Small-Angle X-ray Scattering Study of the Interaction of Poly(ethylene oxide)-*b*-Poly(propylene oxide)-*b*-Poly(ethylene oxide) Triblock Copolymers with Lipid Bilayers

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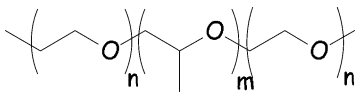
The relationship between molecular architecture and the nature of interactions with lipid bilayers has been studied for a series of poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO−PPO−PEO) triblock copolymers using small-angle X-ray scattering (SAXS) and thermal analysis (differential scanning calorimetry, DSC). The number of molecular repeat units in the hydrophobic poly(propylene oxide), PPO, block has been found to be a critical determinant of the nature of triblock copolymer−lipid bilayer association. For dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)-based biomembrane structures, polymers possessing a PPO chain length commensurate with the acyl chain dimensions of the lipid bilayer yield highly ordered, swollen lamellar structures consistent with well-integrated (into the lipid bilayer) PPO blocks. Triblock copolymers of lesser PPO chain length yield materials with structural characteristics similar to a simple dispersion of DMPC in water. Increasing the concentration (from 4 to 12 mol %) of well-integrated triblock copolymers enhances the structural ordering of the lamellar phase, while concentrations exceeding 16 mol % result in the formation of a hexagonal phase. Examination of temperature-induced changes in the structure of these mesophases (complex fluids) reveals that if the temperature is reduced sufficiently, all compositions exclude polymer and thus exhibit the characteristic SAXS pattern for hydrated DMPC bilayers. Increasing the temperature promotes better insertion of the polymers possessing PPO chain lengths sufficient for membrane insertion. No temperature-induced structural changes are observed in compositions prepared with PEO−PPO−PEO polymers that feature PPO length insufficient to permit full incorporation into the lipid bilayer.

Introduction

The structural characteristics of aqueous solutions of amphiphilic, nonionic block copolymers have been studied extensively during the last several years. Of particular interest have been poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) triblock copolymers (abbreviated as PEO−PPO−PEO or $EO_n-PO_m-EO_n$ (**I**), *n* and *m* representing the number of repeat units in the polymer) distributed



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commercially under the names Pluronic (BASF) or Poloxamer (ICI). These biocompatible polymers are among a select group of surfactants approved by the United States Food and Drug Administration for use in food, pharmaceuticals, and a wide range of consumer products.[1] In addition, PEO−PPO−PEO have recently emerged as potentially important agents in biotechnology and molecular medicine.[2,3] To date, in fact, more than 1000 articles have been published on the applications of these polymers in the medical and pharmaceutical fields alone.[4]

Although early research with these polymers focused primarily on their self-assembly in aqueous solution and the rich variety of mesophase structures arising from their diverse range of compositions,[5−8] recent interest has shifted to investigations of their interactions with biological materials, including substrate-supported lipid monolayers,[9] phospholipid vesicles, and liposomes.[10−12] This interest stems primarily from the potential application of these polymers as inexpensive, readily available substitutes for more expensive lipid-grafted polymers in the preparation of sterically stabilized ("Stealth") liposomes for drug delivery. (Steric stabilization has been demonstrated as an efficient means by which to prolong drug circulation times in the bloodstream.[13]) The use of PEO−PPO−PEO triblock copolymers for the sealing of damaged or permeabilized cell membranes following trauma is another area of considerable promise. First demonstrated for electrical injury,[14,15] membrane sealing has since been expanded to include a diverse range of injuries, including heat shock,[16] high-dose radiation,[17]ischemia-reperfusion injuries,[18] and the treatment of neurotoxic events.[3]

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Although work to date (which has been primarily clinical) has demonstrated marked improvement in cellular/tissue healing via membrane sealing, a clear understanding of the molecular-level mechanism of the process is lacking. For this approach to serve as an effective therapy for the restoration of structural integrity of the cell membranes in damaged tissue, basic research into the molecular-level interactions of these surfactants with cell membranes is needed.

A better understanding of the details of the association of PEO−PPO−PEO triblock copolymers with biological nanostructures (such as lipid bilayers) is also required in another emerging area of interest, the potential of these compounds as the basis for biomimetic scaffolds in the production of functional nanostructures. In prior work, we demonstrated that poly(ethylene glycol)-grafted, lipid-based complex fluids, which are biomimetic, can be employed as scaffolding for the formation of organized arrays of either soluble or membrane proteins.[19] In related work, these materials were shown to serve as a scaffolding for the formation of inorganic nanoparticle arrays.[20] Furthermore, a simple modification of the composition (i.e., increasing the number of repeat units on the lipid-appended PEG moieties) was shown to offer a facile means to modulate the interactions of encapsulated nanoparticles, as reflected in changes in their optical and electronic properties. Given that the properties (e.g., packing arrangement of encapsulated guest species, viscosity, etc.) of these materials are partly dependent upon the number of molecular repeat units in the PEG chain, it is clear that far greater tunability of materials properties could be achieved by developing similar formulations employing PEO−PPO−PEO triblock copolymers, which are available in a wider range of molecular architectures.[21]

In this report, we evaluate the effect of PEO−PPO−PEO triblock copolymer molecular architecture on their mode of interaction with a model biomembrane, a modification of the quaternary, PEGylated lipid-based system previously developed in this laboratory described above.[22] This system can serve as a robust model biomembrane that can adopt a lamellar structure comprising alternating layers of water and biomolecular layers of phospholipid/cosurfactant (Figure 1A). Here, we employ small-angle X-ray scattering to probe the structural perturbations caused by introducing PEO−PPO−PEO triblock copolymers of various molecular architectures (compositions). Of particular interest are the effect of the molecular weight (length of the central PPO block) and the flanking PEO units and the impact of their association as a function of concentration and solvent quality (as modulated by variations in temperature).

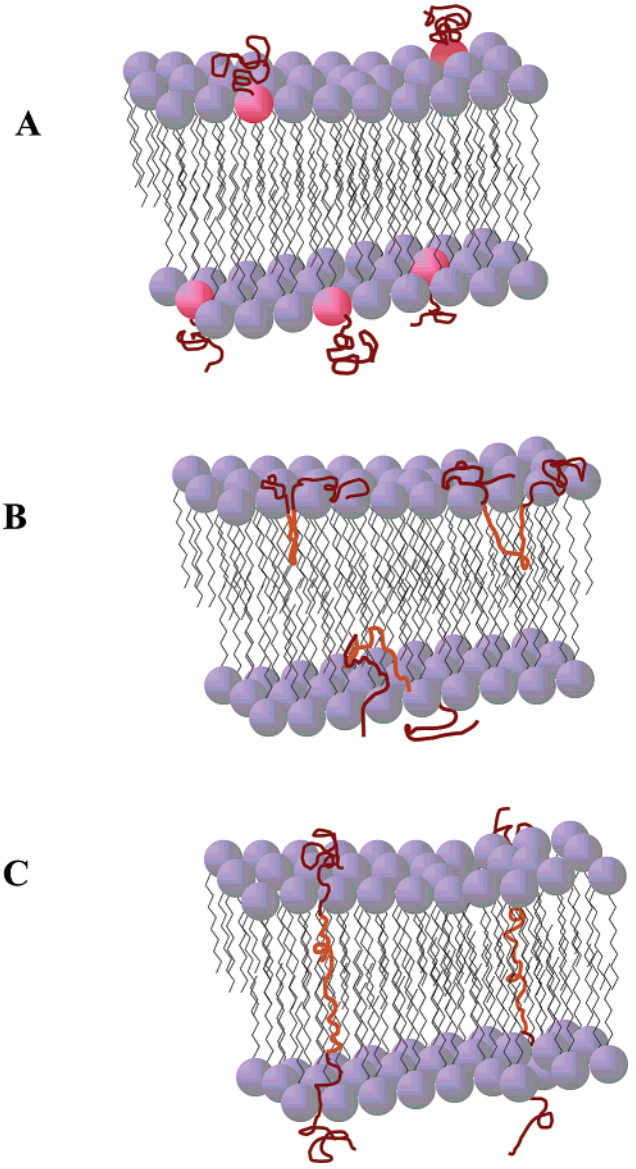

Figure 1. Schematic of possible modes of Pluronic interactions with lipid bilayers: (A) PEG terminally grafted to the lipid headgroup (PEG−DMPE); (B) partial insertion of PO unit into the acyl chain portion of the lipid bilayer; (C) full insertion of PO chains by spanning the lipid bilayer.

Table 1. Properties of PEO−PPO−PEO Triblock Copolymers Used in This Work

Pluronic	MW (g/mol)	no. of PO	no. of EO	PO/EO
F38	4700	15	2 × 43	0.17
F68	8400	30	2 × 75	0.20
F88	11400	39	2 × 103	0.19
P85	4600	40	2 × 25	0.80
F98	13000	47	2 × 117	0.20

Experimental Section

Materials and Methods. Lyophilized dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-poly(ethylene glycol) (DMPE-EO_{114}) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Lauryl dimethylamine-*N*-oxide (LDAO) was purchased from Calbiochem-Novabiochem Corp. (LaJolla, CA). All Pluronics were obtained from BASF Corporation (Mount Olive, NJ) and used as received. Table 1 summarizes the characteristics of these polymers. Milli-Q (18 MΩ) water was used for sample preparation.

Sample Preparation. Samples were prepared as quaternary compositions as previously described.[22] All samples were prepared such that the polymer-to-phospholipid ratio was held between 4 and 20 mol %, as specified in the text. Hydration of the solid components in either 20 mM HEPES,

150 mM NaCl (pH 7.4) buffer, or deionized water was accomplished by repeated cycles of heating (50 °C), vortex mixing, and cooling on an ice bath until sample uniformity was achieved.

Physical Methods. Thermal properties were measured by differential scanning calorimetry (DSC) at heating rates ranging from 5 to 10 °C/min on a Perkin-Elmer Pyris instrument. Instrument calibration was performed using an indium standard. Weighed amounts (2−9 mg) of the samples were sealed in aluminum pans and equilibrated at the starting temperature for 15 min prior to initiation of the scans. Small-angle X-ray scattering (SAXS) measurements were made using the instrument at undulator beamline 12ID-C of the Advanced Photon Source at Argonne National Laboratory. The sample-to-detector distance was such as to provide a detecting range for momentum transfer of $0.0025 < \mathbf{q} < 0.6$ Å^{-1}. The scattering vector, **q**, was calibrated using a silver behenate standard at $\mathbf{q} = 1.076$ Å^{-1}. The 2-D scattering images were first corrected for spatial distortion and sensitivity of the detector and then radially averaged to produce plots of scattered intensity, $I(\mathbf{q})$, versus scattering vector, **q**. The value of **q** is proportional to the inverse of the length scale, Å^{-1}. Samples were sealed in 1.5 mm quartz capillaries. Temperature control of samples was achieved using a custom-built Peltier cooler.

Results and Discussion

To gain insight into the mode of interaction of amphiphilic, nonionic triblock copolymers with lipid bilayers, PEO−PPO−PEO triblock copolymers of varying molecular architectures (Table 1) were examined to determine the mode of association with model membranes, adsorption at the bilayer surface, partial insertion (Figure 1B), or full spanning of the bilayer (Figure 1C). The molecular architecture of the polymers selected allows for the assessment of the effect of the hydrophobic block length (molecular weight) and the EO chain length on the nature of association and the effect of the concentration of the incorporated triblock copolymer on the steric barrier produced and thus the aggregate structure and the effect of temperature on the mode of insertion. The SAXS profiles collected on two well-characterized systems, a simple aqueous dispersion of a zwitterionic phospholipid, DMPC, and the PEGylated lipid-based mesophase, at 37 °C are presented in Figure 2, panels A and B, respectively. The scattering profile of the dispersion of DMPC in water (30% (w/v)) is dominated by two equally spaced Bragg reflections of integral order spacing ($\mathbf{q} = 0.099$ and 0.199 Å^{-1}) indicative of one-dimensional lamella with a periodicity, *d*, of 63 Å. This pattern is consistent with a sheetlike structure composed of lipid bilayers that are separated by a water channel.[23,24] The addition of other components (e.g., polymer, cosurfactants) to this simple binary mixture is known to modify the balance of forces between the lipid molecules producing new phases, often with interesting physical properties that can be exploited in the development of new materials.[22,25] For example, prior work in this laboratory has explored the structure and physical properties of various polymer-grafted, lipid-based mesophases that consist of a quarternary mixture of a phospholipid DMPC, a polymer introduced as poly(ethylene glycol) (PEG) terminally grafted onto a phospholipid headgroup (dimyristoylphosphatidylethanolamine, DMPE), and a zwitterionic cosurfactant (*N*,*N*-dimethyldodecylamine-*N*-oxide, LDAO) in water. This material undergoes a thermoreversible phase transition at 16 °C, converting between a structured, elastic solid (gel) lamellar phase (Lα*g*) and a low-viscosity, 2-D hexagonally ordered array of prolate micelles (H_1).[22,26]

A typical SAXS curve for such quaternary compositions prepared with PEG5000-DMPE at a grafting density of 10 mol % at 37 °C is shown in Figure 2B. The scattering curve features five distinct diffraction peaks at integral order spacing ($\mathbf{q} = 0.0244$, 0.0448, 0.0732, 0.0976, and 0.123 Å^{-1}), consistent with a highly ordered lamellar structure featuring a significantly increased (vs the DMPC aqueous dispersion) repeat distance of 257 Å. The observed ∼195 Å expansion of the lattice spacing in these materials (vs the simple aqueous dispersion of DMPC) is believed to arise from the steric pressure produced by the grafted PEG chains. (That is, a stable material is formed when the intervening water channel is large enough to accommodate a swollen polymer coil.) The scattering curves of the aqueous dispersion of DMPC and the DMPC−PEG5000-DMPE−LDAO in water mixture can now serve as standards against which to evaluate the effect of replacing the PEG−lipid conjugate with the PEO−PPO−PEO triblock copolymers on aggregate structure.

Influence of PO Architecture. The effect of the incorporation of a nonionic triblock copolymer (PEO−PPO−PEO) on the membrane structure was assessed by replacing the PEG5000-DMPE component of the quaternary phases (described above) with 10 mol % of any one of several PEO−PPO−PEO triblock copolymers (e.g., Pluronic F38, F68, F88, and F98) of varying PPO block molecular weight (chain length). Compositions prepared with Pluronics F38 or F68 were found to self-organize to form optically opaque samples, while the use of F88 or F98 yielded materials that are optically transparent at room temperature (25 °C). At room temperature, under polarized light, all of the complex fluid formulations are optically birefringent, exhibiting Schlieren textures with numerous topological defects, indicative of a liquid crystalline phase.

The first of these copolymers, F38, (Table 1) possesses a very short propylene oxide (PO) chain length (only 15 molecular repeat units). The radius of gyration (R_g) of the PPO block of F38 can be calculated (assuming a Gaussian chain), as approximately 7 Å, a length less than the dimension of the hydrophobic part of the lipid bilayer, which is estimated to be ca. 20 Å.[6,27] A typical SAXS profile collected on the F38-formulated sample at 37 °C is presented in Figure 2C. The scattering curve is dominated by two broad diffraction peaks at $\mathbf{q} = 0.111$ and 0.219 Å^{-1}, corresponding to one-dimensional lamellae with periodicity of 57 Å, a dimension similar to that observed in a simple DMPC−water dispersion. The significant increase in the width of the diffraction peaks compared to those found for the DMPC−water dispersion indicates poor spatial coherence (i.e., structural heterogeneity), perhaps due to a perturbation of

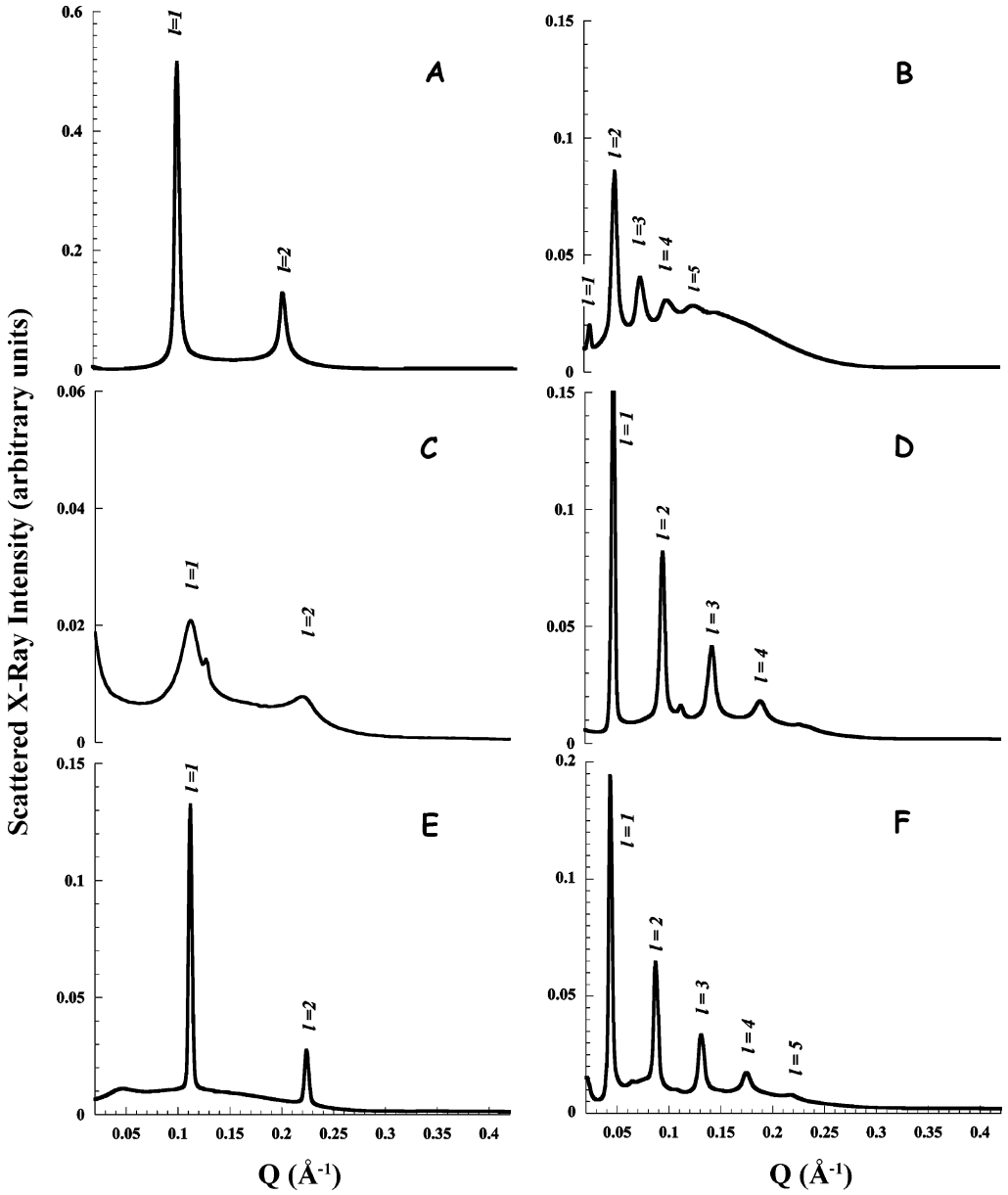

Figure 2. Synchrotron small-angle X-ray scattering profiles collected for (A) 30 wt % DMPC dispersion in water and for quaternary mixture employing (B) 10 mol % PEG5000−DMPC conjugate, (C) 10 mol % F38 polymer, (D) 10 mol % F88 polymer, (E) 10 mol % F68 polymer, and (F) 10 mol % F98 polymer. All measurements were made at 37 °C.

the lipid bilayer packing by introduction of the triblock copolymer. Such disruption of DMPC packing may occur so that the short, hydrophobic PPO chains can preferentially associate with the alkyl chains of the phospholipid. Because the hydrophobic block, PPO, is not of sufficient length to span the lipid bilayer, most likely, it simply inserts into the membrane in such a way that both ethylene oxide (EO) blocks reside on the same side of the membrane (Figure 1B). The loose integration of F38 into the bilayer may yield a structure in which the EO chains "decorate" the membrane (i.e., dangle into the aqueous regions) by adopting a primarily flat or "lying down" configuration. Such a PEO conformation would force the polymer chains to extend laterally along the membrane rather than protruding into the water channels perpendicular to the lipid bilayer, as is the case for the PEG chains introduced by grafting them onto the lipid headgroup. Thus, poor integration of the PPO block and the accompanying flattened conformation of the PEO units may account for the lack of formation of a swollen lamellar phase, such as is observed with the use of PEGylated lipid that incorporates similar EO chain lengths.

Figure 2E presents the results of SAXS studies to determine the effect of doubling the PO chain length to 30 repeat units (Pluronic F68) on the structure of the complex fluid. Here again, the SAXS profile features two diffraction peaks at $\mathbf{q} = 0.111$ and 0.223 Å^{-1}, corresponding to a lamellar structure with a d spacing of 57 Å. In contrast to the results for F38, however, a higher degree of long-range translation order is observed, as evidenced by the narrower diffraction peaks. In addition, the enhancement in spatial coherence is greater than that observed for DMPC−water dispersions (Figure 2A). Although the origin of the significant increase in spatial coherence is unclear, it may arise from better anchoring of the PO chains to the membrane structure, because the increased length may allow for a greater degree of incorporation. It is also noted that the

scattering curve (Figure 2E) exhibits a broad feature in the low **q** region ($\mathbf{q} = 0.046$ Å^{-1}). Thus, this particular composition of PEO−PPO−PEO (which is the one most often used in clinical studies of the sealing of permeablized membranes) yields an aggregate structure similar to a simple DMPC aqueous dispersion, suggesting poor PPO integration and leading to PEO chains that are most likely directed laterally along the membrane surface.

Figure 2D shows the SAXS profile collected on compositions prepared using Pluronic F88, a polymer comprising 39 repeat PO units. With 39 repeat units, the molecular architecture of Pluronic F88 is such that the PPO segment may span (fully insert into) the lipid bilayer. Here, the R_g of the PPO block is ca. 15 Å, a dimension commensurate with the hydrophobic portion of the bilayer. The scattering curve features four diffraction peaks at $\mathbf{q} = 0.0467$, 0.094, 0.142, and 0.188 Å^{-1}, consistent with a swollen lamellar structure with an interlamellar distance of 134 Å. In addition, a second considerably weaker set of two Bragg peaks is observed at $\mathbf{q} = 0.110$ and 0.220 Å^{-1} ($d = 57$ Å), indicating the coexistence of the lamellar structure for DMPC−water. These results indicate that the broad feature observed at $\mathbf{q} = 0.046$ Å^{-1} in the F68-based quaternary system signals the emergence of the swollen lamellar phase.

The effect of employing Pluronic F98, in which the length of the PO block is increased to 47 repeat units, is shown in Figure 2F. Here, a SAXS pattern with five sharp Bragg peaks of integral order spacing ($\mathbf{q} = 0.0433$, 0.0867, 0.133, 0.174, and 0.216 Å^{-1}) is observed, indicating a lamellar structure with an increased lattice spacing of 145 Å. Interestingly, increasing the length of the PPO block by only eight molecular repeats appears to enhance the structural ordering of the swollen lamellar phase (as evidenced by the appearance of a resolved fifth-order Bragg peak) and suppress formation of the DMPC−water lamellar phase. The slight increase in the interlamellar spacing may arise from both the increase in the length of the number of PEO repeat units (vide infra) and the more favorable orientation of the PEO due to improved insertion (and therefore, orientation) of the PPO block. Although the length of the PPO block is sufficient to span the lipid bilayer, in reality, it is most likely that a distribution of modes of membrane association (i.e, membrane spanning, as well as "harpooning") exists.

Taken together, the results for the four Pluronics indicate that triblock copolymers incorporating a PPO block possessing a sufficient chain length (i.e., one that has dimensions to span the acyl chain region) can produce swollen lamellar phases that are structurally analogous to those produced using PEG−lipid conjugates. Most importantly, the results suggest that molecular architecture of the polymer is a critical factor determining the strength of "anchoring" and therefore, the orientation of the polymer relative to the membrane.

The Influence of EO Block Architecture. The influence of the molecular architecture of the two symmetric PEO blocks that flank the central PPO unit is another parameter that can be systematically varied in the triblock copolymers. Prior work with polymer-grafted lipid-based complex fluids has shown that the length of the lipid-appended PEG unit can be used to tune both the interlamellar spacing (d) and

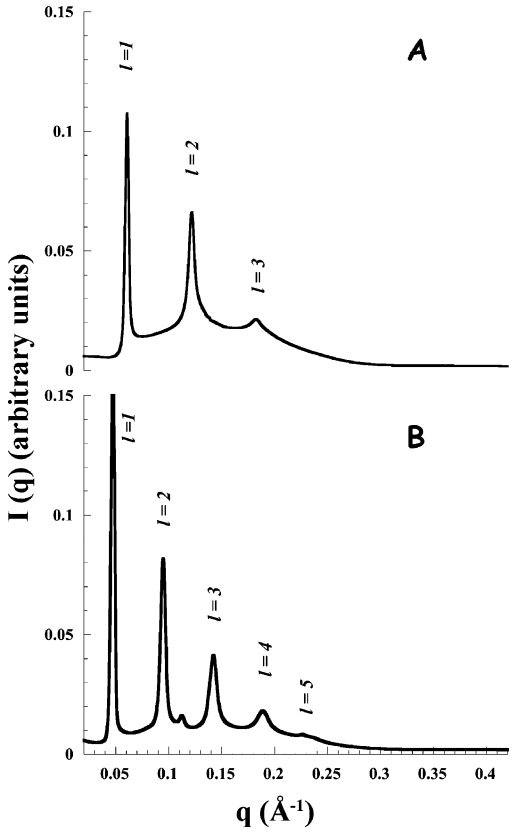

Figure 3. Synchrotron small-angle X-ray scattering profiles collected for quaternary mixture prepared with PEO−PPO−PEO triblock copolymers (A) 10 mol % P85 and (B) 10 mol % F88. All measurements were made at 37 °C.

the mechanical properties of these materials.[20] In other work, the length of the PEG unit has been shown to be an important parameter in determining the physical properties (e.g., circulation time due to steric stabilization) of Stealth liposomes.[28] It is believed that in both materials the extension of the PEO chains from the bilayer surface contributes to the observed changes in physical properties. That is, if the bilayer structure is stable, the lateral pressure between opposing polymer/amphiphile leaflets will be felt as an isotropic tension in the bilayer, causing an area expansion of the bilayer. Because experimental and theoretical studies on steric repulsion caused by incorporation of PEG or PEO chains on colloidal surfaces (i.e., steric barrier) indicate that the range and magnitude of the repulsion depend on both the length of the polymer chain and the concentration (i.e., the number of polymer chains per unit surface area of the substrate, here the lipid bilayer), the influence of both parameters on mesophase structure was probed by SAXS.

First, the effect of PEO molecular weight (chain length) on the structure of the formed materials was explored by evaluating the use of polymers that have a PPO length previously determined to be sufficient for tight anchoring to the lipid bilayer (∼39 molecular repeats). Figure 3 shows the effect on the X-ray scattering profiles of increasing the PEO chain length by a factor of 4 at a constant PO repeat (40 units) and constant concentration (10 mol % P85). Samples prepared using a triblock copolymer that possesses only 25 EO repeats produce a scattering pattern (Figure 3A) consistent with a lamellar structure with a lattice spacing of

103 Å, as evidenced by three diffraction peaks that occur at integer multiples of the first-order reflection ($\mathbf{q} = 0.061$ Å^{-1}). Although compositions prepared with F88 (Figure 3B), which contains a 4-fold increase in EO repeat units ($n = 103$) vs P85, display the same periodic lamellar structure, several differences are readily apparent in comparing the diffraction patterns. First, the number of resolvable diffraction peaks increases from three to five ($\mathbf{q} = 0.047$, 0.094, 0.142, 0.189, and 0.227 Å^{-1}), indicating a marked improvement in long-range ordering in the sample prepared using F88. Furthermore, a notable increase in the lattice spacing is observed (to 134 Å), reflecting a ca. 31 Å expansion in the lattice dimensions. Because the ionic strength and temperature have been held constant in these experiments, only the increased steric pressure exerted by the larger PEO chains confined between opposing bilayers should influence the structural ordering and lamellar periodicity.[28] These results are consistent with prior findings in the PEGylated-phosopholipid compositions, whereby increasing the appended PEG chain length from 45 repeats to 114 resulted in a near 100 Å increase in the lattice spacing.[20]

These experiments demonstrate that with appropriate selection of the triblock copolymer molecular architecture (i.e., sufficient PPO block length), materials that exhibit essentially the same properties as those prepared with polymer-grafted lipids can be produced. Furthermore, the work suggests a simple way to tune the lattice dimensions of these biomimetic nanostructures over a wide range by controlling the PEO chain length. The clinical efficacy of triblock copolymers featuring molecular architectures with sufficient PO lengths for tight anchoring to the lipid bilayer and increased PEO chain length to seal permeabilized membranes is unknown at present and represents an important area for future investigation.

The effect of PEO concentration on the aggregate structure was studied similarly by SAXS (Figure 4). Prior work on planar EO-functionalized surfaces has shown that the steric barrier can be easily modified by controlling the amount (i.e., the grafting density) of EO or PEG.[28,29] Here, the effect of introducing increasing amounts of F98, a Pluronic that features sufficient PPO chain length (47 molecular repeat units) for insertion into the lipid bilayer and a large PEO block (117 molecular repeat units), which should produce a large steric repulsion, was examined at a range of concentrations (4−20 mol %). All of the samples contain amounts of the triblock copolymers in which the PEO polymer chains should be above the dilute, nonoverlapping regime (i.e., "mushroom") and well within the dense, overlapping regime (i.e., "brush"). The surface density of polymer required to transition from the "mushroom" regime to the "brush" regime can be calculated according to de Gennes' scaling theory[30] using the Flory radius, R_F, assuming a value of 0.65 nm^2 for the average area per lipid molecule.[31] The transition occurs when the distance between adjacent EO chains decorating the lipid bilayer, *s*, is equal to or less than the Flory radius. For samples prepared using F98, the Flory radius is ca. 61 Å, indicating that the mushroom−to−brush transition would occur at concentrations exceeding 1.75%. The lowest concentration of F98 used, 4 mol %, therefore places the sample

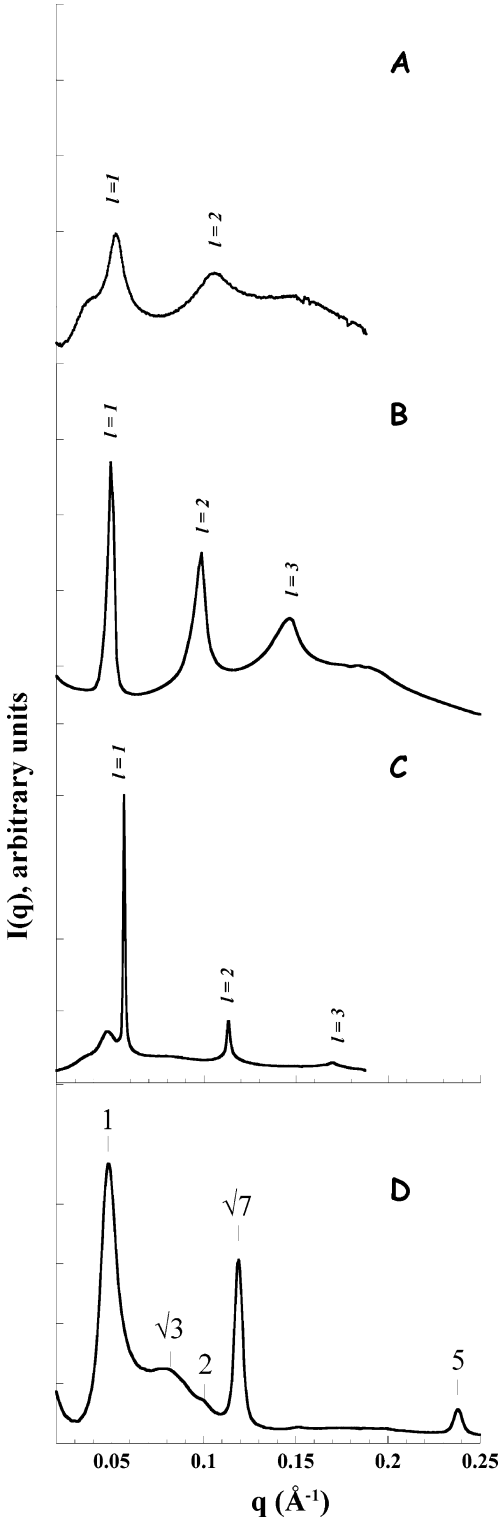

Figure 4. Synchrotron small-angle X-ray scattering profiles collected for quaternary mixture prepared with increasing concentration of PEO−PPO−PEO triblock copolymers: (A) 4 mol % F98; (B) 8 mol % F98; (C) 12 mol % F98; (D) 20 mol % F98. All measurements were made at 37 °C.

well within the "brush" regime with its extended PEO chains. At this same concentration, a SAXS pattern (Figure 4A) exhibiting only two broad diffraction peaks (q =0.052 and 0.105 Å^{-1}) and consistent with a weakly ordered lamellar structure having an interlamellar spacing of 120 Å is observed. Upon doubling of the concentration of F98 to 8 mol % to yield a high surface-coverage state subject to

significant lateral tension in the polymer/headgroup region, both a sharpening of the first-order Bragg peak and the emergence of harmonics higher than those found for compositions containing 4 mol % are observed in the scattering profile, reflecting a significant improvement in the structural ordering (Figure 4B). This scattering curve also reveals a minor (~8 Å) increase in the lattice spacing to 128 Å. This improvement in structural ordering continues in compositions prepared with 10 mol % polymer (Figure 2F), for which the enhancement is accompanied by an increase in the *d* spacing to 145 Å. For compositions containing 12 mol % polymer (Figure 4C), in which dense, large brushes are formed, the complex fluid adopts a highly ordered lamellar structure, as demonstrated by a substantial narrowing of the diffraction peaks. The lattice spacing, however, is reduced to 112 Å. Furthermore, a small but discernible shoulder appears at $\mathbf{q} = 0.047$ Å^{-1}, which may be due to the formation of a second phase. At 16 mol %, a mixed lamellar and hexagonal phase is observed in the diffraction pattern (data not shown). Finally, at 20 mol % (Figure 4D), the hexagonal phase emerges, as indicated by a $1:\sqrt{3}:2:\sqrt{7}$ diffraction pattern. The formation of a two-phase (lamellar + hexagonal) region has been previously reported for lecithin/PEO−PPO−PEO/water systems.[27] The abrupt changes in the fluid structure at 12 mol % suggests that at polymer concentrations exceeding 10 mol % the steric tension has reached a saturation limit, leading to a transition from a bilayer into a mixed bilayer and then, at higher concentrations, to a hexagonal phase. These finding are in good agreement with those reported by McIntosh et al.[28] in which Stealth liposomes formulated with large concentrations of both PEG-2000 and PEG-5000 were found to be detrimental to aggregate structures. Moreover, the range of the steric barrier provided by the incorporation of PEG−lipid in stealth liposomes was shown to reach a maximum at ~10 mol % PEG−lipid.[28] Comparable studies on supported bilayers composed of 1−10 mol % EO_{45}−DSPE also clearly exhibit an increase in lateral stress on the bilayer with increasing polymer concentration.[32]

The changes in structure observed for compositions using F98 contrast with those prepared with F68, a Pluronic that does not have a sufficient number of PO molecular repeat units to achieve a membrane spanning configuration. The Flory radius for samples prepared with F68 is 46.7 Å, and therefore, at concentrations of 2.98% and higher, dense overlapping PEO chains ("brush" regime) would occur. For these compositions, however, we find that the SAXS patterns are essentially invariant with F68 concentration (data not shown), suggesting the importance of the orientation of the tethered/anchored PEO chains in producing steric repulsion between opposing bilayers. That is, for compositions prepared with F68, the PEO chains are not believed to be well oriented with respect to the membrane surface.

Effect of Temperature on Structure. Additional insights into the nature of the triblock copolymer−biomembrane interactions can be obtained by studying the influence of temperature on aggregate structure. First, thermal analysis by differential scanning calorimetry (DSC) was used to understand the phase transitions in these materials. The baseline-corrected DSC curves obtained for quaternary mixtures prepared with several PEO−PPO−PEO triblocks are presented and compared to those of an aqueous dispersion of DMPC and the DMPC/PEG5000-DMPE/LDAO mixture in Figure 5, panels A and B, respectively. Unlike the heating curve for the aqueous dispersion of DMPC alone (Figure 5A), which is dominated by a narrow endothermic phase transition centered at 23.8 °C (T_m) corresponding to the hydrocarbon chain melting transition (i.e., lamellar gel phase, Lβ, to a lamellar fluid phase, Lα), the differential heating scan collected on the quarternary mixture with grafted PEG chains (Figure 5B) exhibits a complex endothermic phase transition in the temperature range 13−25 °C. Prior work has shown that the width of the transition is indicative of the size of the cooperative unit (i.e., the molecular unit) that is undergoing the transition.[22] A highly cooperative thermal phase transition (i.e., one involving a large cooperative unit) is characterized by a very sharp transition such as that observed for the chain melting transition in the DMPC−water system (full width at half-maximum (fwhm) = 1.78 ± 0.02 °C). Conversely, the broader peak (fwhm = 6.04 ± 0.01 °C) observed for the quaternary phase system is most likely the result of a less cooperative thermal transition.

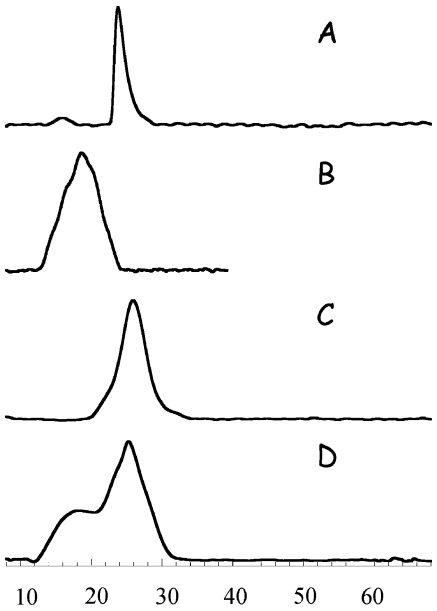

Figure 5. Calorimetric scanning curves: (A) DMPC dispersion in water; (B) 10 mol % PEG5000-grafted lipid-based complex fluid composition; (C) 10 mol % F68 Pluronic-based complex fluid composition; (D) 10 mol % F98 Pluronic-based complex fluid composition. Heating rate was 5 °C/min.

Compositions prepared using Pluronic F68 and F98 (Figure 5, panels C and D, respectively) show no significant shift in the position of the main endothermic peak but do exhibit significant broadening of the transitions (fwhm = 4.37 ± 0.02 °C for F68). In addition, the curve for F98 exhibits a broad, prominent shoulder at 17.5 °C, which compares well with the thermal transition recorded for the PEG-grafted quaternary mixtures. These results provide evidence consistent with the SAXS results indicating that the nature of association of the F98 involves tighter anchoring in the lipid bilayer and a mesophase with properties similar to formulations prepared using PEG-grafted lipids.

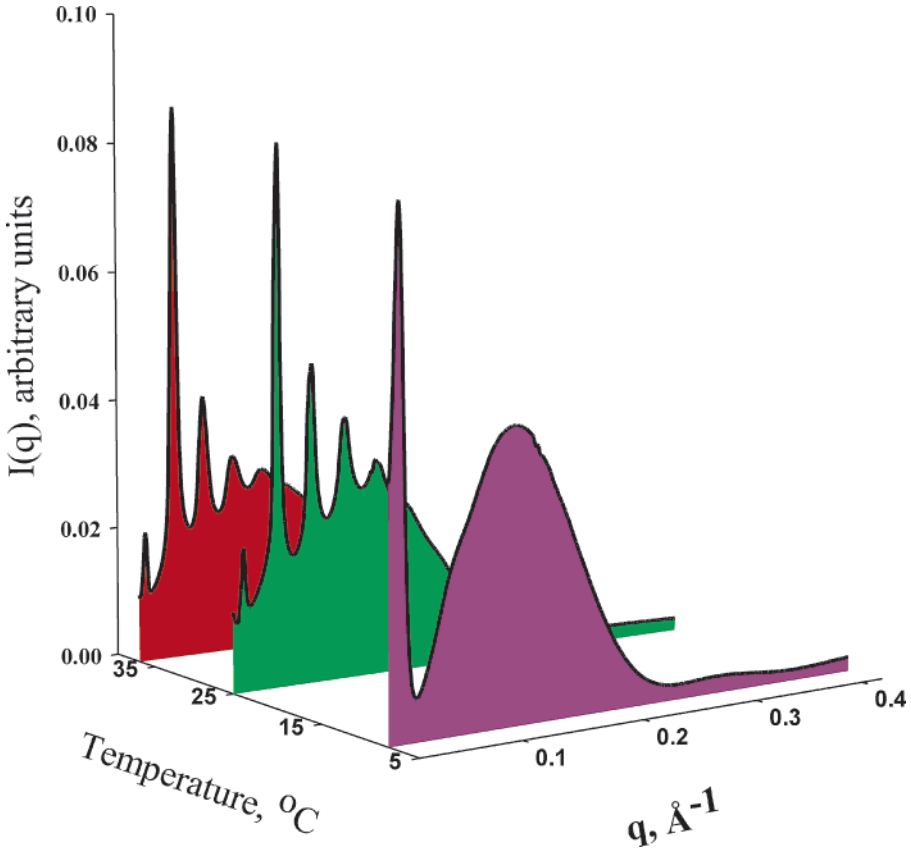

Figure 6. SAXS profiles on quaternary compositions prepared with 10 mol % PEG5000−DMPE at 8, 25, and 37 °C.

Polymer aggregation processes leading to supramolecular structures depend not only on molecular architecture, but also on polymer−solvent interactions (i.e., solvent quality). The aqueous solubility of PPO exhibits dramatic temperature dependence. Below ca. 15 °C (at ambient pressure), water is a good solvent for PPO, while at higher temperatures, PPO becomes more hydrophobic and hence less soluble in water.[6] Conversely, PEO is hydrophilic and remains water soluble within the temperature range from 0 to 100 °C but with the reduced solvent quality that accompanies increasing temperature causing chain contraction.[29] Although there have been several reports studying the effect of temperature on micelle formation and structure in aqueous solutions of PEO−PPO−PEO triblock copolymers,[5,7,8] considerably fewer studies have been reported evaluating their temperature-dependent association with lipid bilayers.[10] The effect of temperature on the structure of the Pluronic-based systems was studied at three temperatures: 5−8 °C, 25 °C, and (because of their potential for in vivo applications) at physiological temperatures, 37 °C. These results were then compared to those obtained for compositions prepared using PEGylated phospholipids.

Figure 6 shows the temperature-dependent SAXS profiles collected on compositions prepared with PEG5000−DMPE. Below the phase transition in the low viscosity state at a sample temperature of 5 °C, the scattering curve exhibits a single broad Bragg peak and significant scattering in the low **q** region. Prior work employing small-angle neutron scattering[22,26] has shown that this scattering pattern is consistent with a cold-phase structure comprising a 2-D hexagonal array of prolate micelles. At room temperature, in the gel state, six diffraction peaks with a *d* spacing ratio of integral order (**q** = 0.028, 0.053, 0.084, 0.110, 0.131, and 0.160 Å^{-1}) are observed. This pattern indicates a lamellar structure with periodicity of 224 Å. The resolvable Bragg peaks reside on top of significant background scattering believed to arise from the sample retaining some of the cold-phase structure. At a sample temperature of 37 °C, the structure remains relatively unchanged except for a minor increase in lattice dimensions (to $d = 251$ Å). This material, which features a novel inverted thermoreversible phase transition (that is, a lower viscosity state at reduced temperature) is believed to arise from temperature-induced alterations in the conformation of the grafted PEG chains that are confined in a nanospace environment (water channel). That is, at reduced temperature (<16 °C), upon modest improvement in solvent quality, the anchored PEG chains expand and the induced bilayer stress is reduced by transformation into a normal hexagonal micellar phase.[22] These mesophases, which feature tightly anchored PEG chains, can be compared to the temperature-induced structural changes observed for the triblock copolymer-based systems.

The results of SAXS investigations of the temperature-dependent structural changes in samples prepared with 10 mol % F98, which as demonstrated above is believed to fully insert into the lipid bilayer, are presented in Figure 7. At a reduced temperature, 8 °C, the scattering profile is characterized by a pair of broad diffraction peaks at **q** = 0.100 and

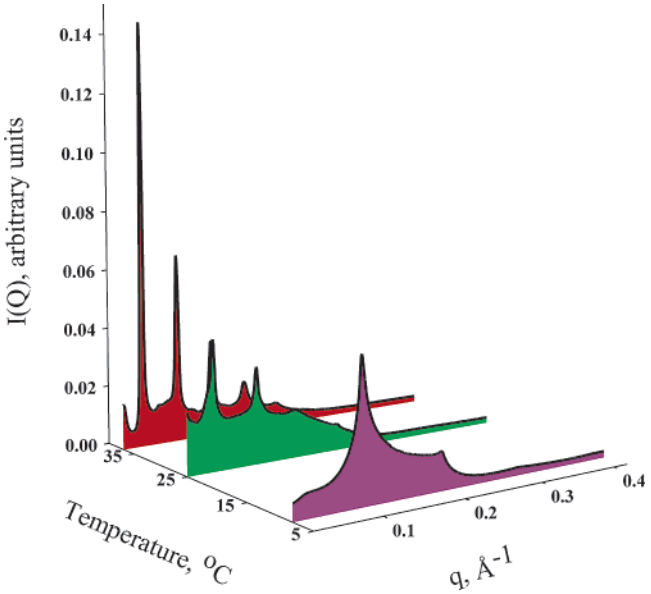

Figure 7. SAXS profiles on quaternary compositions prepared with 10 mol % F98 at 8, 25, and 37 °C.

0.201 Å^{-1} that correspond to a lamellar structure with a *d* spacing of 63 Å. Such a SAXS pattern is consistent with a simple DMPC dispersion in water (Figure 2A) and with complete exclusion of F98 from the lipid bilayer. The lack of polymer integration into the lipid bilayer suggests that as the PPO block becomes more water-soluble, it is easily removed from the hydrophobic acyl chain region of the membrane. Although it is possible that the increased alkyl chain ordering in the Lβ state may also promote exclusion of the triblock polymer, it is unlikely because similar experiments on certain compositions of PPO−PEO diblock copolymers indicate that they are not excluded at reduced temperature (i.e., in the Lβ state).[33] As the temperature is increased to 25 °C, a weakly ordered lamellar structure (**q** = 0.0501, 0.102, and 0.150 Å^{-1}) featuring a significantly larger lattice spacing, 126 Å, emerges, reflecting reinsertion of the PO block into the bilayer. Finally, at 37 °C, five sharp Bragg peaks (**q** = 0.043, 0.082, 0.130, 0.174, and 0.220 Å^{-1}) are observed, indicating that an ordered structure has formed with PPO fully inserted into the bilayer. The more efficient absorption of PEO−PPO−PEO triblock copolymer with increasing temperature has been observed previously in TEM studies of various polymers with egg phosphatidylcholine liposomes.[10] These results underscore both the utility of temperature as a means of regulating the aggregate structure and the significantly enhanced temperature sensitivity of the less well-anchored triblocks vs the more highly integrated PEG−lipid conjugates. In addition, these results indicate that unlike the quarternary phases that are prepared by introduction of PEG via a lipid conjugate, in which temperature-induced solvent conditions affect only the PEG chain conformation, for the triblock copolymers, changing solvent quality here yields pronounced changes in the PPO block. Moreover, these changes most likely result from the poor integration of the triblock copolymers with the lipid bilayers because certain PPO−PEO diblock copolymers do not exhibit such pronounced temperature-induced structural changes.[33]

Next, the temperature dependence of the scattering profiles for complex fluid compositions comprising F68, which as determined previously does not have sufficient PPO chain length to allow for membrane spanning and thus is only

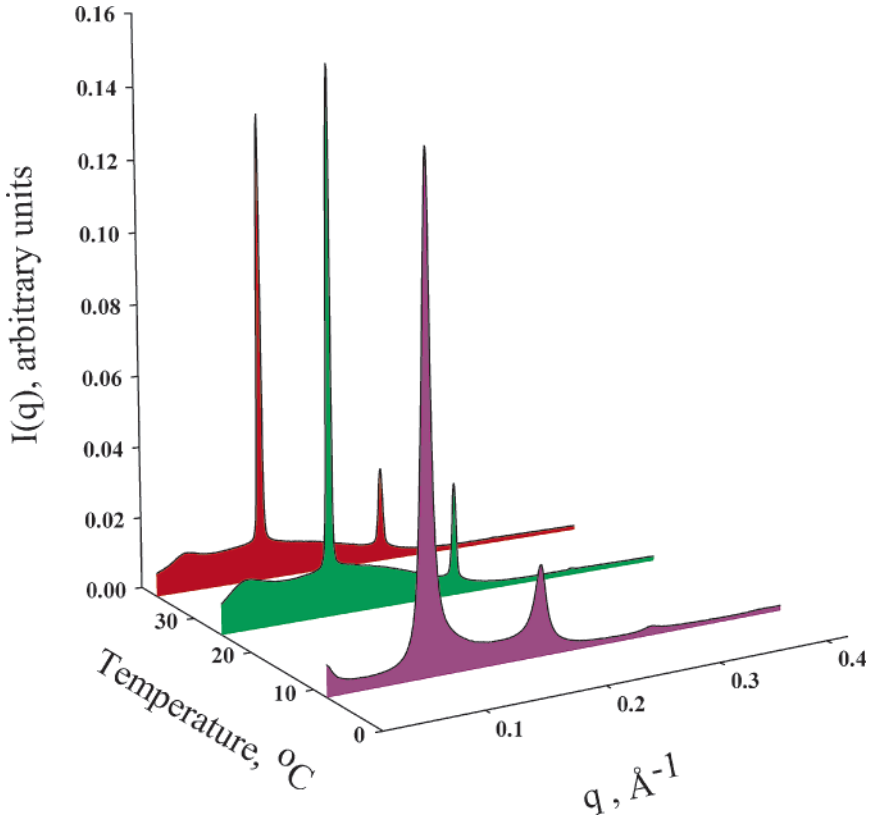

Figure 8. SAXS profiles on quaternary compositions prepared with 10 mol % F68 at 8, 25, and 37 °C.

weakly associated with the lipid bilayer (Figure 8), was determined. As for F98 compositions, at 8 °C, a scattering curve typical for a simple DMPC aqueous dispersion is observed ($\mathbf{q} = 0.099$ and 0.198 Å^{-1}), indicating lack of polymer integration into the lipid bilayer. Upon increase of the temperature to 25 °C (and thereby increase of the hydrophobicity of PPO), a small correlation peak emerges in the low **q** region at ca. 0.4 Å $^{-1}$, reflecting possible formation of a minor component such as the swollen lamellar phase. Further increasing the sample temperature to 37 °C does not induce any significant changes in the SAXS pattern.

The observed temperature-induced changes in the SAXS profiles illustrate a major difference in materials prepared using the triblock copolymers vs the PEG−lipid conjugates. That is, although at elevated temperatures lamellar structures with large lattice dimensions equivalent to those achievable with formulations using PEG-grafted lipids are attainable with the appropriate triblock copolymers, at reduced temperatures, the latter materials do not convert to a 2-D hexagonal structure. Rather, they retain a lamellar structure with a significantly reduced lattice spacing, presumably from expulsion of the copolymer.

Conclusions

The results presented here demonstrate that certain nonionic, amphiphilic triblock copolymers of PEO−PPO−PEO can be employed as an alternative to more costly and architecturally limited PEG−lipid conjugates for producing biomimetic nanostructures. In particular, the data suggest that the molecular architecture of the triblock copolymer, specifically, the length of the PO block, is a critical determinant of the mode of interaction (insertion) of the copolymer with the lipid bilayer. When the PPO block length is less than the bilayer leaflet, poor integration of the polymer is observed, allowing the PEO chains to adopt a more flat configuration (i.e, laterally directed orientation), which may serve to more effectively seal defects on the surface of the membrane. On the contrary, the need for robust biomimetic nanostructures for use in the development of functional materials or sterically stabilized drug-delivery vehicles necessitates strong "anchoring" of the polymer (PPO block) to the membrane. The data further suggest that to achieve a strongly anchored triblock copolymer, the PPO chain length must approximate the dimensions of the acyl chain region of the lipid bilayer. Full PPO insertion into the lipid bilayer is accompanied by an arrangement of PEO chains normal to the membrane surface and into the water channels. Pluronics having this molecular architecture produce systems having the structure and physical characteristics similar to those prepared employing a PEG−lipid conjugate, but there are significant differences in the influence of temperature on their structures. That is, independent of the molecular weight of the PPO block, all Pluronics investigated in this study were found to exclude polymer at reduced temperature. This temperature-triggered expulsion of the triblock copolymers at reduced temperatures hints at a possible approach to drug delivery via controlled release. The dependence of structure on temperature, however, indicates that even with optimized PPO chain length (to promote bilayer insertion), the PEO−PPO−PEO triblock copolymers are generally more weakly held in the lipid bilayer than the corresponding PEG-grafted lipids and may therefore not be ideal for use in the steric stabilization of vesicles. These studies underscore the complexity of multicomponent self-assembled materials and represent a step toward a fuller understanding of them, which will ultimately allow for their adaptation in a broad range of technological applications.

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