EFFECT OF CHAIN LENGTHS OF PEO-PPO-PEO ON SMALL UNILAMELLAR LIPOSOME MORPHOLOGY AND STABILITY: AN AFM INVESTIGATION

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ABSTRACT

The morphology and stability of small unilamellar egg yolk phosphatidylcholine (EggPC) liposomes modified with the Pluronic® copolymer (poly (oxyethylene)-poly (oxypropylene)-poly (oxyethylene) (PEO-PPO-PEO)) with different compositions on mica surface have been investigated using atomic force microscopy. Morphology studies reveal significant morphological changes of liposomes upon incorporating the Pluronic® copolymer. Bilayers are observed for Pluronic® with small hydrophilic (PEO) chain lengths such as L81 [(PEO)2(PPO)40(PEO)2] and L121 [(PEO)4(PPO)60(PEO)4]; bilayer and vesicle coexistence are observed for P85 [(PEO)26(PPO)39.5(PEO)26] and F87 [(PEO)61.1(PPO)39.7(PEO)61.1]; and stable vesicles are observed for F88 [(PEO)103.5(PPO)39.2(PEO)103.5], F127 [(PEO)100(PPO)65(PEO)100], and F108 [(PEO)132.6(PPO)50.3(PEO)132.6]. The micromechanical properties of Pluronic®-modified EggPC vesicles were studied by analyzing AFM approaching force curve. The bending modulus ($k_c$) of the Pluronic®-modified EggPC vesicles increased several-fold compared with that of the pure EggPC vesicles. The significant difference is due to the enhanced rigidity of the EggPC vesicles as a result of the incorporation of PPO molecules and PEO chains. Based on the analysis of onset point by AFM and diameters of vesicles by light scattering, it was concluded that the favorable model to describe the polymer-bilayer interaction is the membrane-spanning model.

KEYWORDS: Small unilamellar vesicle, PEO-PPO-PEO copolymer, stability, morphology, atomic force microscopy.
INTRODUCTION

Liposome technologies are very promising for sophisticated pharmaceutical products such as drug carriers because of their good biocompatibility and low toxicity [1]. There are several technological obstacles to the extensive application of the liposome: short blood circulation time resulting from rapid recognition; rapid sequestration of encapsulated drugs by macrophages, often eliminating the intended beneficial effects and posing considerable risk of toxicity; lack of control over drug release rate; lack of means to override biological barriers (i.e. skin, blood-brain barrier); therapeutically efficient active targeting and others [1, 2]. Thus, liposomes used or intended for drug delivery are often sterically stabilized to prolong their circulation time in the blood stream both in vitro and in vivo [1, 3].

Although some liposome or liposome-like products are on the market, fundamental investigations of sterically stabilized liposomes were started recently [3]. Such knowledge is important to understand and optimize sterically stabilized liposome systems. The stability of the liposomes can be enhanced by biocompatible polymer systems with covalent attachment, such as polyethyleneglycol (PEG) or peptides [3, 4]. PEG is the most commonly used stabilizer. It has been reported that polymer-coated liposomes exhibit a smooth, slow release rate, and remain relatively stable for a longer period of time than others, while, most other technologies exhibit a sharp peak with an immediate drop off or immediate tapering effect. Thus, blood circulation time is increased after incorporating PEG into liposomes [1, 5].

Another alternative to steric stabilization of liposomes is accomplished by adsorption of water-soluble poly (ethylene oxide) - poly (propylene oxide) –poly (ethylene oxide) (PEO-PPO-PEO, Pluronic®) nonionic triblock copolymers. These are characterized by good stabilization ability, low cost,
and biocompatibility [6-16]. PEO-PPO-PEO consists of two hydrophilic PEO chains and a hydrophobic PPO chain, and is commercially available in a number of different segment lengths.

Two preparation methods to introduce the Pluronic® copolymers into liposomes have been investigated [6-10]. The first method is based on incorporation, that is, vesicles were prepared in the presence of copolymer molecules in the hydration and sonication steps. In the second method, the lipid vesicles were first formed, and after sonication they were diluted with copolymer solutions to the desired concentration. It was reported that a more improved sterically stabilized vesicle system with a dramatic increase in vesicle size was obtained by the incorporation method [7-9]. Thus, Kostarelos et al. [7, 10] concluded that it is possible to improve steric stabilization of PC liposomes for drug delivery application by incorporating Pluronic®. However, significant morphological changes were observed by cryo-TEM for this system. It was found that Pluronic® with a large PEO chain length induced the formation of small bilayer fragments, or bilayer disks at low polymer-to-lipid molar ratios [11]. Thus, it is doubtful whether PEO-PPO-PEO triblock copolymers can be incorporated into the liposome without causing serious structural changes. It is quite possible that the structure of the vesicles would rupture or fuse, especially upon adsorption on a substrate. The adsorption of vesicles represents an essential step for many processes such as the transport of small vesicles through large membrane surfaces. To the best of our knowledge, no report was found that investigated the stability and morphology of triblock copolymer modified vesicles on substrate.

Physical stability, in terms of cation-induced flocculation, aggregation, and resistance to osmotic swelling and size change, was found to be qualitatively improved upon incorporation of Pluronic® [9, 10]. An increase in the blood circulation time was also observed for liposomes treated with an aqueous solution of the triblock copolymer, F108 [(PEO)_{132.6}(PPO)_{50.3}(PEO)_{132.6}] [14]. Most of these studies attributed stability enhancement primarily to the PEO repulsive effect, while the PPO chain effect is not addressed extensively. In order to elucidate the effects of PEO and PPO chains on stability
enhancement, a more quantitative approach is desired to measure the micromechanical properties of the individual liposome. AFM force plots were used to investigate the micromechanical properties of small vesicles on a mica surface [17-19].

Two models, a membrane-spanning and an adsorption model, were used to illustrate the interaction between the Pluronic® molecule and the lipid membrane in Kostarelos et al.’s earlier studies [6]. In their subsequent NMR study, they concluded that the PPO was buried inside the vesicle bilayer thus following the membrane-spanning model [7-9]. However, Johnsson et al. suggested that the preferred position of the polymers is still not fully elucidated, and they believe it is possible that both models can occur [11].

In this paper, a series of Pluronic® with different hydrophilic chain (PEO) lengths (2, 4, 26, 61, 100, 103, 132) and different hydrophobic chain (PPO) lengths (40, 50, 60, 65) were selected to study the effect of PEO and PPO chain lengths on the morphology and stability of EggPC vesicles upon adsorption. EggPC lipid was chosen since stable vesicles on a flat mica surface without fusion was observed by AFM [20]. The liposome was prepared using the incorporation method. The size distribution in solution was characterized using light scattering. The morphology characterizations were performed by means of AFM imaging. The micromechanical property, such as bending modulus, was calculated from the AFM force curve. Based on our data, we attempted to address the following questions: 1) Can the Pluronic® copolymer enhance the steric stability of liposomes without serious structural change? 2) What are the important factors for the Pluronic® copolymer to function effectively as a steric stabilizer? and 3) What is the mechanism of copolymer incorporation?
EXPERIMENTAL SECTION

Materials. EggPC with 99% purity purchased from Sigma (St. Louis, MO) was used in this study. Pluronic® was obtained from BASF Corp. (Mount Olive, NJ). The nominal composition and the molecular weight of Pluronic® used in this study are listed in Table 1. Sodium chloride (ACS grade), chloroform (99.9%), methanol (HPLC grade), and acetone (HPLC grade) were purchased from Fisher Chemicals (Fairlawn, NJ). Grade V5 ruby muscovite mica from Ted Pella, Inc. (Redding, CA) was used as the substrate because it is molecularly smooth. In aqueous solutions, the mica cleavage surface becomes negatively charged. Deionized water with a resistivity of 18 MΩ-cm was obtained from a Barnstead Nanopure water purification system (Dubuque, IA). All glassware used in this study was cleaned, and rinsed thoroughly with deionized water prior to use.

Preparation of the Pluronic®-modified EggPC vesicles. The incorporation method was used to prepare the Pluronic®-modified EggPC vesicles [6, 7, 10]. EggPC lipid was used since the incorporation was more readily obtained with a partially unsaturated lipid membrane [15]. The EggPC concentration was kept at 0.5 mg/ml. The concentration of all Pluronic® studied was fixed at 0.02 % w/w (well below the critical micelle concentration (CMC)) [21]. It has been reported [6,10] that Pluronic® with 0.06 % w/w or less can be fully incorporated into the vesicles, therefore, a conservative concentration of 0.02 % w/w Pluronic® was used to ensure complete incorporation. Pluronic®-modified EggPC liposomes were prepared by dissolving the EggPC and the Pluronic® in chloroform/methanol (2:1 v/v). The solvent was removed with nitrogen, followed by drying in a desiccator connected with a rotary vacuum pump for 30 minutes. A multilamellar vesicle (MLV) solution was obtained by redissolving the EggPC and the Pluronic® mixture in a 20 mM NaCl solution. Sonicated unilamellar vesicles (SUVs) were produced from the MLV suspension by sonication to clarity (about 4 hours) in a
Branson 2200 bath sonicator (Danbury, CT). Sonicated samples were centrifuged for one hour at 16,000 rpm to remove large lipid fragments in a Sorvall OTD70B ultraspeed centrifuge (Wilmington, DE).

**Light scattering.** Particle size distribution of vesicles in a buffer solution (20 mM NaCl) was measured using a Nicomp 380 ZLS light scattering instrument (Santa Barbara, CA). Polystyrene Nanospheres™ with 32 ± 1.3 nm diameters from Duke Scientific Corp. (Palo Alto, CA) were used as a calibration standard. The light scattering cell is from VWR Scientific (West Chester, PA). A He-Ne laser (632.8 nm) was used. Data were taken at 23 °C.

**AFM characterization.** AFM imaging and force measurement were conducted using a Nanoscope IIIa atomic force microscope from Digital Instruments Company (Santa Barbara, CA) equipped with an E scanner with a maximum scan area of 16 µm². The scanner was calibrated following the standard procedures provided by Digital Instruments. The fluid cell (Digital Instruments) was washed with deionized water, ethanol, and deionized water before each experiment. Freshly cleaved mica was mounted on a stainless steel disk using a sticky tab (Latham, NY). After the substrate was brought close to the AFM tip, the freshly prepared vesicle solution was injected through silicone rubber tubing into the fluid cell, sealed by a silicone rubber O-ring. The vesicle solution was allowed to incubate on mica at room temperature for one hour. Excess vesicles were removed by flushing the fluid cell with buffer solution followed by pure water, and the images were obtained in pure water because a higher image contrast can be obtained [22]. The microscope was allowed to equilibrate thermally for thirty minutes before imaging. Scanning rate between 1 to 5 Hz was used. Room temperature was maintained at 22 ± 1 °C. All the images are flattened and lowpassed unless specified.

Images were recorded in fluid by tapping mode using standard silicon nitride (Si₃N₄) integral tips (NP type) (Digital Instruments) mounted on cantilevers with a manufacturer-specified spring constant of 0.22 N/m, length of 120 µm, width of 15 µm, and a nominal tip radius curvature between 20
and 40 nm. The radius of the tip was calibrated to be $33 \pm 7$ nm by imaging the TGT01 gratings from Mikromasch Inc. (Portland, OR) [23]. The spring constant of the cantilever was calibrated using the deflection method against a reference cantilever purchased from Park Scientific Instruments Company (Santa Barbara, CA) of known spring constant (0.157 N/m) [24]. A calibrated spring constant of $0.17 \pm 0.05$ N/m was used in all force curve calculations. Force curves were obtained in contact mode only. Multiple force curves were obtained around and on the vesicle to ensure that the force curve represented the interaction between the tip and the vesicles. In our experiments, we found that the retraction force curve is quite complicated because it involves the resealing of portions of the bilayer, the adhesion force between the tip and the vesicles, as well as the elongation of bilayer-vesicle molecules. On the other hand, the approaching force curve provides a wealth of stepwise micromechanical deformation events on the bilayer or liposomes. Thus, only the approaching force curves were discussed. Based on our previous experiences [18-19], force curves without the characteristic behavior attributed to bilayer (one jump-in point) and to vesicles (two jump-in points) were rejected. The characteristic force distance curves are quite consistent, and at least five representative force curves on each sample were averaged to determine the onset of forces and the mechanical properties. The total time for a complete cycle was ~ 1 s. The force calibration plot is converted to a force versus distance plot by defining the zero force point and zero separation point [25].

**Data analysis.** The data analysis has been described elsewhere [17] and will be briefly summarized here. Force curve data was fitted to the Hertz contact model by assuming a spherical shape for the tip [17, 26]. The indentation, $\delta$, from the difference between the cantilever distance $z - z_o$ and cantilever deflection $d - d_o$ is described as Equation 1

$$|z - z_o| - (d - d_o) = \delta = A(d - d_o)^{2/3} = 0.825 \left[ \frac{k^2 (R_{tip} + R_{ves})(1 - v_{ves}^2)}{E_{ves}^2 R_{tip} R_{ves}} \right]^{1/3} (d - d_o)^{2/3}$$ (1)
Where $E_{ves}$ is the Young’s modulus of the vesicle, $R_{tip}$ and $R_{ves}$ are the radius of the tip and vesicle, respectively, $\nu_{ves}$ is the Poisson’s ratio of the vesicle, and $k$ is the cantilever spring constant. The Poisson’s ratio for the vesicle is assumed to be 0.5 [17, 27, 28]. The spring constant and the tip radius were calibrated to be 0.17 N/m and 33 nm, respectively. The vesicle radius is taken to be equal to $z_0/2$. Experimental data can be fitted to Equation 1 with two fitting parameters $A$ and $z_0$ using SigmaPlot®. In general, the fitted $z_0$ value was found to be consistent with the visually examined contact point. Thus, $z_0$ in our experiment is determined by the onset point of the repulsive force. $E_{ves}$ is then computed from $A$ (obtained by least square fitting).

Bending modulus $k_c$ is deduced from Young’s modulus based on Equation 2 [29]

$$k_c = \frac{E_{ves} h^3}{12(1 - \nu_{ves}^2)}$$

(2)

where $h$ is the bilayer thickness.
RESULTS

Size characterization of Pluronic®-modified EggPC vesicles. Table 2 shows the mean vesicle diameters measured by light scattering for different Pluronic®-modified EggPC vesicles. Generally, there is an increase in vesicle size after copolymer incorporation compared with pure EggPC. For the Pluronic®-modified vesicles (L81, P85, F87, and F88) containing the same PPO chain length (~40 units), a slight increase of diameter was observed with the increasing of PEO chain length from 2 (L81) to 103.5 (F88). For another set of modified vesicles (L81, F108, and L121) with different PEO, PPO chain length, a significant size change was observed as a function of PPO chain length. For example, the vesicle diameter of L121-modified vesicles (PPO = 60) increased ~ 30 nm compared with L81 (PPO = 40). Kostarelos et al. reported a number of light scattering studies on Pluronic®-modified vesicles [6 – 10] and found that the vesicle size increased after introducing Pluronic® during vesicle preparation. They initially claimed that PEO hydrophilic chains seem to be a deciding factor for changes in the vesicle size observed. Higher PEO chain length leads to a much higher increase in vesicle diameter [6]. In their subsequent work, they revised their previous conclusion that the vesicle size change is determined by factors other than simply PEO chain length [10]. Our data showed that a hydrophilic PEO chain has a slight effect on vesicle size; and a hydrophobic PPO chain with short PEO chain lengths exhibits a more significant effect on the vesicle size. The hydrodynamic radius of vesicles increased with PEO chain lengths because of the dangling PEO chains outside of vesicles. However, the increase levels off when the PEO chain length reaches a certain value. It is because a dangling PEO chain tends to coil randomly. On the other hand, when a PPO chain is incorporated into the lipid bilayer, the bilayer structure expands and thus increases the vesicle size. Comparing the vesicle’s size for EggPC/L81 and EggPC/L121, both have a short PEO chain (2 and 4 for L81 and L121, respectively), the difference in vesicles size (from 40 nm to 74 nm) can be attributed to PPO chain length (40 and 60 for L81 and L121, respectively). However, the same trend was not observed when we compared F88 and F127, which have different PPO chain lengths with similar PEO chains. The possible
reason is that the longer PEO chain length of these systems can act as a tighter net on the outside of vesicles, thus limiting the increase of vesicle size due to PPO chain incorporation into the bilayer. The size of the adsorbed vesicles remaining intact on a mica surface was also studied by the AFM image. The measured vesicle diameter for EggPC/F127, EggPC/F88, and EggPC/F108 is 37.0 ± 6.0 nm, 35.0 ± 7.0; and 41.0 ± 12.0 nm, respectively. The vesicle diameters measured by the AFM image are smaller than vesicles in solution measured by light scattering. This can be attributed to the fact that light scattering measured the hydrodynamic diameter of vesicles, for which the PEO chain has significant effect on vesicle size; while AFM measured the vesicles adsorbed on a mica surface.

**Morphology of Pluronic ®-modified EggPC vesicles.** Kostarelos et al. reported on the successful incorporation of Pluronic® on vesicles and called attention to potential drug delivery applications in a series of publications about Pluronic®-modified lipid systems [6-10]. All their work was done in solution. This study used AFM to characterize the morphology of a series of Pluronic®-modified EggPC vesicles on a mica substrate. Figure 1 shows representative AFM images from the respective lipid/polymer (0.02% w/w ratio) systems upon adsorption to illustrate different liposomes morphologies on a mica surface. It is obvious that the morphology of the modified liposomes depends critically on the composition (PEO/PPO chain length) and the molecular weight of the respective copolymer. As shown in Figures 1a and 1b, large bilayer patches, with other fragments were detected for EggPC/L81 and EggPC/L121. When the hydrophilic block (PEO) chain length increased, both bilayers and vesicles (spherical particles in the images) were observed for EggPC/P85 (figure not shown) and EggPC/F87 (Figure 1c). Further increase in PEO chain length resulted in primarily spherical vesicles as shown in Figure 1d (EggPC/F127), Figure 1e (EggPC/F88), and Figure 1f (EggPC/F108).

Morphological changes of liposomes after copolymer incorporation were also observed by Cryo-TEM [11]. Cryo-TEM visualizes the aggregation morphology in dilute aqueous solutions. Table 3 compared morphological changes of Pluronic®-modified EggPC systems observed by AFM in this study
and by c-TEM reported by Johnsson et al. [11], the results are quite different. For example, formation of bilayer disks and liposomes were observed for F87, F108, and F127, and compacted liposomes were observed for P85 and P105 in solution by cryo-TEM. Whereas in our study, both bilayer and liposomes were observed for P85, and only vesicles were observed for F127 and F108.

It was reported that the AFM force curves on lipid bilayer and on the individual vesicle were very different [18]. The force curve on a bilayer is characterized by one jump-in point and short repulsive force, while the force curve on a vesicle is characterized by two jump-in points and two long range repulsive force regimes [18]. The jump-in point on the approaching force curve was interpreted as a penetration of the AFM through the bilayer as described elsewhere [30]. AFM force curves were obtained to further illustrate whether the tip is on vesicle or on bilayer of the Pluronic®-modified EggPC system. Figure 2 shows typical force curves observed for EggPC, EggPC/L81, EggPC/L121, EggPC/P85, and EggPC/F87. At a large separation, no force was detected. As the separation distance was gradually reduced, a repulsive force became measurable and increased in magnitude. The repulsion reached a maximum value and was immediately followed by a discontinuity. At the end of the discontinuity, the repulsion force increased rapidly with further compression. These are typical characteristic properties of a force curve on lipid bilayers previously reported [18, 30]. Comparing the force curves of the Pluronic®-modified EggPC bilayer, it was found that the onset point of the repulsive force increased as a function of the PEO chain length.

To characterize the properties between different bilayer systems, force curves were analyzed to obtain onset points of repulsive force and jump-in distance data for EggPC [18], EggPC/L81, EggPC/L121, EggPC/P85, and EggPC/F87 bilayers (Figure 3a). The onset point is defined as the separation distance at which the tip begins to feel the repulsive force of the bilayer during approaching [18]. This repulsive force encountered can be attributed to the steric repulsion that originates from the force necessary to confine and desolate the PEO chain of copolymers. However, the onset of this steric
repulsion corresponds to the undisturbed thickness of the Pluronic®-modified EggPC bilayer sandwiched between the tip and the substrate (Figure 3b). The discontinuity in the force curve, often called the jump-in process, corresponds to the bilayer thickness (Figure 3c). Figure 3a shows that the onset point from the AFM force curve on the Pluronic®-modified EggPC bilayer depends on the composition of the Pluronics®. A clearly increasing trend of the onset point of the repulsive force was observed with increasing PEO chain length. It indicated that the undisturbed bilayer thickness as measured by AFM was modified after Pluronic® incorporation and the change reflects the contribution from the PEO chain. On the other hand, the difference between the jump-in points was insignificant, which suggested that the bilayer is flexible and the dangling PEO chains are easily compressed. Wang et al. also used the AFM force curve measurement to study the steric interaction between the Pluronic® copolymer adsorbed at the interface of an n-octadecyltrichlorosilane monolayer surface [31]. A similar finding about the barrier thickness increasing steadily with the PEO chain lengths was reported.

Figure 4 is the force curve captured on EggPC/F88, EggPC/F127, and EggPC/F108 vesicles. These force curves have a similar characteristic to the force curve of the EggPC vesicles with two repulsive regimes and two jump-in points [18]. The two repulsive regimes are interpreted as the tip feels the upper and lower bilayer of vesicles, and the two discontinuities are inferred as an abrupt jump of the AFM tip across the top and bottom portion of the bilayer during the approach process [18]. The slope of the first repulsive force regime can be used to calculate the elastic properties of a sample. The slope of EggPC/F88 and EggPC/F127 (~ 100 PEO units) is found to be lower than that of EggPC/F108 (132 PEO units). The slope of EggPC/F127 is close, but slightly higher than that of EggPC/F88. Both F127 and F88 have similar PEO size while the PPO chain of F127 is longer (Table 2).

**Stability of Pluronic®-modified EggPC vesicles.** For the Pluronic®-modified vesicle system, the physical stability was found to be improved qualitatively due to the PEO repulsive effect [9, 10]. The PPO chain effect is not addressed extensively. In order to elucidate both the effect of PEO and PPO
chains on the stability enhancement, AFM force curves were obtained to investigate the micromechanical properties of the Pluronic®-modified EggPC vesicles. The mechanical properties of phospholipid membranes can be related to many aspects of the behavior of phospholipid vesicles, such as their formation, stability, size, shape, fusion, and budding processes. Bending rigidity is a fundamental, characteristic mechanical property of vesicles which is related to the stability and strength of the bilayer [32].

The slope of the first repulsive force regime in Figure 4 was used to calculate the elastic properties of the Pluronic®-modified EggPC vesicles based on Eq. 1 and Eq. 2. Table 4 lists the bending modulus of pure EggPC [18], EggPC/F88, EggPC/F127, EggPC/F108 (only those systems which form vesicles were considered), and EggPC/Cholesterol (1:1 molar ratio) [19]. The bending modulus of the Pluronic®-modified EggPC systems was increased by an order of magnitude compared with that of pure EggPC. The magnitude of increase is comparable with cholesterol/EggPC vesicles. These data show that the bending modulus of the Pluronic®-modified EggPC is a function of PEO and PPO chain lengths. The dramatic bending modulus increase can be attributed to the PPO block incorporation and the PEO chain on the surface.
**DISCUSSION**

**Copolymer and lipid bilayer interaction.** Liposomes studied in this paper were prepared using the incorporation method, i.e., the copolymers were presented during liposome preparation. The hydrophobic PPO chain is incorporated into the liposome bilayer membrane, and the hydrophilic PEO chain extends into the surrounding solution in different possibilities, as shown schematically in Figure 5a. There are two possible configurations for the copolymer and bilayer interaction [6, 10, 11]. In model A, or the membrane-spanning model, the PPO chains align themselves parallel to the acyl chains of the lipid layers, leaving the PEO chains at both sides of the vesicles bilayer. In model B, or the adsorption model, the PPO chains are incorporated inside the bilayer in a flat configuration, leaving two PEO chains dangling on the same side of the lipid bilayer [10, 11]. It has been suggested that the configuration follows the adsorption model (Model B) when the addition method was used to prepare copolymer-modified liposome [10]. Using NMR, the broadening effect of the $^{31}$P signals (phosphatidylcholine) and the $^{13}$C NMR signals of the methyl groups (PPO) indicated molecular mobility was restricted due to copolymer incorporation inside the bilayer [8, 9]. Moreover, an increase in hydrodynamic radius and a reduction in the $\zeta$ potential were observed suggesting incorporation of the PPO chain into the bilayer [10]. On the other hand, Johnsson et al. suggested no concrete evidence was provided to indicate whether one of the models, or any combination of these models, is likely to be accurate. Thus, the preferred configuration of the polymers is not, at present, conclusive [11].

Before discussing the most likely interaction model between copolymer and lipid molecules, it is helpful to understand the possible PEO chain conformations. For the PEO chains, there are two possible conformations (Figure 5b), depending on the average distance, $d$, between grafting sites, and the Flory radius [33], $R_F$, for a coiled chain. $R_F$ can be expressed as $aN^{3/5}$ in a good solvent with $N$ monomer units of length $a$, where $a = 0.35$ nm (CH$_2$CHO). When the distance $d > R_F$, the chains develop as
random coils or mushrooms, whereas for $d < R_F$, the chains adopt a fully extended state or brush regime. The average grafting distance, $d$, is a function of polymer concentration. Thus, there is a transition concentration determining the conformation of the PEO chain. At a low concentration (below transition concentration) the hydrophilic PEO polymer chains are in a random-coil conformation, while an extended brush conformation is formed at a high grafting concentration (above the transition concentration) because of lateral repulsion [33, 34]. The transition concentration could be calculated for different copolymers based on the following assumptions [11, 33]: 1) the number of PEO chains per unit area $T$ is approximately given by $T = 1/R_F^2$, and 2) an average area per lipid molecule is 0.65 nm$^2$ [35].

Table 5 shows two series of theoretical transition concentrations for different copolymers. The first series was based on model A (membrane-spanning model, Figure 5a), and the second series was based on model B (adsorption model, Figure 5a). In model B, if the two PEO chains are dangling on the same side of the lipid bilayer, then the transition concentrations should be less than those adopting model A. The PEO chains would be present on both sides of the vesicle. However, more copolymer molecules tend to be on the outside layer of the vesicles because of curvature and chain repulsion, as well as a larger surface area. A conservative estimate of the distribution of the polymer chain can be calculated based only on the inner and periphery vesicle bilayer surface areas using the following assumptions. Assuming a typical vesicle with a 20 nm radius and a vesicle bilayer thickness of 5 nm, the inner radius is around 15 nm. Thus,

the surface area of the interior of the vesicle ($S_{\text{interior}}$) is: $S_{\text{interior}} = 4\pi r_{\text{interior}}^2$

the surface area of the periphery of the vesicle $S_{\text{outer}}$ is: $S_{\text{outer}} = 4\pi r_{\text{outer}}^2$

and the possible distribution ratio of the polymer chains is: $\frac{S_{\text{outer}}}{S_{\text{interior}}} = \frac{4\pi r_{\text{outer}}^2}{4\pi r_{\text{interior}}^2} = \frac{16}{9}$

On the other hand, the distribution ratio of the PEO chains based off of Model A is 1/1 because of “membrane spanning”. Thus, the calculated theoretical transition concentrations are less than those adopted by model A.”
From Table 5, it is obvious that the PEO chain of L81 and L121 will adopt a random coil conformation since the experimental concentration is lower than the theoretical transition concentration based on both models. If the interaction between the polymer molecules and the lipid bilayer follows model B, then all the experimental concentration for P85, F87, F88, F127, and F108 is much higher than the theoretical transition value. Thus a fully extended chain is expected for those systems based on model B. On the other hand, if model A is dominant, P85 will be in random coil; and for F87, F88, F127, and F108, the experimental concentrations are very close to the transition concentration, and thus, PEO chain conformations can be in random coil or fully extended. Based on the above analysis, if experimental data obtained suggested predominately random coil configuration, then model A dominates, and vice versa.

Figures 6 and 7 compare experimental data for the onset point of bilayer (Figure 3a) and the diameter of vesicles from light scattering (Table 2) with calculations based on a fully extended chain and random coil configurations of the PEO, respectively. The theoretical thickness of the undisturbed bilayer and the vesicle diameter are calculated using following equations:

- Fully extended chain length, $L_f$:
  \[ L_f = a \times N \]

- Random coil chain length, $L_r$:
  \[ L_r = a \times N^{3/5} \]

where $a$ is the monomer chain length ($a = 0.35$ nm for (CH$_2$CHO)), $N$ is the number of monomer unit; therefore,

- Pluronic$^\text{®}$-modified EggPC bilayer thickness: $L_{f\text{bilayer}} = L_0 + 2L_f$ (nm);

  or $L_{r\text{bilayer}} = L_0 + 2L_r$ (nm)

where $L_0$ is the undisturbed EggPC bilayer thickness, $L_{f\text{bilayer}}$ is the bilayer thickness with a fully extended PEO chain, and $L_{r\text{bilayer}}$ is the bilayer thickness with a random coiled PEO chain.

As for the vesicle diameter:

- The diameter of the Pluronic$^\text{®}$-modified EggPC: $D_{f\text{vesicle}} = D_0 + 2L_f$ (nm);
or $D_{\text{vesicle}}^r = D_0^r + 2L_r$ (nm).

where $D_0^r$ is EggPC vesicle diameter incorporating the estimated effect of the PPO chain, $D_{\text{vesicle}}^r$ is the vesicle diameter with a fully extended PEO chain, and $D_{\text{vesicle}}^r$ is the vesicle diameter with a random coiled PEO chain.

Figure 6 and 7 show that experimental data points are in good agreement with calculated values based on the random coil chain. For L81 and L121, since the PEO chain length is very short (2 and 4 respectively), it is not surprising to find the data based on a random coil chain consistent with the data based on a fully extended chain. While for P85, F87, and F88, the data from the onset point of repulsive force and/or light scattering show that the PEO chains should be in a random coil instead of being fully extended. This strongly suggests membrane-spanning (model A) represents the most likely interaction model between lipid and copolymer molecules. Moreover, all Pluronics® used in our study have sufficient PPO chain length (> 40 PPO repeat unit) to span the lipid bilayer [36].

**Morphology change upon adsorption.** Different morphologies and morphology changes of liposomes were observed upon Pluronic® modification (see Table 3). The transition from vesicles to open structure and to an intermediate domain in which both vesicles and bilayer coexist were also observed and described for phospholipids/cholesterol/PEG-PE [37] and phospholipid/Pluronic® systems [11]. The transition mechanism was explained in terms of vesicle-to-micelle transition [37]. Our AFM results on the liposome morphology do not fully agree with those obtained by cryo-TEM [11]. In their experiment, bilayer and liposomes were observed for a copolymer with a long PEO chain length, while for P85 only liposome existed. Some possible reasons for this inconsistency can be attributed to the difference in the copolymer concentration and the physical state of liposomes. The high concentration in their system led to bilayer formation of EggPC/F87, EggPC/F127 and EggPC/F108. Moreover, the morphology of liposomes in solution is likely to be different from that on a surface. Cryo-TEM visualizes the aggregation morphology in dilute aqueous solutions directly, while AFM observes the
morphology of vesicles upon adsorption. It is believed the vesicles undergo a non-trivial adhesion transition from a free to a bound state upon adsorption [38].

With the insertion of PPO chains, the geometric packing structures of the phospholipid bilayer changes. From the light scattering results, EggPC/L81 and EggPC/L121 was found to maintain vesicle shape in solution. However, bilayers were observed when these vesicles were adsorbed on the mica substrate [Figure 1a and 1b]. The morphological change upon adsorption can be due to the relatively higher molar concentration that was used for L81 and L121 (12.2 mol % and 8.2 mol %, respectively). High PPO chain concentration resulted in stronger lateral tension in the polymer/headgroup region. The tension can be relaxed by curving the surface or by a transition into a bilayer disk. As the liposome itself depends on physical forces for retaining its structure, it is sensitive to compositional changes imposing any kind of packing disorder [11]. Thus, high grafting concentrations could affect the structural properties of the liposome and eventually lead to its disruption because of the higher lateral repulsion.

Figure 8 shows a schematic of the steric stabilization effect of a copolymer on a liposome. A block copolymer may aggregate to form micelles (core/shell structure) consisting of a swollen core of insoluble blocks surrounded by a flexible fringe of soluble block [39]. As an analogy to the polymer micelle, we proposed that the random coil PEO chains dangling outside of vesicles have a shell-like effect to provide steric stabilization to the vesicle. The shell-like structure also serves as a kind of “net” or “coating” of the vesicles [3]. When the individual modified vesicle was adsorbed on the mica surface, the stabilization effect was not significant for a copolymer with a short PEO chain length (EggPC/L81, EggPC/L121), since PEO chains are too short to provide an effective steric barrier (shell-like) in the form of a random coil polymer chain (Figure 8a). Thus, bilayers were found for those systems upon adsorption on mica. On the other hand, with longer PEO chain length (>19 units), a steric barrier can be formed steadily [31]. For EggPC/P85 and EggPC/F87 (Figure 1c and 1d), both bilayer and vesicles coexist. For these systems, the random coils served as a rough shell or “coating” to enhance the stability
of the vesicle [Figure 8b]. However, for a system with mid-size PEO chain length, such as P85 and F87, the shell-like structure may not be strong enough to keep all liposome intact upon adsorption. Thus, bilayer and vesicles coexisted under these circumstances. For a copolymer with longer PEO chain length (F88, F108 and F127), the shell-like wall structure was strong enough to keep all liposome intact upon adsorption.

**Stability improvement.** It is well known that the stability of polymer-coating liposome is improved in terms of steric repulsive force between the polymer chains dangling outside of the liposome. In earlier studies, the steric stabilization effect was described by aggregation and fusion between intervesicles in the solution [5-10]. Kostarelos et al.’s drew a similar conclusion by comparing prolonged vesicle durability against flocculation and ζ-potential change [9]. On the other hand, our results showed the micromechanical properties of the Pluronic®-modified EggPC membrane also increased dramatically (Table 4). It was reported that the bending and mechanical properties of vesicles from a diblock polymer exceeds those of phospholipid liposomes by a factor of 5 or more [40]. Thus, the significant bending modulus improvement can be attributed to PEO (incorporated) and PPO chains (dangling outside of EggPC vesicles). As discussed earlier, the shell-like structure due to long PEO chains dangling outside of vesicles would make vesicles more rigid, thus resulting in a higher bending modulus, as shown in Table 4 for F88/EggPC, F127/EggPC, and F108/EggPC.

On the other hand, since the preferred interaction between copolymer molecules and lipid is the membrane-spanning model; the hydrophobic PPO chain orients itself parallel to the acyl chains of the lipid. Thus, a more ‘rigid’ bilayer was constructed due to restrictions of the lipid acyl chain mobility. This phenomenon was similar to cholesterol-containing liposomes, in which the bending modulus was observed to increase dramatically [19]. The PPO block incorporated inside the bilayer, sandwiched between lipid molecules, can result in a similar rigidifying effect that is responsible for the observed bending modulus improvement. For the PPO effect on bending modulus improvement, F127 was used
to compare with F88. Both have similar PEO chain lengths and different PPO chain lengths. The bending modulus obtained on F127 vesicles was \((1.64 \pm 0.28) \times 10^{-19}\) J, slightly larger than that of F88 \(((1.50 \pm 0.40) \times 10^{-19})\). The increase can be attributed to the longer PPO chain length of F127. While comparing the bending modulus of F127 and F108, the circumstances are somewhat complicated since both have different PPO and PEO chain lengths. The bending modulus of F108 is higher than that of F127 although the PPO chain length of F108 is smaller. However, F108 has a longer PEO chain and the stiffer shell-like “coating” on the vesicles seems to contribute to the bending modulus increase of the EggPC/F108 system. By comparing the bending modulus for EggPC/F88, EggPC/F127 and EggPC/F108, it can be postulated that the improvement of the bending modulus of the Pluronic®-modified EggPC system is due to the PPO chain insertion into lipid bilayer and shell-like structure over the vesicles. Furthermore, the bending modulus results indicated that the copolymer provides not only a steric stabilization of liposome by polymer chain repulsion but also an enhancement of the micromechanical properties of bilayer.

**CONCLUSIONS**

Atomic force microscopy (AFM) imaging and force curve were employed successfully to provide a direct observation and quantitative measurement of adsorbed Pluronic®-modified EggPC vesicles morphology and stability as a function of PEO and PPO chain lengths. The results in this paper should provide some guiding principles for selecting the appropriate Pluronic® copolymer to provide steric stabilized vesicles and to tailor the lipid membrane stiffness. The following conclusions are obtained:

1. Morphology of adsorbed Pluronic®-modified EggPC depends mainly on PEO chain length. Pluronic® with longer PEO chain length is desirable for the Pluronic®-modified EggPC systems to obtain stable vesicle shape without rupture upon adsorption. Bilayer
formation is observed for Pluronic® with a small PEO chain. Vesicles and bilayers coexistence are also observed for the intermediate PEO chain.

(2) Stabilization enhancement as shown by the bending modulus was found to correlate with two factors: one is a shell-like structure effect over the liposome; the other is the increased rigidity of the bilayer as a result of PPO block incorporation.

(3) Based on transition concentration calculations, AFM, and light scattering results, the interaction between Pluronic® and lipid molecules should follow the membrane-spanning model.
ACKNOWLEDGMENTS

We thank the Petroleum Research Fund for grants 36149-ACS and 33036-ACS, administered by the American Chemical Society, the Michigan Life Science Corridor, and the Institute for Manufacturing Research at Wayne State University for financial support. We also thank Jerome S. Jourdan at BASF Wyandotte for help with the light scattering measurements.
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Table 5. Theoretical transition concentration and experimental concentration for different Pluronic® copolymers (% mol/mol)
FIGURE CAPTIONS

Figure 1 AFM amplitude images of typical Pluronic®-modified EggPC systems. a) EggPC/L81, bilayer patches; b) EggPC/L121, bilayer patches; c) EggPC/F87, bilayer patch and vesicles coexist; d) EggPC/F127; e) EggPC/F88; f) EggPC/F108. Spherical vesicles are dominant for EggPC/F127, EggPC/F88, and EggPC/F108. Z scan size is 30 nm.

Figure 2 Force curves on EggPC, EggPC/L81, EggPC/L121, EggPC/P85 and EggPC/F87 bilayer. There is one repulsive regime with one jump-in point. The EggPC bilayer was prepared by extrusion method [Reference 18].

Figure 3 a) Comparison of onset point (square) and jump-in point (circle) for EggPC, EggPC/L81, EggPC/L121, EggPC/P85 and EggPC/F87 bilayer systems. The error bar is calculated based on the standard deviation of jump-in distance and onset point from a number of force curves. b) Schematic of onset point of repulsive force on bilayer (undisturbed bilayer thickness); c) Schematic of jump-in point (bilayer thickness). The onset point of the repulsive force increased with increasing PEO chain length and the jump-in point is around 4 ~ 5 nm.

Figure 4 Force curves on EggPC/F88, EggPC/F127 and EggPC/F108 vesicle. The empty triangle is the force curve on EggPC/F88, the full square is the force curve on EggPC/F127, and the empty circle is the force curve on EggPC/F108. The force curves on vesicles have two repulsive regimes with two jump-in points.

Figure 5 a) Schematic view of two polymer/lipid interaction models. Model A is membrane-spanning which hydrophilic PEO chains reside on either side of lipid bilayer. In model B, adsorption model, both PEO chains stick out on the same side of the lipid bilayer. b) Schematic view of two PEO chain
conformations. One is a random coil ($d>RF$), and the other one is fully extended ($d<RF$). (Reference 6, 9, 10)

Figure 6 Comparison of onset point distance based on experimental and theoretical calculations. The number in the parenthesis followed by sample name in X-axis is the PEO chain length. The straight line and dotted line are theoretical calculations of the vesicle’s diameter based on fully extended and random coil configurations of PEO chains, respectively. The onset point is calculated as $L_{f,\text{bilayer}} = \text{undisturbed EggPC bilayer thickness } L_0 + 2 L_f (\text{nm})$ (fully extended); or $L_{r,\text{bilayer}} = L_0 + 2 L_r (\text{nm})$ (random coil). The scatter square is the onset point measured by AFM force curves. The experimental data lie in the vicinity of the calculated size based on the random coil configuration.

Figure 7 Comparison of vesicles size based on experimental and theoretical calculations. All the copolymers have similar PPO chain length. The number in the parenthesis followed by the sample name in the X-axis is the PEO chain length. The straight line and dotted line are theoretical calculations of the vesicle’s diameter based on fully extended and random coil configurations of PEO chains. The diameter of the Pluronic®-modified EggPC was calculated as $D_{f,\text{vesicle}} = D_0' + 2 L_f (\text{nm})$ (fully extended); or $D_{r,\text{vesicle}} = D_0' + 2 L_r (\text{nm})$ (random coil). The scatter square is the vesicles size measured by light scattering. The experimental data lie in the vicinity of the calculated size based on the random coil configuration.

Figure 8 Schematic of steric stabilization of a copolymer on liposome. a) System with short PEO chain: higher PPO concentration make the structure of liposome change and possible rupture happens upon adsorption; b) System with long PEO chain: shell-like structure formed as a results of longer PEO random coil chain outside of liposome makes the liposome stiff and rigid.
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Table 1. Molecular weight and composition of Pluronic® copolymers provided by BASF

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>Composition</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>L81</td>
<td>(PEO)$<em>2$(PPO)$</em>{40}$(PEO)$_2$</td>
<td>2750</td>
</tr>
<tr>
<td>L121</td>
<td>(PEO)$<em>4$(PPO)$</em>{50}$(PEO)$_4$</td>
<td>4400</td>
</tr>
<tr>
<td>P85</td>
<td>(PEO)$<em>{26}$(PPO)$</em>{39.5}$(PEO)$_{26}$</td>
<td>4600</td>
</tr>
<tr>
<td>F87</td>
<td>(PEO)$<em>{61.1}$(PPO)$</em>{39.7}$(PEO)$_{61.1}$</td>
<td>7700</td>
</tr>
<tr>
<td>F127</td>
<td>(PEO)$<em>{100}$(PPO)$</em>{65}$(PEO)$_{100}$</td>
<td>12700</td>
</tr>
<tr>
<td>F88</td>
<td>(PEO)$<em>{103.5}$(PPO)$</em>{39.2}$(PEO)$_{103.5}$</td>
<td>11400</td>
</tr>
<tr>
<td>F108</td>
<td>(PEO)$<em>{132.6}$(PPO)$</em>{50.3}$(PEO)$_{132.6}$</td>
<td>14600</td>
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</table>
Table 2. Diameter of Pluronic®-modified EggPC vesicles (0.02% w/w copolymer) measured by light scattering

<table>
<thead>
<tr>
<th>Sample</th>
<th>PEO chain units</th>
<th>PPO chain units</th>
<th>Diameter (nm)</th>
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<tbody>
<tr>
<td>EggPC *</td>
<td>/</td>
<td>/</td>
<td>37.0 ± 8.0</td>
</tr>
<tr>
<td>EggPC/L81</td>
<td>2</td>
<td>40</td>
<td>41.0 ± 6.0</td>
</tr>
<tr>
<td>EggPC/P85</td>
<td>26</td>
<td>39.5</td>
<td>46.0 ± 5.0</td>
</tr>
<tr>
<td>EggPC/F87</td>
<td>61.1</td>
<td>39.7</td>
<td>40.0 ± 11.0</td>
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<tr>
<td>EggPC/F127</td>
<td>100</td>
<td>65</td>
<td>48.0 ± 10.0</td>
</tr>
<tr>
<td>EggPC/F88</td>
<td>103.5</td>
<td>39.2</td>
<td>53.0 ± 3.0</td>
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<td>EggPC/F108</td>
<td>132.6</td>
<td>50.3</td>
<td>49.0 ± 9.0</td>
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<tr>
<td>EggPC/L121</td>
<td>4</td>
<td>60</td>
<td>74.0 ± 14.0</td>
</tr>
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</table>

* Data from ref. 18
Table 3. Morphology observed for Pluronic®-modified EggPC by AFM in this study and by c-TEM by Johnsson et al.

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>Composition</th>
<th>Morphology</th>
<th>Conc. (mol%)</th>
<th>Method</th>
<th>State</th>
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<tr>
<td>L81</td>
<td>2-40-2</td>
<td>Bilayer</td>
<td>12.2</td>
<td>AFM</td>
<td>On mica, this work</td>
</tr>
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<td>L121</td>
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<td>8.2</td>
<td></td>
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<td>7.4</td>
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<td>4.3</td>
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<tr>
<td>F127</td>
<td>100-65-100</td>
<td>Liposome</td>
<td>3.0</td>
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<td></td>
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<td>F88</td>
<td>103.5-39.2-103.5</td>
<td>Liposome</td>
<td>2.9</td>
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<tr>
<td>F108</td>
<td>132.6-50.3-132.6</td>
<td>Liposome</td>
<td>2.4</td>
<td></td>
<td></td>
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<tr>
<td>P85</td>
<td>26-40-26</td>
<td>Liposome</td>
<td>5.0</td>
<td>c-TEM</td>
<td>In aqueous solution *</td>
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<td>P105</td>
<td>37-56-37</td>
<td>liposome, open pore</td>
<td>5.0</td>
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<tr>
<td></td>
<td></td>
<td>liposome</td>
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<td>61-40-61</td>
<td>bilayer, liposome</td>
<td>5.0</td>
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<tr>
<td>F127</td>
<td>100-65-100</td>
<td>bilayer, liposome</td>
<td>5.0</td>
<td></td>
<td></td>
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<tr>
<td>F108</td>
<td>132-50-132</td>
<td>bilayer, liposome</td>
<td>5.3</td>
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* From ref. 11
Table 4. Bending modulus of Pluronic®-modified EggPC liposomes measured by AFM force curve

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of PEO chain</th>
<th>No. of PPO chain</th>
<th>Bending modulus ($k_c \times 10^{19}$ (J))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EggPC *</td>
<td>/</td>
<td>/</td>
<td>0.21 ± 0.08</td>
</tr>
<tr>
<td>EggPC/F88</td>
<td>103.5</td>
<td>39.2</td>
<td>1.50 ± 0.40</td>
</tr>
<tr>
<td>EggPC/F127</td>
<td>100</td>
<td>65</td>
<td>1.64 ± 0.28</td>
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<tr>
<td>EggPC/F108</td>
<td>132.6</td>
<td>50.3</td>
<td>2.06 ± 0.38</td>
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<tr>
<td>EggPC/Cholesterol (1:1) **</td>
<td>/</td>
<td>/</td>
<td>1.81 ± 0.41</td>
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</tbody>
</table>

* Data from ref. 18

** Data from ref. 19
Table 5. Theoretical transition concentration and experimental concentration for different Pluronic® copolymers (% mol/mol)

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>Theoretical transition concentration (Model A)</th>
<th>Theoretical transition concentration (Model B) *</th>
<th>Experimental concentration.</th>
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<tbody>
<tr>
<td>L81</td>
<td>231.4</td>
<td>~180</td>
<td>12.2</td>
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<tr>
<td>L121</td>
<td>101.6</td>
<td>~79</td>
<td>8.2</td>
</tr>
<tr>
<td>P85</td>
<td>10.65</td>
<td>~8.3</td>
<td>7.4</td>
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<tr>
<td>F87</td>
<td>3.81</td>
<td>~2.98</td>
<td>4.3</td>
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<tr>
<td>F127</td>
<td>2.11</td>
<td>~1.64</td>
<td>3.0</td>
</tr>
<tr>
<td>F88</td>
<td>2.03</td>
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<td>2.9</td>
</tr>
<tr>
<td>F108</td>
<td>1.51</td>
<td>~1.18</td>
<td>2.4</td>
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</table>

*An estimate distribution of the polymer chain was calculated based on the following assumption: for a vesicle with 20 nm radius, then the outside layer is 20 nm and the inner side is around 15 nm, given the vesicle bilayer is 5 nm. Thus the possible distribution of the polymer chains based on surface area is $16/9 \left(4\pi r_{outer}^2/4\pi r_{interior}^2\right)$; and the calculated theoretical transition concentrations of Model B are less than those adopted for Model A.
Figure 2
Figure 3a
Figure 3b, 3c

PEO chain
PPO chain

Undisturbed bilayer thickness
(Onset point of repulsive force)

Bilayer thickness
(Jump-in point)

Figure 3b, 3c
Figure 4
Figure 5
d
Random coil
Model B: Adsorption
Model A: Membrane-spanning

a) Polymer/Lipid interaction model

PEO chain
PPO chain
Fully extended

b) PEO chain conformation

$d$ Random coil
Figure 6
Figure 7
System with short PEO chain
Tend to rupture
EggPC/L81, EggPC/L121

System with long PEO chain
Shell-like wall structure
EggPC/F108, EggPC/F127