical micelle concentration to pre-formed liposomes, liposomes are disrupted into flat discs at temperatures above T_m but remain as spherical shells at temperatures below T_m , as evidenced by cryo-EM. With relatively low concentrations of poloxamers introduced during liposome formation, spherical poloxamer-lipid vesicles are formed at $T > T_m$; The spherical-shell structure of binary poloxamer-lipid liposomes changes to flat discs over a short time scale when the temperature is dropped below T_m . These flat discs are capable of reverting to the spherical vesicular structure when the temperature is raised above T_m , though a much longer time is needed. Understanding the effects of the bilayer phase in lipid-poloxamer interactions can help improve the design of poloxamers for pharmaceutical use.

Introduction

Poloxamers, also known as Pluronics, are non-ionic triblock copolymers composed of a central hydrophobic poly(propylene oxide) (PPO) chain capped by two hydrophilic chains of poly(ethylene oxide) (PEO). Poloxamers have gained increasing attention due to their abilities in repairing biological membranes damaged by trauma and diseases,^{1–12} in sterically stabilizing liposomes for drug delivery,⁶ in improving cell survivability in gene therapy, and in inhibiting drug efflux from drug resistant cancer cells *via* interaction with membranes.¹³ Despite the importance of poloxamers, the fundamental mechanism of their interactions with membranes is not yet fully understood. For example, despite the application of poloxamers as membrane sealants, there have been contradictory reports that poloxamers disturb lipid bilayer structural integrity.^{14,15} It was observed that poloxamers accelerated the permeation of the entrapped anti-tumor drug doxorubicin through lipid bilayers. Moreover, it is not clear why mouse tumor cells accumulated approximately 3 times more poloxamer P181 and P235 than normal murine blood

cells.¹⁶ Apparently poloxamer-cell interaction depends on the cell type.¹⁷

Different biological membranes vary in lipid composition, packing and fluidity (or membrane microviscosity). Due to the complexity inherent in biological membranes, we use lipid bilayers as simplified models to investigate the poloxamer-membrane interactions. We hypothesize that the mechanism of the action of poloxamers, either as a transport enhancer or as a membrane sealant, is affected by how much poloxamer can be accumulated in the lipid bilayers before reaching their saturation limit above which the poloxamers solubilize phospholipids to form micelles. This poloxamer-bilayer interaction depends on both the hydrophilic-hydrophobic balance of poloxamers, and the fluidity of the lipid bilayer. In detail, a certain poloxamer can insert into the more fluid cell membranes and alter the molecular packing of the bilayers to cause the permeabilized structures to reform into continuous ones; but for less fluid cell membranes, the same amount of poloxamer can overload the bilayers and solubilize some lipids into micelle-like structures and stabilize the pore formation, and hence make bilayers leaky. To confirm this, we investigate the role of the fluidity of lipid bilayers in the poloxamer-bilayer interactions by changing the bilayer phase structure. Previous studies^{18,19} reveal that lipid packing density regulates the incorporation of poloxamer into Langmuir monolayers. This is achieved by allowing poloxamers to insert into lipid monolayers only with a packing density below that in intact bilayers;^{20,21} once inserted the poloxamer can eventually be eliminated from the lipid monolayer when the lipid packing density increases beyond a threshold.^{22,23} However, the lipid

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† Electronic supplementary information (ESI) available: Particle size distribution determined from dynamic light scattering; cryo-EM micrographs. Both show that the particle size is more uniform with the presence of poloxamer.

monolayer has been a controversial model for lipid bilayers, and it is shown that the lateral ordering in bilayers is significantly less than that of an monolayer with equivalent surface pressure.²⁴ The fundamental question is whether the distinct effects of lipid packing in lipid monolayers on controlling the poloxamer-bilayer interactions can be observed in the case of bilayers.

Here we report our experimental findings which clearly demonstrate that the manner in which poloxamers partition into the lipid bilayer is determined by the underlying phase state of the liposome. Our isothermal titration calorimetry (ITC) results indicate that poloxamers only partition into fluid-phase liposomes and hardly ever into gel-phase ones. As the phase state of the liposome is temperature-dependent, so is the interaction between poloxamers and liposomes. In the presence of poloxamers, lipids can change their self-assembled structure from intact spherical vesicles to flat discs when experiencing either decreased or increased temperature depending on whether the poloxamer is incorporated before or after the formation of the liposomes, respectively. The understanding of self-assembly structure-property relationship may clarify the mechanisms of poloxamer-membrane interactions and help improve poloxamer design for pharmaceutical use.

Results and discussion

Effects of membrane phase structure

It has been well established that the lipid packing is dependent on bilayer phases. The area per lipid molecule increases by about 15% to 20% upon the gel-fluid transition.²⁵ We investigate the

effect of membrane phase structure by studying the interaction between poloxamers and liposomes as a function of the temperature. To closely examine the interaction, we use a simple model system with synthetic dimyristoylphosphocholine (DMPC) and poloxamer 338 (P338, (EO)₁₃₂-(PO)₅₀-(EO)₁₃₂, molecular weight = 14600 g/mol, polydispersity $M_w/M_n = 1.2$)²⁶ to represent phospholipids and poloxamers, respectively. Using isothermal titration calorimetry (ITC), we have quantitatively studied the partitioning of poloxamers between lipid bilayers and water at temperatures above and below the main phase transition temperature, $T_m = 24\text{ }^\circ\text{C}$, of DMPC. At temperatures above T_m , the DMPC liposome is in the fluid-phase. ITC results at $37\text{ }^\circ\text{C}$ demonstrate that P338 is incorporated into the fluid-phase DMPC bilayer (Fig. 1A and B) in a fashion similar to other non-ionic detergents which partition into liposomes.²⁷ Each injection of the fluid-phase DMPC liposomes into the P338 solution produces an endothermic heat of reaction which decreases with injections. This endothermic phenomenon is consistent with the partitioning process of detergent into stable bilayers with little curvature strain.²⁸ The decay of reaction heat is due to the fact that with time less and less free P338 is available in the aqueous environment to associate with liposomes. Eventually, the heat flow simply resembles the heat of dilution as all free P338 has been used up and incorporated into the liposomes, leaving almost no free polymer for further association (Fig. 1A, after 70 minutes). In Fig. 1B, it is evident that the simple thermodynamic partitioning model by Heerklotz²⁹ gives an excellent description of the high sensitivity calorimetric results of the poloxamer partitioning between water and the fluid-phase membrane. The fitting gives the molar enthalpy of partitioning, $\Delta H = 12 \pm 1$

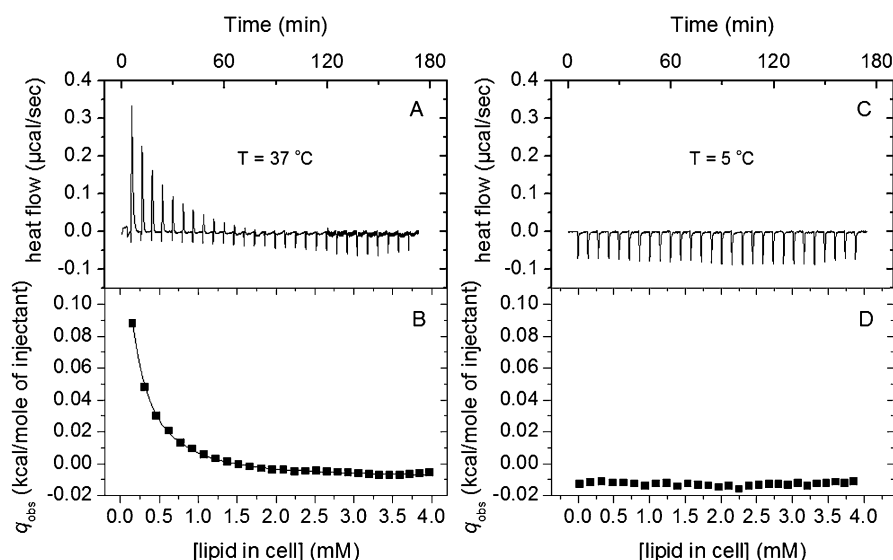


Fig. 1 Isothermal titration calorimetry data comparing fluid- and gel-phase DMPC liposomes reacting with P338. A series of 15 mg/ml DMPC liposomes (10 μl each) are injected into a cell of volume 1.4045 ml, filled with P338 solution of (A, B) 0.05 mg/ml at $37\text{ }^\circ\text{C}$ (C, D) 0.1 mg/ml at $5\text{ }^\circ\text{C}$. (A, C) Heat flow vs. time; (B, D) integrated heat per injection normalized with respect to the number of moles of DMPC injected. In each experiment, both liposomes and P338 solutions are equilibrated at the same desired temperatures. At $37\text{ }^\circ\text{C}$, DMPC liposomes are in the fluid-phase and incorporation of P338 into the bilayer generates endothermic reaction heat. The solid line is a nonlinear least-square fit of the equilibrium partitioning model,²⁹ assuming impermeability of the bilayer for P338 (P338 interacts with the outer leaflet of the bilayer and no transbilayer migration occurs) within the experimental time scale. The fitting parameters are $\Delta H = 12 \pm 1\text{ kcal/mol}$, $K = (28 \pm 2) \times 10^4$. The permeable model has also been thoroughly tested but turns out to be invalid in the titration time scale for our system.³² At $5\text{ }^\circ\text{C}$, DMPC liposomes are in the gel-phase. The small, constant, and exothermic heat flows are due to simple dilution, suggesting no incorporation of poloxamer into the gel-phase lipid bilayer.

kcal/mol, and the partition coefficient, $K = (28 \pm 2) \times 10^4$, at 37 °C. Consequently, the entropy of P338 partitioning into the fluid-phase lipid bilayer at 37 °C can be calculated to be 64 cal/mol. Similar results are obtained when the temperature is decreased to 30 and 26 °C at which point the DMPC liposomes are in the fluid-phase. However, at these reduced temperatures, less heat of reaction is generated (data not shown), indicating less poloxamer insertion. The partition coefficient, K , indicates the spontaneity of the process: when $K > 1$, the process of poloxamer partitioning into the lipid bilayer is spontaneous; and the higher the K value, the higher the tendency is for the process to occur. A comparison of the fitting parameters at 30 °C of $\Delta H = 13 \pm 1$ kcal/mol and $K = (6 \pm 1) \times 10^4$ to those at 37 °C shows that the higher temperature gives rise to stronger interactions between poloxamers and the lipid bilayer. More poloxamers are incorporated into bilayers at elevated temperatures possibly because the fluidity of the bilayer, as well as the hydrophobicity of the poloxamer increase.^{30,31} In the future, the amounts of poloxamer inserted into vesicles can be obtained by solving the equation defining the partition coefficient (eqn (1) in the Experimental session, for the value of $\frac{\text{poloxamer in bilayer}}{\text{lipid}}, \frac{P_b}{L}$), and their subsequent correlation with vesicles' size can also be studied.

When the system is cooled to temperatures below T_m , however, ITC results show that poloxamers do not partition at all into gel-phase liposomes. When the titration was performed at 5 °C, the heat flow was small and exothermic, independent of the amount of liposomes injected (Fig. 1C and 1D). The integrated heat normalized by the injected amount of DMPC gives an average value of -0.013 kcal/mol, which is equivalent to the total dilution heats from DMPC liposome and P338 solutions (under similar conditions, the measured heats of dilution are $q_{\text{dil}}(\text{DMPC}) = -0.010$ kcal/mol and $q_{\text{dil}}(\text{P338}) = -0.004$ kcal/mol). Systematic experiments were performed at temperatures between 5 and 22 °C, and similarly, only dilution heats were generated with titration of DMPC liposomes despite the presence of poloxamer in the experimental cell. These results indicate that the gel-phase bilayer completely inhibits the poloxamer incorporation. This inhibition is independent of the exact temperature, as long as it is below T_m .

It is worth noting that the change of temperature affects the physical properties of poloxamers as reflected by their inverse temperature dependence in solubility. A decrease in temperature causes the PPO chains to be more polar and hydrated and hence the poloxamers to be less hydrophobic.³⁰ To confirm that it is indeed the phase structure of the liposome rather than changes in the properties of the poloxamer with temperature that is affecting the interaction, we have also tested fluid-phase liposomes at 5 °C. Palmitoyl-oleoylphosphocholine (POPC) liposomes have a $T_m = -2$ °C and hence are in a fluid-phase at 5 °C. At this low temperature, the injection of 15 mg/ml POPC liposomes into a 2 mg/ml P338 solution produces similar data as those in Fig. 1A and 1B with fitted parameters $\Delta H = 0.23 \pm 0.02$ kcal/mol and $K = (76 \pm 7) \times 10^4$ (Fig. 2). The observation of endothermic reaction heat clearly shows that at 5 °C P338 partitions into fluid-phase POPC liposomes. The fact that P338 partitions into disordered, fluid-phase POPC liposomes instead of ordered, gel-phase DMPC liposomes allows us to conclude that the phase state of the membrane is crucial for the interaction between

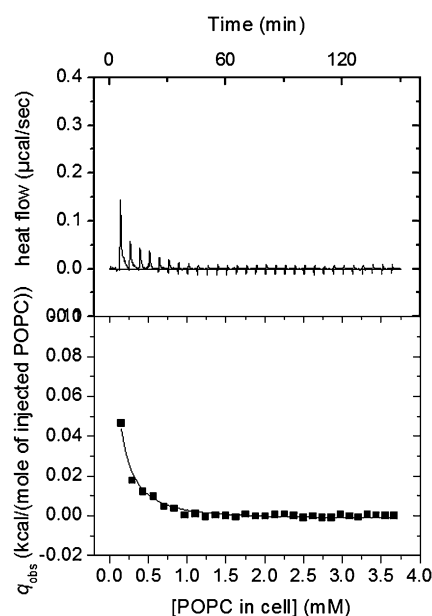


Fig. 2 Isothermal titration calorimetry data showing poloxamer P338 partitions into fluid-phase POPC liposomes at 5 °C. A series of 15 mg/ml POPC liposomes (10 μ l each) are injected into a P338 solution of 2 mg/ml. (A) Heat flow vs. time; (B) integrated heat per injection normalized with respect to the injected number of moles of POPC. The solid line is a nonlinear least-square fit of the equilibrium partitioning model,²⁹ assuming impermeability of the bilayer for P338 (P338 interacts with the outer leaflet of the bilayer and no transbilayer migration occurs) within the experimental time scale. The fitting parameters are $\Delta H = 0.23 \pm 0.02$ kcal/mol and $K = (76 \pm 7) \times 10^4$.

poloxamers and lipid bilayers. Our data in Fig. 1 provide further evidence for the insertion of the poloxamer inside the fluid-phase lipid bilayer rather than just having the poloxamer adsorbed onto the surface of the liposome, otherwise there would not have been such a distinction in the heat of reaction between the gel- and the fluid-phase liposomes.

In the context of using poloxamers as sealants to repair structurally compromised membranes, the ability of poloxamers to distinguish subtle differences in the lipid packing density allows them to associate preferentially with damaged cell membranes (owing to a reduction in their lipid packing density) while not interfering with normal ones (with tightly packed lipid molecules). This finding is consistent with conclusions from earlier studies on monolayers,^{18,22} where poloxamers were found to insert into monolayers only when lipid packing density is low. As the level of poloxamer interaction depends on the phase state of the membrane which in turn is temperature-dependent, one can envisage using this temperature-sensitive nature of the lipid-poloxamer interaction to protect healthy tissues *via* poloxamer-assisted cancer chemotherapy. Poloxamers are among the most potent sensitizers of drug resistant cancer cells¹³ and are able to reduce anti-cancer drug efflux from tumor cells. In chemotherapy, if the surrounding normal tissues are slightly cooled, the effect of poloxamer on membranes of these tissues would be minimized. As a result, the normal cells would be unaffected by the poloxamer while the cancer cells can be selectively targeted.

Morphological changes by adding poloxamer to pre-formed liposomes

The effects of bilayer phases on lipid-poloxamer interactions are clearly visible from the different morphologies of self-assembled structures resulting from the addition of poloxamers at concentrations above the critical micelle concentration (CMC) to pre-formed liposomes. At concentrations above CMC, the poloxamer is referred to as being at high concentration in this report. The micellization of poloxamer is not a sharp transition, but spans a wide range of concentrations at a given temperature due to its polydispersity as well as the presence of impurities. Depending on the methods used, a wide range of CMCs have been reported for P338, from 0.03 to 0.1 mg/ml at 37 °C, 0.3 to 45 mg/ml at 25 °C, and ≥ 1 mg/ml at 20 °C.^{33,34}

The morphology of the mixed system was monitored by cryo-EM. Stock solutions of 10 mg/ml pre-formed DMPC liposomes and 200 mg/ml P338 were mixed to give a solution with a final concentration of 7.5 and 50 mg/ml for DMPC and P338, respectively. The mixing experiment was conducted at either 4 or 37 °C and the mixed solution was stored at the corresponding temperature. The sample stored at 4 °C was allowed to equilibrate at 21 °C for 15 minutes prior to cryo-EM specimen preparation. Cryo-EM images show that after the addition of P338, DMPC liposomes remain as spherical vesicles at 21 °C (Fig. 3A), but those stored at 37 °C are disrupted into discs with diameters ranging from 30 to 100 nm (Fig. 3B). The side view of these floppy discs is captured as shown in Fig. 3C-a, revealing two bilayer leaflets with a 5.5 nm thickness, which is consistent with that of the DMPC bilayer.^{35,36} The high electron density shown as a relatively darker color in the leaflets is attributed to the phospholipid headgroups. In Fig. 3A, the existence of intact

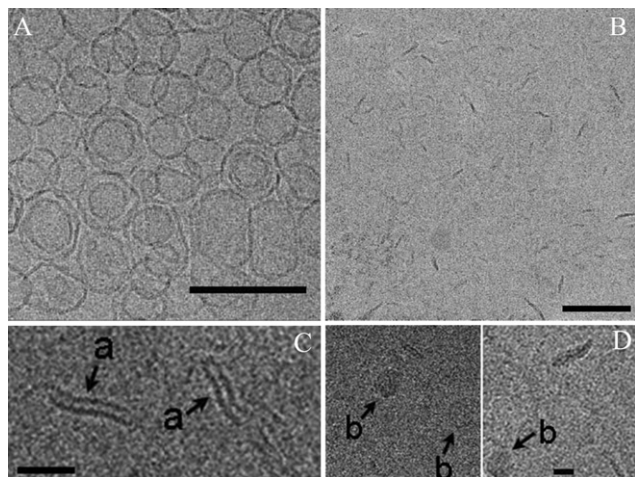


Fig. 3 Cryo-EM images comparing the morphologies of objects in solution when mixing the gel- and the fluid-phase DMPC liposomes with P338. The solutions were vitrified 25 minutes after mixing 10 mg/ml DMPC liposomes and 200 mg/ml P338 (the final concentrations are 7.5 and 50 mg/ml for DMPC and P338, respectively) (A) at 21 °C, and (B)–(D) at 37 °C. With the addition of P338 at a final concentration of 50 mg/ml, the DMPC liposomes remain as spherical vesicles at 21 °C, but transform to bilayer discs at 37 °C. (C) and (D) are high magnification images of the sample in (B) displaying relatively large bilayer discs as observed edge-on (a) and face-on (b). In (A) and (B), bar = 200 nm; in (C) and (D) bar = 20 nm.

DMPC liposomes at 21 °C despite the presence of a high concentration of poloxamers is due to the low level of interaction between poloxamers and liposomes. These results clearly demonstrate that it is not necessarily relevant whether the poloxamer concentration in the bulk solution is above CMC or not; what really matters is the concentration of the poloxamer accumulated in the membrane which determines the changes of the self-assembled structure.³⁷ These observations corroborate the fact that poloxamers partition into disordered, fluid-phase lipid bilayers rather than ordered, gel-phase bilayers.

Morphological transformation of pre-mixed DMPC/P338 liposomes with temperature

Cryo-EM further reveals the morphology change of the self-assembly of lipid and poloxamer as a function of temperature with the poloxamer at a moderate concentration (below CMC). Pre-mixed DMPC-P338 liposomes were prepared by drying P338 together with DMPC from a chloroform solution at a concentration of 10 mg/ml for each component then hydrating with water. As a result, poloxamers were incorporated into liposomes during the self-assembly process. The temperature of the solution was controlled to be 10 to 15 °C above the T_m of the DMPC liposomes during the hydration and extrusion steps in preparation as well as in storage. When the temperature was above T_m , the incorporation of poloxamers into the bilayer resulted in a narrower size-distribution of the spherical liposomes as indicated by the cryo-EM images and the particle size measurements from dynamic light scattering (ESI†). This increase in monodispersity has been previously reported for PEG-lipid analog grafted liposomes.³⁸

The incorporation of poloxamers into the liposomes can facilitate a temperature-triggered structural change which does not occur in the absence of poloxamers. When cooled through T_m to 4 °C, DMPC liposomes with no poloxamers do not show much change and retain their spherical-shell structure at 4 °C. The structure observed at this low temperature is similar to that above T_m , except for the formation of faceted surfaces on the vesicle (Fig. 4) which is consistent with the fact that DMPC liposomes exist in the more rigid gel-phase.^{39,40} However, when binary DMPC-P338 liposomes are cooled from 37 to 22 °C (below their $T_m = 24$ °C) and stored for 30 minutes, a pronounced difference in the turbidity of the dispersion is observed. The sample stored at 37 °C remains opalescent, while the one stored at 22 °C becomes translucent (Fig. 5). The decrease in turbidity clearly suggests a decrease in particle size. The same phenomenon is observed when the sample is stored at 4 °C at which temperature the sample changes from opalescent to translucent within a shorter time (~ 15 minutes).

Cryo-EM was used to examine the size and morphology change of pre-mixed DMPC-P338 liposomes stored at temperatures below (Fig. 6A) and above (Fig. 6B) T_m . Below T_m , a dramatic change in the morphology was observed where spherical vesicles present at higher temperatures disappeared, instead there were bilayer discs (Fig. 6A) with uniform diameters of approximately 30 nm. The smaller size of these discs is consistent with the decrease in the observed sample turbidity. This decreased size is also confirmed by dynamic light scattering (data not shown).

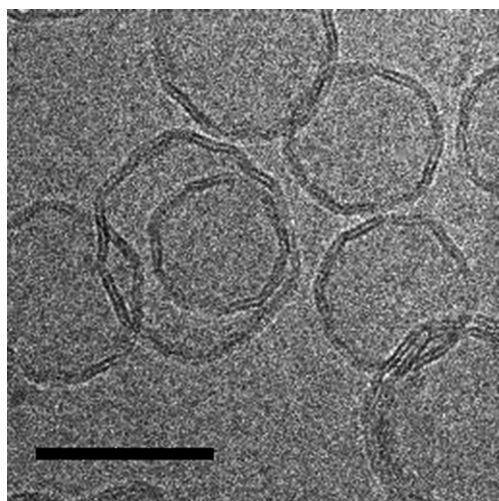


Fig. 4 Cryo-EM image of DMPC (10 mg/ml) liposomes stored at 4 °C revealing spherical vesicles with faceted surfaces. Scale bar = 100 nm.



Fig. 5 Temperature effect on pre-mixed DMPC/P388 (10/10 mg/ml) liposomes. The DMPC/P388 liposome solution was aliquoted into two vials and stored for 0.5 hour at 37 °C (a) and 22 °C (b), respectively. The sample stored at 37 °C remained opalescent while the one stored at 22 °C became translucent. The loss of turbidity was caused by a decrease in particle size in the solution.

When the temperature of the DMPC-P388 bilayer discs (in Fig. 6A) was raised back above T_m to 31 °C and equilibrated for 2 weeks, a fair amount of spherical vesicles re-appeared and co-existed with bilayer discs (Fig. 6B). The diameters of these vesicles varied from 50 to 200 nm. Meanwhile, the remaining bilayer discs grew bigger and spanned a larger size range (Fig. 6B), in contrast to a uniform size of 30 nm (Fig. 6A) when the temperature was first brought below T_m . It is worth noting that the structural transformations, from vesicles to bilayer discs and vice versa, take place over two rather different time scales. Complete transition from intact vesicles to bilayer discs occurs within 30 minutes while the reverse process takes 2 weeks to transform only a fraction of the discs to vesicles.

The sharp transition of binary DMPC-P388 spherical vesicles to bilayer discs upon cooling, is due to lipid solubilization by poloxamer. The phase separation between poloxamers and gel-phase lipid domains causes the disintegration of liposomes into small bilayer discs. The structure of bilayer discs at temperatures below T_m is composed of a 2D crystalline lipid core with poloxamers decorating the edge.⁴¹ To avoid the unfavorable free energy of exposing hydrophobic phospholipid tails to water, the poloxamer with its flexible hydrophobic and hydrophilic moieties acts to stabilize the 30 nm planar lipid bilayer. Meanwhile, the

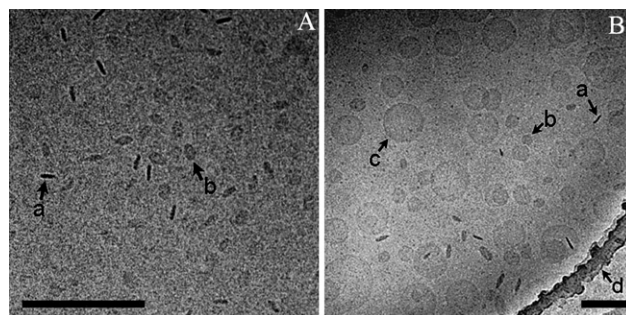


Fig. 6 Cryo-EM images showing the effect of temperature on pre-mixed DMPC/P388 liposomes. Both DMPC and P388 are at 10 mg/ml. After sample preparation at a temperature above 24 °C, the pre-mixed DMPC/P388 liposome solution was then (A) stored at 4 °C for 0.5 hour and imaged by cryo-EM. The solution changed from opalescent to translucent, and uniform bilayer discs with a diameter of 30 nm were revealed by the cryo-EM image. The discs were randomly oriented so that both the top-view and side-view of the discs are captured; (B) the translucent solution is then heated and kept at 31 °C for two weeks. The solution changed from translucent to opalescent, with most discs reverting to spherical vesicles. Arrows (a, b) denote bilayer discs as observed edge-on (a) and face-on (b), arrow (c) denotes a spherical vesicle, and arrow (d) points to the edge of the carbon film of the EM grid. In (A–B), bar = 200 nm.

poloxamer cannot partition into the crystalline lipid bilayer phase that exists at temperatures below T_m . This is based on our previous X-ray scattering and simulation studies,^{18,22,42} which shows that poloxamers phase-separate from the ordered lipid phase due to the hydrophobic mismatch between the PPO block in poloxamers and the acyl chains in lipids; this is further corroborated by our current ITC experiments which show that poloxamers do not incorporate into gel-phase lipids. Therefore, the only feasible way for the poloxamer to stabilize the small bilayer discs is to reside at the edge of bilayer discs. As the lipid bilayer undergoes the fluid-to-gel transition when the temperature is decreased below T_m , poloxamers originally mixed with fluid lipid molecules phase-separate from the ordered lipid domains and accumulate at the gel-phase domain boundaries. The enhanced rigidity of the gel-phase lipid and the accumulated poloxamers at domain boundaries eventually cause the vesicular structure to break up and adopt a thermodynamically favored disc configuration with the ordered lipids occupying the center portion and the poloxamers decorating the edge of the bilayer, thereby stabilizing the otherwise hydrophobic rim of an open lipid disc. This structure has been demonstrated for other lipid/detergent mixed discs as evident from NMR, calorimetry and X-ray scattering data,⁴¹ and resembles the highly monodisperse phospholipid bilayer nanodiscs.⁴³

Similar nanosized bilayer discs have been recently reported on lipid mixtures containing phosphatidylethanolamine-PEG (PE-PEG).^{44,45} These nanodiscs are promising model membranes in drug partition studies. However, conjugation of PEG to PE involves a carbamate linkage that results in a net negative charge on the phosphate group of the PE-PEG and this extra charge could be a concern.⁴⁵ Since poloxamers are uncharged non-ionic polymers, they are good candidates to replace expensive PE-PEG for the preparation of uniform nanodiscs without inducing unwanted charges and are available at very reasonable prices.

Furthermore, the size homogeneity found in our system can also be useful.⁴⁴

The formation of bilayer discs when the system is cooled to $T < T_m$ can be thought of as a nucleation process where the lipids form “crystalline” gel domains. Such a process involves first the nucleation and then the subsequent growth of gel-phase domains. We conjecture that the uniform size of the bilayer discs (Fig. 3A) is the result of successive nucleations. This means that the diffusion rates of poloxamers and lipid molecules inside the bilayer are slow compared to the quick increase of the number of nucleation sites; hence the process is nucleation dominated. The uniform length scale observed in the bilayer discs formed here is a common feature of processes which occur by successive nucleations.^{46–49}

When the temperature is increased beyond T_m , the lipid bilayer changes to fluid-phase and the poloxamer partitions again into the bilayer and no longer prefers to stay on the open edge. Subsequent fusion of these more flexible bilayers results in the re-formation of spherical vesicles which is apparently the favored structure at the elevated temperature though the time scale for this transition is much slower.

Our on-going studies reveal that within the time scale of ITC experiments (typically 200 min), poloxamer partitions primarily into the outer leaflet of the fluid-phase lipid bilayer instead of permeating through the bilayer into the inner leaflet.⁵⁰ Therefore, the poloxamer is likely to have adopted a different configuration in pre-mixed DMPC/P338 liposomes (where P338 can access both the inner and outer leaflets) compared with that added to the pre-formed liposomes (which only has access to the outer leaflet of the bilayer).

Conclusions

In this work we demonstrate that the temperature-sensitive interactions between lipids and poloxamers, as well as the resulting self-assembled structures crucially depend on the lipid bilayer phase. ITC and cryo-EM results reveal that the poloxamer is only incorporated into fluid-phase lipid bilayers rather than gel-phase ones. When the poloxamer is at a concentration above CMC, it disrupts the spherical lipid vesicles into discs at temperatures higher than T_m ; while leaving the vesicles intact at lower temperatures. When the poloxamer concentration is below CMC, the pre-mixed fluid-phase lipid/poloxamer liposomes change from spherical vesicles to bilayer discs when cooled through T_m , due to the phase separation between poloxamers and gel-phase lipids and the fact that the latter is stabilized by poloxamer at the edges. These bilayer discs can reversibly transform back to spherical vesicles upon heating through T_m under which conditions poloxamers and fluid-phase lipid bilayer re-mix. Both liposomes and poloxamers are promising agents for drug encapsulation and delivery, and elucidation of their interactions can improve their design for pharmaceutical use. The bilayer phase-dependant interaction between lipids and poloxamers suggests that the localized heating or cooling can be used to promote or suppress the poloxamer insertion into different cells. In reality, the biological membranes are always in the fluid-phase. Nature uses different lipid compositions to regulate the fluidity or membrane microviscosity in various cells. One important component is cholesterol. Cholesterol can affect

membrane microviscosity and decrease the lipid membrane permeability and is often included in liposomes for drug delivery. Mixing with cholesterol can change the phase behavior of the bilayers and split bilayers into liquid ordered and liquid disordered phases.⁵¹ In the future, model membranes with more realistic compositions such as cholesterol and unsaturated lipids will be used to explore the membrane-poloxamer interactions.

Experimental

Liposome preparation

Liposomes were prepared *via* the freeze-thaw extrusion procedure. Dimyristoylphosphocholine (DMPC) and palmitoylphosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The dry lipid was hydrated by Milli-Q water and vortexed. After ten freeze-thaw cycles, the suspension was passed through a polycarbonate filter having a pore size of 100 nm with the aid of a lipid mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL). During hydration and extrusion, the temperature of the lipid solution was maintained higher than T_m .^{52–54} The size distribution of the resulting liposomes was determined by dynamic light scattering (PD2000DLS, Precision Detectors, Franklin, MA), and was typically found to center at a diameter ~ 140 nm with a standard deviation of 30 nm. The liposomes were stored for at least 4 hours at a desired temperature before a given experiment. Poloxamer 338 (P338) was provided by BASF (Parsippany, NJ). The pre-mixed lipid-poloxamer vesicles were prepared by drying a chloroform solution containing both lipid and poloxamer, then hydrating the mixture film at the desired concentration.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was carried out using a VP isothermal titration calorimeter from MicroCal (Northampton, MA). Each ITC experiment consisted of a series of injections of 10 μ l of the DMPC (or POPC) liposome suspension from a 298.67 μ l syringe into the 1.4045 ml cell loaded with P338 solution at a concentration below its CMC. Both the syringe and the cell equilibrate at the same temperature. The differential power needed to compensate the reaction heat to maintain zero temperature difference between sample and reference cells after the injection of liposome titrant is recorded as a function of time. Integration of the individual calorimeter traces yielded the heat of binding reaction, h_i , of each injection step.

To analyze our data, nonlinear least-square curve fitting was conducted on the model suggested by Heerklotz^{29,55} for the partition of poloxamers into lipid membranes. Briefly, in Heerklotz's model the partition coefficient K is defined in terms of mole fractions:^{29,56}

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

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

The normalized heat q_{obs} , resulting from the total amount of lipid and poloxamer introduced upon the injection is expressed as:

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

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 bilayers: $\Delta H = h_p^l - h_p^w$, X_P^{syrr} denotes the total poloxamer mole fraction in the syringe. The term q_{dil} is the molar heat of dilution of the injectant, and can be measured separately by a control experiment.

Electron cryo-microscopy (cryo-EM)

Particles suspended across a thin layer of vitreous ice were prepared by rapidly plunging into liquid ethane after excess liquid of the specimen solution was blotted off by a filter paper⁵⁷ using the Vitrobot (FEI company, Oregon). To study the morphology of self-assemblies as a function of temperature, the solution was maintained at the desired temperature during transfer and was kept at a pre-set temperature inside the Vitrobot chamber prior to freezing. The specimen grid travelling time between the chamber and the liquid ethane container, as well as the vitrification time in the liquid ethane, is short enough that the particle morphology would be preserved.⁵⁸ Cryo-EM imaging was performed on a JEM 1200 microscope operating at 100 kV with a Gatan liquid nitrogen specimen cryo-holder under a low dose condition. The images were collected on Kodak SO163 films developed in full strength D19 at 20 °C. The films were subsequently digitized in a Nikon Super Coolscan 8000 ED scanner at 6.35 μm /pixel step size. High magnification images were acquired using a JEOL2010F electron microscope (JEOL, Tokyo Japan) with a field emission gun operated at 200 kV and were recorded by a Gatan US4000 4k \times 4k CCD camera (Gatan, Pleasanton CA).

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