Cross-Linking Polymerizations in Two-Dimensional Assemblies

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Received July 25, 1996; Revised Manuscript Received October 1, 1996[⊗]

ABSTRACT: Polymerization of monomeric lipids in an assembly proceeds in a linear or cross-linking manner depending on the number of polymerizable groups per monomeric lipid. Lipids that contain a single reactive moiety in either of the hydrophobic tails or associated with the hydrophillic head group yield linear polymers. Polymerization of lipids with reactive groups in each hydrophobic tail generally yield cross-linked polymeric networks. This report describes three approaches to the characterization of the gel point for polymerizations constrained by the two-dimensional nature of lipid bilayers. The gel point for two-dimensional lipid assemblies was determined by correlation of the onset of significant changes in the physical properties of the polymerized bilayers with the bilayer composition. The properties examined in this study were the lateral diffusion of a small molecule probe of the bilayer, the stability of polymerized bilayer vesicles in the presence of surfactants, and the solubility of lipid polymers isolated from the bilayers after removal of water. Each of the three methods used indicated that a substantial mole fraction (0.25−0.35) of the bis-substituted lipid was necessary to cause cross-linking of the bilayer. The general agreement between the methods provides confidence that these results accurately indicate the relative inefficiency of the cross-linking process in bilayers composed of lipids having a reactive group at the hydrophobic terminus of the lipid tail(s). The possible explanations for the inefficient nature of the lipid bilayer cross-linking are discussed with regard to the preferred conformation of monomeric lipids in the bilayer, the motions of the lipid tails, and competing side reactions. These studies provide a new insight into the behavior of polymerizations in organized assemblies, which will aid in the design of new materials based on bilayers or other types of assemblies, e.g. inverted hexagonal or bicontinuous cubic phases.

Introduction

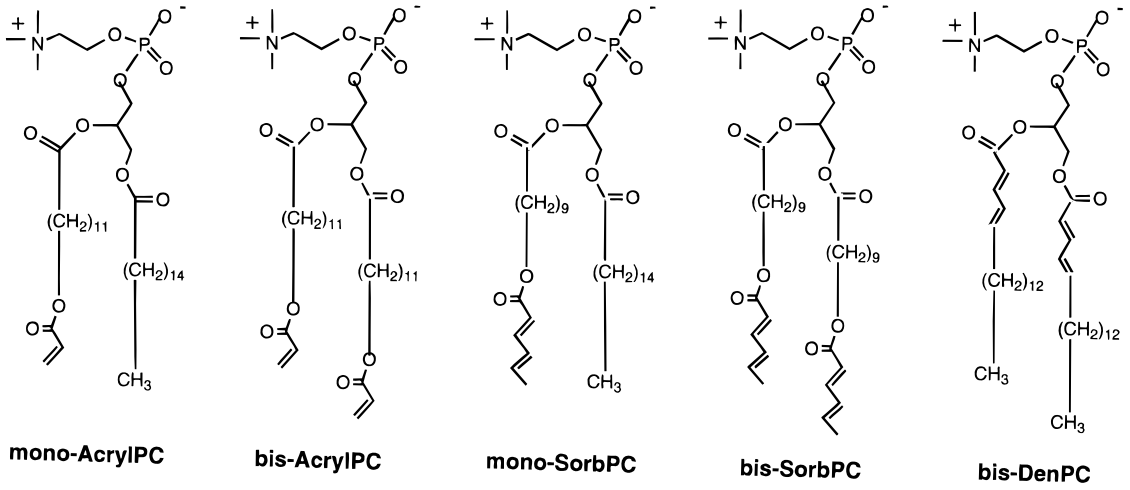
The self-assembly of amphiphilic molecules in water yields lamellar or nonlamellar supramolecular assemblies depending on their structure and the variables of temperature, concentration, and pressure.[1] The polymerization of these assemblies is an effective means to modify their chemical and physical properties. The reactivity of the polymerizable amphiphilic monomer in two-dimensional arrays of molecules, such as hydrated lipid bilayers, is dependent on the mode of initiation, the polymerizable group, and the position of the reactive group in the amphiphile.[2] Unlike monomers in isotropic media, the motions of hydrated amphiphilic monomers in assemblies is limited by the two-dimensional fluid of tens of thousands of amphiphiles in which the hydrophilic head groups are exposed to water and the hydrophobic tails are aggregated to minimize water contact. In certain hydrated lipid phases, the amphiphiles rapidly diffuse within the assembly, which affords ample opportunity for monomers to react with the propagating chain end.[3] Thus the assembly is highly ordered yet sufficiently dynamic for efficient chain polymerizations. Numerous studies of bilayer polymerizations have reported the design of reactive lipids based upon acryloyl, methacryloyl, dienoyl, sorbyl, lipoyl, styryl, vinyl, and other groups suitable for radical chain polymerizations.[4−6] Significant differences between bilayer and solution or bulk polymerizations were revealed through systematic investigations of radical initiated polymerizations in bilayers composed of acryloyl (Acryl), methacryloyl (Meth), and sorbyl (Sorb) substituted lipids.[7−10] At high conversions, the polymer chains are likely to be terminated by reaction with initiator fragments, i.e. primary radical termination. Primary termination occurs in isotropic media in viscous solvents and/or at very high initiator concentrations. Relatively large degrees of polymerizations, X_n, were observed for the radical polymerizations of acryloyl-, methacryloyl-, and sorbyl-substituted lipids in bilayers. There are sufficient lipid monomers in a vesicle, even a small 100 nm diameter vesicle has 8×10^4 lipids, to support the growth of exceptionally long linear polymers. Differences in the reactivity of the propagating radical are also reflected in the size of the polymers obtained from the polymerization of bilayers of AcrylPC and SorbPC (Chart 1).

Polymerization of monomeric lipids in an assembly proceeds in a linear or cross-linking manner, depending on the number of polymerizable groups per monomeric lipid. Lipids that contain a single reactive moiety in either of the hydrophobic tails or associated with the hydrophillic head group yield linear polymers. Polymerization of lipids with reactive groups in each hydrophobic tail generally yields cross-linked polymers. Two-dimensional polymeric networks in condensed phases have also been reported for polymerizations in smectic liquid crystals,[11,12] at interfaces,[13] and in the hydrophilic regions of cast multilayer films.[14] Other cross-linking strategies employed in mono- and multilayers include the addition of small molecule cross-linkers[15] and the use of bifunctional amphiphiles.[16] Linear and cross-linked polymeric assemblies have significantly different physical properties, e.g. permeability, chemical stability, solubility. Polymerized vesicles show a decrease in bilayer membrane permeability.[17−19] Polymerization of lipid bilayer vesicles composed of mono-substituted lipids yields linear polymers in the bilayer, causing a moderate decrease in the permeability of water soluble solutes, e.g. glucose or sucrose, to ca. 0.3 of the unpolymerized bilayer membrane.[17,18] In constrast, the polymerization of bis-substituted lipids to yield cross-

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.

S0024-9297(96)01104-7 CCC: $12.00

Chart 1



linked poly(lipid) vesicles can decrease the solute permeability by 2 orders of magnitude.[17,18] The stability of vesicles to solubilization increases from those that are unpolymerized to those that were polymerized in a manner to yield linear polymers, and finally to cross-linked bilayer vesicles.[20] The polymerization of vesicles composed of the lipid bis-DenPC (Chart 1) were shown to be resistent to solubilization by either sodium dodecyl sulfate or Triton X-100.[4,21] The increased chemical stability can be attributed to the cross-linking of the lipids into a covalently linked monodomain polymeric vesicle.

Although these earlier studies demonstrated that cross-linking polymerizations could occur in bilayers, they did not provide a basis of comparison of cross-linking in the constrained environment of organized assemblies vs isotropic polymerizations. In this report we describe three approaches to the characterization of the gel point for polymerizations constrained by the two-dimensional nature of lipid bilayers. These methods described here rely on experimentally observed changes in lipid lateral diffusion, polymer solubility, and vesicle stability. A preliminary report of the lipid lateral diffusion method has previously appeared.[22]

Results and Discussion

The gel point for two-dimensional lipid assemblies was determined by correlation of the onset of significant changes in the physical properties of the polymerized bilayers with the bilayer composition. The properties examined in this study were the lateral diffusion of a small molecule probe of the bilayer, the stability of polymerized bilayer vesicles in the presence of surfactants, and the solubility of lipid polymers isolated from the bilayers after removal of water. We utilized fluorescence photobleaching recovery to obtain the lateral diffusion coefficient of a fluorescent probe within the bilayer following the procedures previously developed for unpolymerized lipid assemblies.[23,24] The chemical stability of bilayer vesicles to surfactants was determined using quasi-elastic light scattering (QELS) to detect particle size changes resulting from vesicle conversion to mixed micelles.[25] The weight percent solubility of the polymer was measured by attempting to dissolve the dehydrated lipid polymer, removing any insoluble material, and weighing the recovered soluble polymer after removal of the solvent. The bilayer composition was controlled by varying the molar ratio of mono-substituted and bis-substituted lipids, where the mono-substituted lipid yields linear polymers and the bis-substituted lipid served as a cross-linking agent. The specific lipids used in this study are phosphatidylcholines (PC) with the polymerizable groups Acryl and Sorb incorporated into the terminal end of the lipid hydrocarbon chains (Chart 1). The phase behavior and polymerization characteristics of these lipids have already been described.[8−10,26] In certain cases the effect of varying the polymerization conditions was also investigated by using thermal or redox generation of the initiating radicals, or by direct photoactivation of the monomeric lipids. A comparison of radical vs photoactivated polymerization for the SorbPC lipids allowed an examination of the effect of polymer size on the cross-linking efficiency, because the degree of polymerization for radical initiation was ca. 50−300 (depending on the [M]/[I], the ratio of the concentrations of polymerizable groups and initiator) and 5−10 for direct photolysis.[10]

Fluorescence Photobleaching Recovery. The lateral or translational diffusion of membrane components in the plane of the bilayer was predicted by the fluid mosaic model of biological membranes. Shortly after the appearance of the model, both Edidin[27] and Cone[28] reported experimental confirmation of lateral diffusion of membrane proteins. The ability to estimate diffusion of lipids became possible with the advent of techniques to photobleach fluorescently labeled lipid probe molecules, e.g. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphoethanolamine (NBD-PE), which were incorporated into the bilayer membrane. These techniques, which are known as fluorescence recovery after photobleaching (FRAP) or fluorescence photobleaching recovery (FPR), have allowed the determination of lateral diffusion coefficients, D, for a wide variety of model and biomembranes.[29] The D of lipids in model bilayer membranes has been reported by several authors to be between 1 and 5 $\mu m^2/s$, when the sample temperature is above the lipid phase transition temperature, T_m, i.e. the hydrated lipids are in the L_α phase.[29] However when the experimental temperature is below the T_m, the D is reduced by at least 2 orders of magnitude.

Since the lateral diffusion of lipids and other small molecules in the plane of the lipid bilayer is sensitive to the phase and composition of the bilayer, it was expected that polymerization of the bilayer would affect diffusion as well. Lateral diffusion coefficients for NBD-PE in linear or cross-linked polymeric hydrated lipid

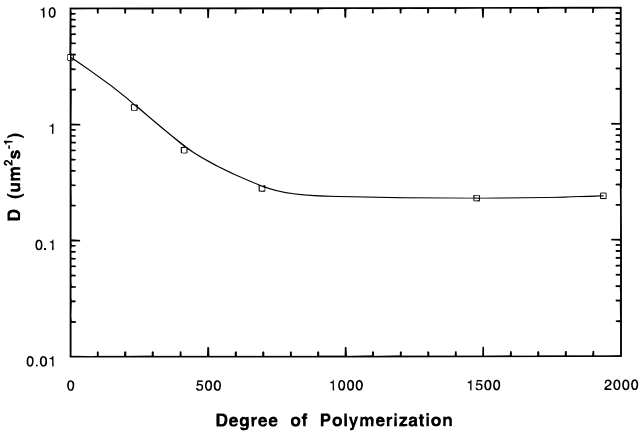


Figure 1. Measured lateral diffusion coefficeint (D, $\mu m^2\ s^{-1}$) of NBD-PE in polymerized mono-AcrylPC bilayers as a function of the degree of polymerization. Measurement temperature, 35 °C; [M]/[I] = 5−40; lipid/NBD-PE, 1000:1.

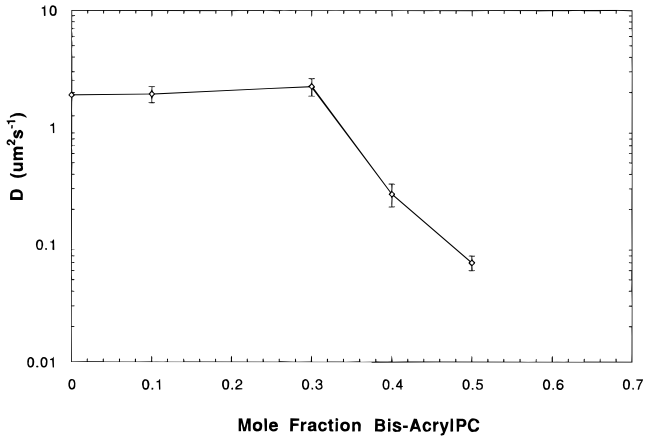


Figure 2. Measured lateral diffusion coefficient (D, $\mu m^2\ s^{-1}$) of NBD-PE in polymerized mono-AcrylPC/bis-AcrylPC bilayers as a function of the mole fraction of bis-AcrylPC. Measurement temperature, 35 °C; [M]/[I] = 5; lipid/NBD-PE, 1000:1.

bilayers were measured, where the mole fraction of NBD-PE was 10^{-3}. The lipids selected for this study were mono-AcrylPC and bis-AcrylPC, respectively. The experimentally determined D for NBD-PE in unpolymerized bilayers of mono-AcrylPC at 40 °C was 3.8 ± 0.6 $\mu m^2\ s^{-1}$. The nonpolymerizable dimyristoylPC (DMPC) was used as a reference lipid in these studies. The measured D for NBD-PE in bilayers of DMPC was 3.9 ± 0.6 $\mu m^2\ s^{-1}$ at 32 °C. In both cases the measurements were performed at 8 °C above the T_m of the respective lipids. Thus the motion of lipids in the L_α phase of unpolymerized mono-AcrylPC appears to be comparable to that of other commonly used phospholipids. The D for NBD-PE in mono-AcrylPC bilayers was studied as a function of temperature and found to significantly decrease at 32.0 ± 0.5 °C. This temperature agrees with the T_m value of 31.8 °C obtained by differential scanning calorimetry.[26]

The diffusion coefficient at 40 °C of NBD-PE in linearly polymerized mono-AcrylPC bilayers varies with the degree of polymerization (Figure 1). Polymerizations were initiated by thermal decomposition of AIBN at 70 °C. The degree of polymerization was controlled by selection of the appropriate [M]/[I] ratio, as described earlier by Sells and O'Brien.[7,8] The diffusion coefficients for these linearly polymerized bilayers were rather insensitive to temperature over the range of 15−40 °C, which is not surprising since the linearly polymerized bilayers of mono-AcrylPC do not exhibit a phase transition over this temperature range. The lack of phase transition indicates that the polymer chains inhibit the cooperative interaction of lipid tails associated with the thermotropic transition from a solid-analogous to liquid-analogous phase. The data in Figure 1 show that increasing the degree of polymerization of linear poly(mono-AcrylPC) progressively decreased the diffusion coefficient for the small molecule probe. The decrease was approximately an order of magnitude as the bilayer was changed from unpolymerized to one composed of many linear chains with a number-average degree of polymerization of ca. 700. Further decreases in D were not observed if the polymer size was increased beyond this number of repeat units.

After establishing the effect of polymer size on the lateral diffusion of NBD-PE, the effect of bilayer composition on the diffusion coefficient was examined. The mole ratio of mono-AcrylPC and bis-AcrylPC in the bilayer was varied and the bilayers were polymerized with AIBN at a [M]/[I] of 5, where bis-AcrylPC was counted as two acryloyl groups. The number-average degree of polymerization for pure mono-AcrylPC polymerized at a [M]/[I] of 5 was comparatively small, i.e. 233 ± 29, and the diffusion coefficient of NBD-PE was high at 1.4 ± 0.4 $\mu m^2\ s^{-1}$. This relatively fast diffusion was observed in polymerized bilayers prepared from mono-AcrylPC and bis-AcrylPC as long as the mole fraction of bis-AcrylPC was 0.3 or less. However, the data in Figure 2 show that polymerized bilayers with higher mole fractions of bis-AcrylPC exhibited a significantly reduced diffusion coefficient for NBD-PE. The FPR measurements show a significant decrease in the dynamic motion of the the NBD-PE probe at an initial bilayer composition of ca. 2:1 mono- and bis-AcrylPC. This inhibition of small molecule mobility within the bilayer signals a change in the polymerized bilayer structure, which we ascribe to gelation. In order to test this hypothesis, other methods of characterizing the polymerized bilayer and the polymers themselves were utilized as described next.

Solubility of Polymeric Lipids. Linear and cross-linked polymers can be distinguished by their different physical properties, e.g. permeability, viscosity, and solubility.[17,18,30] Solubility is frequently used to determine whether a polymer is cross-linked.[20,31] In order to test for the cross-linking of bilayers composed of various mole fractions of mono- and bis-substituted PCs, the resulting polymers were isolated and their weight percent solubility determined. The polymerized lipids were recovered from the hydrated bilayer by freeze-drying the sample after completion of the polymerization and then dispersing the remaining solid in an organic solvent in an attempt to dissolve the polymer. A preliminary examination of the effectiveness of various solvents for polymerized zwitterionic PC lipids indicated that 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was the most effective solvent for these polymers. HFIP is an excellent solvent for polymerized lipids due its high hydrogen-bond-donating capability and weak self-association.[32,33] These properties make it a good solvent for a variety of zwitterionic polymers. HFIP has been used as a solvent for polymers which contain a phosphocholine moiety such as found in the lipids used in this study.[34] In addition, the capability of HFIP to form hydrogen bonds with carbonyl functionalities could be important for the poly(acrylates) and poly(sorbates) formed from AcrylPC and SorbPC, respectively.[35]

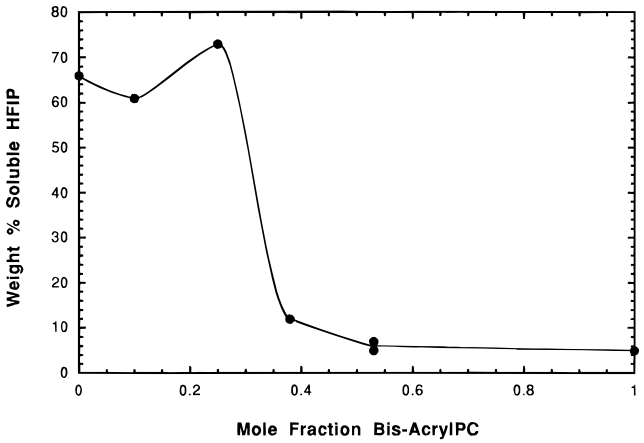


Figure 3. Weight percent solubility of polymerized mono-AcrylPC/bis-AcrylPC bilayers as a function of the mole percent of bis-AcrylPC. Initiation by decomposition of AIBN at 60 ± 2 °C; [M]/[I] = 5.

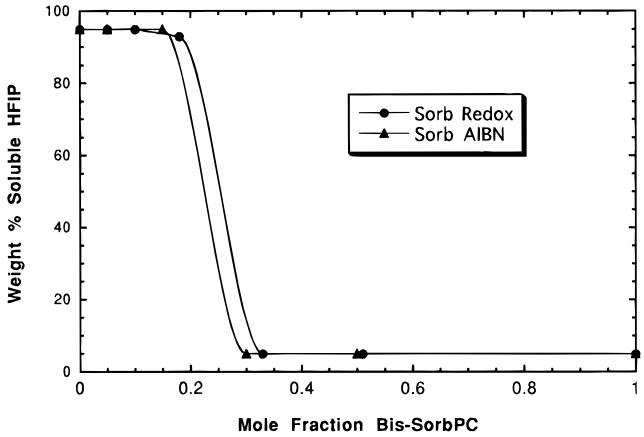


Figure 4. Weight percent solubility of polymerized mono-SorbPC/bis-SorbPC bilayers as a function of the mole percent of bis-SorbPC. (a) Initiation by thermal decomposition of AIBN at 60 ± 2 °C; [M]/[I] = 5. (b) Initiation by $K_2S_2O_8/NaHSO_3$ (1:1) at 60 ± 2 °C; [M]/[I] = 5.

The weight percent of the lipid sample that was soluble in HFIP is shown in Figure 3 as a function of the mole fraction of bis-AcrylPC in the bilayer at the time of the polymerization. The polymerized lipid solubility is drastically different if the mole fraction of bis-AcrylPC is 0.25 (soluble) as opposed to 0.38 (insoluble). This physical change suggests the formation of cross-linked polymers when the mono-AcrylPC to bis-AcrylPC ratio was ca. 2:1, whereas at a molar ratio of ca. 3:1 or higher the polymerized lipids do not appear to be cross-linked. The change in solubility of the isolated poly(AcrylPC) is consistent with the FPR data described above and provides additional evidence that radical chain polymerization of a bilayer composed of ca. 2:1 mono- and bis-AcrylPC bilayer results in gelation of the bilayer.

The data in Figure 3 also show that even when the bilayer composition has insufficient bis-AcrylPC to effectively cross-link the bilayer, the resulting polymer chains were not completely dissolved by the HFIP. The poly(AcrylPC) chains are relatively large and the polydispersity reported by Sells and O'Brien indicate that some of the polymer chains have degrees of polymerization of ca. 10^3, with molecular weights approaching 1 million.[8] High molecular weight chains, such as the larger poly(AcrylPC) chains, can become entangled, thereby preventing complete solubilization.

Lamparski and O'Brien found that the number-average degree of polymerization of mono-SorbPC in bilayers was approximately one-quarter of that found for mono-AcrylPC at the same [M]/[I] using AIBN initiation.[10] Therefore, it was interesting to examine the solubility behavior of polymers obtained from the polymerization of bilayers composed of different proportions of mono-SorbPC and bis-SorbPC. Figure 4 shows the measured HFIP solubility of the isolated polymers as a function of the mole fraction of bis-SorbPC in the bilayer. Note that the smaller linear poly(SorbPC) chains are nearly completely soluble in HFIP, in contrast to the behavior of the poly(AcrylPC) discussed above. At the [M]/[I] of 5 used, the relative number-average degree of polymerization of poly(SorbPC) was 54 with a polydispersity of 1.84. The figure shows a major change in polymer solubility when the mole fraction of bis-SorbPC in the bilayers was increased from 0.15 to 0.30, where the higher fraction of bis-SorbPC rendered the polymerized bilayers insoluble in HFIP. The apparent onset of cross-linking occurs at somewhat smaller mole fraction of the bis-SorbPC (Figure 4) compared to the data obtained for the AcrylPC bilayers (Figure 3), which appears to reflect the difference in the relative number-average degree of polymerization. i.e. 54 and 233, respectively, for the two types of reactive lipids.[8,10]

The polymerization of bilayers composed of mono- and bis-SorbPC was also initiated by hydroxyl radicals generated by redox reaction at 60 °C of potassium persulfate and sodium bisulfite.[36−38] Although the initiating chemistry is water soluble and resides outside the lipid bilayer vesicle, the hydroxyl radical initiating species partitions into the bilayer in sufficient quatitity to polymerize lipids such as SorbPC, where the reactive group is near the center of the hydrophobic region of the bilayer, as well as DenPC,[39] where the dienoyl group is close to the lipid glycerol backbone, which is presumed to interact with water as in the case of dioleoylPC.[40] Although the ratio of monomeric lipid to initiator, i.e. 5, was the same as the experiments with AIBN, the true ratio in the bilayer is unknown since the partition coefficient for hydroxyl radicals between the aqueous phase and the bilayer is unknown as of this writing. In spite of this uncertainty, the solubility characteristics of the polymerized SorbPC bilayers were very similar whether the polymerization was initiated by this redox chemistry or AIBN (Figure 4).

The solubility of linearly polymerized phospholipids in HFIP is great enough to permit the effective use of poly(lipid) solubility to estimate the mole fraction of the bis-substituted lipid required for effective cross-linking of the bilayer. The data for the AcrylPC bilayers agrees well with the lateral diffusion measurements obtained by FRP, thereby providing a high degree of confidence in both procedures since the errors in each are dissimilar. The HFIP solubility protocol is most effective with poly(lipid) chains that have a degree of polymerization less than ca. 10^3. As long as this possible limitation is given proper consideration, the HFIP procedure appears to have broad applicability for the determination of gelation of polymerized assemblies, i.e. bilayers, LB multilayers, nonlamellar phases. The solubility procedure does not require the sophisticated instrumentation necessary for FRP studies, and the amount of lipid required is only dependent on the sensitivity of an available balance.

Surfactant Solubilization of Vesicles. Ease of solubilization can be usefully employed to characterize

the stability of lipid bilayer vesicles in aqueous suspension. Although organic solvents are ineffective for such studies, surfactant micelles are ideal as long as the polymer chains are short enough to be incorporated into a mixed micelle. It has long been known that surfactant micelles are effective at solubilizing lipids and other biomembrane components. Lichtenberg et al. reviewed the structural and kinetic aspects of lipid solubilization.[41] Philippot et al.[42] and Mimms et al.[43] described especially effective methods of forming unilamellar vesicles (LUV) by surfactant dialysis of mixed micelles of phospholipids and nonionic surfactants. Several authors reported that effective solubilization of lipids into micelles requires that the surfactant concentration be greater than its critical micelle concentration (cmc) and that the the surfactant to lipid molar ratio be ca. 3−5.

Regen and co-workers reported that stability of vesicles to surfactant treatment increases in the following order for vesicles that are unpolymerized < linearly polymerized < cross-linked.[20] The increased stability to surfactant action has been attributed to the cross-linking of the lipids into a covalently linked mono-domain polymeric vesicle. The mechanism of surfactant solubilization (lysis) of bilayers initially involves the incorporation of surfactant amphiphiles into the bilayer. When the surfactant concentration predominates, the lipids no longer exist in a vesicle but are incorporated into a mixed micelle of lipid and surfactant. If the lipids are cross-linked in a polymerized vesicle, they cannot be readily extracted by surfactant.

In the experiments described here, factors governing vesicle stabilization, polymer size, and the extent of cross-linking were investigated. The effects of the detergent Triton X-100 on the lysis of polymerized vesicles composed of mono-SorbPC and/or bis-SorbPC were used to examined the above factors. The size of poly(SorbPC) chains could be controlled by the initiator type and concentration. UV-light photopolymerizations of mono-SorbPC yielded oligomers, while much longer polymers were formed by the AIBN-initated polymerizations.[10]

The extent of vesicle lysis by Triton X-100 was determined by QELS. In QELS experiments, the intensity of the scattered light (in photons/second) is measured and the average mean diameter of the particle calculated. The diameter of the vesicle/particle was determined by a variety of different mathematical algorithms (based on a spherical model) which analyze the measured autocorrelation function.[25] Thus, the lysis of poly(lipid) vesicles with detergent results in decreased particle size since the lipids are now solubilized as mixed micelles. Mono- and bis-SorbPC vesicles were polymerized by photoirradiation and then Triton X-100 was added to the suspension (Figure 5). If the vesicles were dissolved to form mixed micelles, the light-scattering intensity decreased due to the smaller particle diameter. Unpolymerized and linear polymerized mono-SorbPC were lysed after the addition of 4 equiv of Triton X-100, whereas the average mean diameter for bis-SorbPC polymerized vesicles remained constant up to at least 15 equiv of Triton X-100. At high surfactant concentrations two different size particles could be observed at ca. 140 and 5−20 nm due to the polymerized vesicles and Triton X-100 micelles, respectively. After cross-linked vesicles become saturated with Triton X-100, excess surfactant forms micelles in the presence of the stabilized vesicles. Scattering intensities can also be used to show the chemical stability of cross-linked

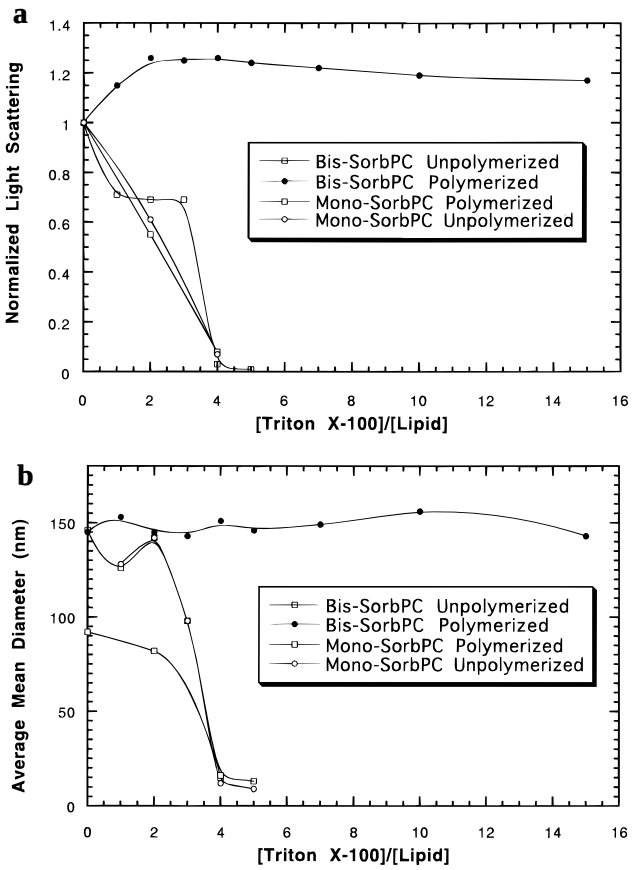


Figure 5. (a) Normalized light scattering for unpolymerized and polymerized mono- and bis-SorbPC LUV as a function of added equivalents of Triton X-100. Samples were polymerized by direct photoirradiation. (b) Average mean diameter for unpolymerized and polymerized mono- and bis-SorbPC LUV as a function of added equivalents of Triton X-100.

vesicles, since larger particles (vesicles) scatter more light than smaller ones (micelles). Scattering intensites for unpolymerized and linearly polymerized mono-SorbPC decreases by an order of magnitude after the addition of 4 equiv of Triton X-100. Normalized scattering intensity of polymerized bis-SorbPC cross-linked vesicles remained unchanged even at 15 equiv of surfactant. Light scattered (in photons/second) varied for each sample and was normalized as described in the Experimental Section.

The normalized scattering intensity after the addition of 15 equiv of Triton X-100 to the polymerized bilayer sample is shown in Figure 6 as a function of the mole fraction of bis-SorbPC in the the bilayer prior to polymerization. A change in vesicle stability to Triton X-100 occurs between vesicle samples having 0.34 vs 0.42 mole fraction bis-SorbPC. The resistence to solubilization of the polymerized vesicles observed at the higher mole fractions of bis-SorbPC is attributed to gelation of the vesicles during the polymerization, because cross-linking of the lipids prevents the extraction of lipid molecules from the vesicle and into mixed micelles.

The stabilities of AIBN-polymerized vesicles of both mono- and bis-SorbPC were examined by detergent lysis. As in the case of UV-polymerized vesicles, the extent of vesicle dissolution was monitored by QELS. The normalized light-scattering data for AIBN-polymerized vesicles of mono-SorbPC and bis-SorbPC are plotted as a function of the ratio of [Triton X-100]/[lipid] in Figure 7. The light scattering intensity of unpolymerized mono-SorbPC vesicles decreased by ca. 40%

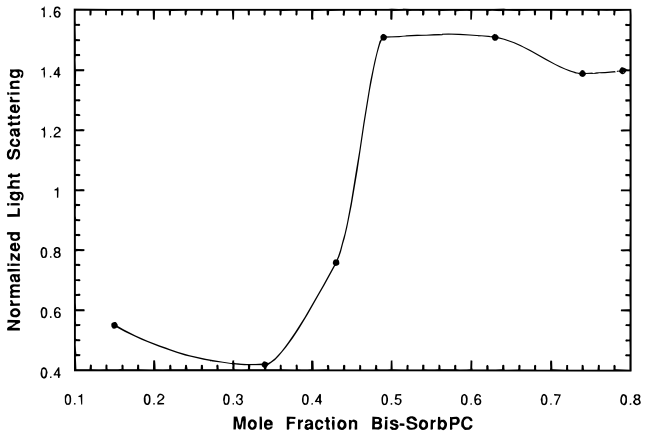


Figure 6. Normalized light scattering of polymerized mono-Sorb/bis-SorbPC LUV after addition of 15 equiv of Triton X-100 as a function of mole fraction bis-SorbPC. Polymerization by direct photoirradiation.

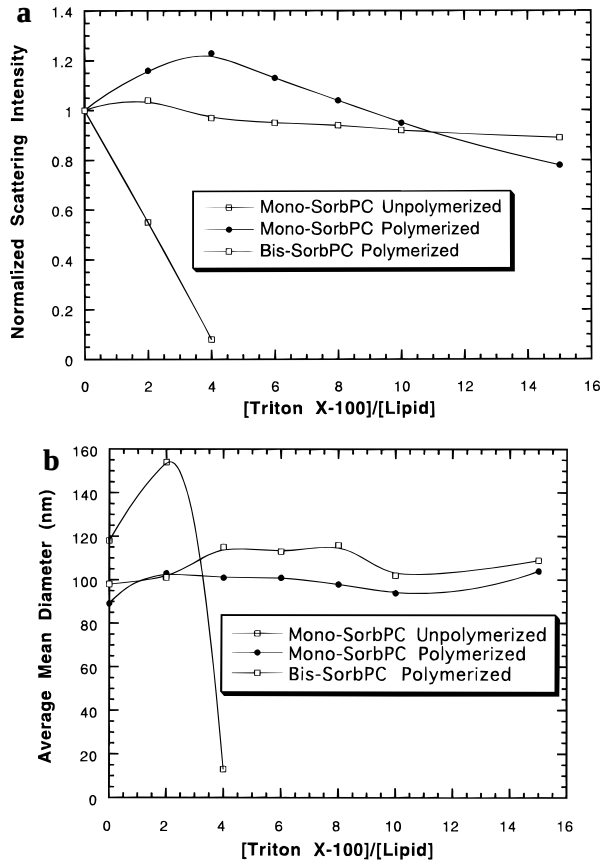


Figure 7. (a) Normalized light scattering for unpolymerized and polymerized mono- and bis-SorbPC LUV as a function of added equivalents of Triton X-100. Polymerization initiated by thermal decomposition of AIBN at 60 ± 2 °C; [M]/[I] = 5. (b) Average mean diameter for unpolymerized and polymerized mono- and bis-SorbPC LUV as a function of added equivalents of Triton X-100.

upon the addition of 2 equiv of Triton X-100, with complete lysis after addition of 4 equiv. The addition of Triton X-100 to poly(lipid) vesicles of mono-SorbPC resulted in an initial increase in the amount of light scattered followed by a decrease at higher amounts of Triton X-100. However, the magnitude of the scattering decrease did not reflect solubilization of the poly(lipid) vesicle. Examination of the particle diameter shows a slight increase in size with no measurable decrease at higher concentrations of Triton X-100. As expected, the AIBN-polymerized bis-SorbPC vesicles did not exhibit any significant change in light-scattering intensity or particle diameter upon the addition of 15 equiv of Triton X-100. Vesicles generally show an apparent increase in size as the surfactant is added. This effect, which is ascribed to the incorporation of surfactant molecules into the bilayer, is usually much smaller for polymerized than unpolymerized vesicles.

These data show that both AIBN-initiated linearly polymerized and cross-linked vesicles of SorbPC were stable with respect to 15 equiv of Triton X-100 (Figure 7). This contrasts with the behavior of photopolymerized vesicles of SorbPC. Photoirradiation of mono-SorbPC yields oligomers.[10] These oligomers are small enough to be dissolved by surfactants and the vesicles are disrupted. Initiation with AIBN yields polymers with a number-average degree of polymerization of ca. 50. These linear polymers apparently are too large to be effectively extracted from the vesicle into a Triton X-100 micelle. Therefore, the vesicle lysis methodology for determination of gelation cannot be employed if the poly(lipids) are too large.

The stability of vesicles to surfactant is greatly enhanced by polymerization of the lipid chains, to the point that lysis does not occur. Furthermore, the surfactant solubilization was dependent upon the degree of polymerization (X_n), extent of cross-linking, as well as location of the reactive group in the lipid. Oligomeric vesicles were efficiently dissolved with Triton X-100, whereas vesicles of longer linear polymers were not solubilized. The mole fraction of a cross-linking lipid necessary for stabilization was 0.3−0.4, which is similar to results from the other methods. It is interesting to compare the behavior of mono-SorbPC polyvesicles to mono-DenPC polyvesicles analyzed by Tsuchida et al.,[21] where both had been polymerized by radical polymerization to similar extents. The poly(SorbPC) vesicles were considerably more stable to surfactant lysis. This difference may reflect greater entanglement of the polymer chains in the middle of the bilayer (SorbPC), where the chains exhibit maximum disorder, whereas in mono-DenPC the polymer is formed near the more ordered glycerol backbone.

General Discussion

Each of the three methods utilized here to characterize the gelation of polymerized bilayers have strengths and weakness. The limitations of surfactant solubilization of vesicles are evident from the experiments involving linear polymers with X_n that were >50. Both the solubility and FRP procedures can be employed with much larger polymers. The HFIP solubility protocol appears to be the most generally useful because of the instrumentation requirements for lateral diffusion measurements.

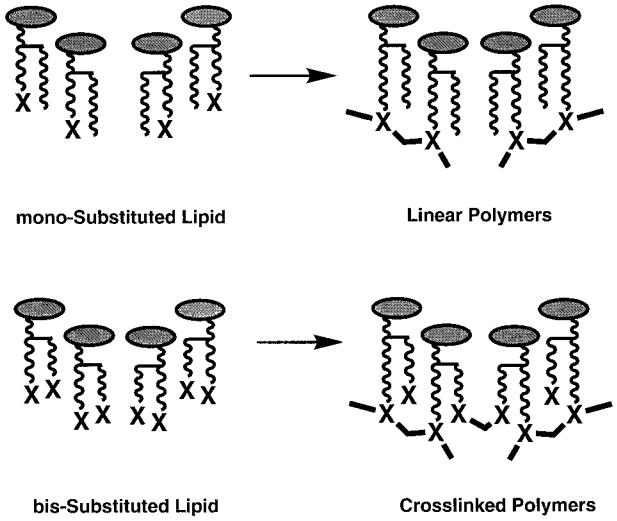
The effect of polymerization on the AcrylPC bilayers on the lateral diffusion of NBD-PE can be usefully compared with earlier studies of the lateral diffusion of small molecule probes in polymerized bilayers. Gaub et al. reported the lateral diffusion in bilayers of a bis-dienoyl ammonium lipid both before and after photopolymerization.[44] Hydrated bilayers of the lipid had a T_m at 30 °C, which increased to about 34 °C after the bilayers are photopolymerized. The unpolymerized bilayers showed a D of 5 $\mu m^2/s$ at 35 °C, which was decreased to nearly 1 $\mu m^2/s$ after the sample bilayers were polymerized. The small increase in T_m, upon photoreaction of the lipid bilayer, indicated that the polymerization had a relatively small effect on the

cooperativity of the lipid chains. This effect could be due to the formation of relatively short polymer chains and/or due to the location of the polymer chain near the lipid−water interface, where it has little direct effect on the motion of the hydrophobic chains. The relatively small effect of the polymerization on *D* suggested that the polymer chains were short with little effective cross-linking. An increase in the average molecular size from monomer to polymer will increase the diffusion pathway of the fluorescent probe. This is observed as an increased recovery time and a smaller *D*. Therefore if the polymer chains are short, then only a moderate increase in the diffusion pathway and corresponding decrease in the lateral diffusion coefficient will be found.

Eggl et al.[45] reported a Monte Carlo simulation of lateral diffusion of fluorescent probes in bilayers of linearly polymerized amphiphiles. The lipids were polymerized through the head groups that interface with the aqueous buffer by either photochemical or thermal initiation. The *D* of a small cyanine dye probe was reduced from 25 $\mu m^2/s$ to about 8 $\mu m^2/s$ upon photochemical formation of polymers in giant vesicles. By assuming that the radius of gyration in two-dimensions scales as $X_n^{3/4}$, they calculated that the X_n was 20 for these polymers. When Eggl et al.[45] studied the lateral diffusion of polymers formed by thermal initiation, the observed *D* was 0.01 that of the unpolymerized bilayers. The X_n for the thermal polymer was estimated to be 300 based on the model. The simulation indicates that lateral diffusion of small molecule probes is very sensitive to the size of linear polymers formed in the bilayer. That prediction agrees with the effect of the degree of polymerization of linear polymers of poly(AcrylPC) on the *D* of NBD-PE, but the magnitude of the effects are smaller in the poly(AcrylPC) system. The differences in sensitivity of *D* to the polymer's degree of polymerization may reflect differences in linear polymer conformation when the polymers are formed at the lipid−water interface vs those formed near the midline of the bilayer interior. The differences could be a consequence of the location of the polymer in the bilayer relative to the fluorescent probe. In the ideal situation, the diffusion path of the probe is increased as the polymer chain length increases, as the probe must diffuse around the polymer. However, it may be possible for the NBD-PE probe to diffuse over the polymer chain of the poly(AcrylPC) because the polymer is near the middle of the bilayer. To the extent that this occurs, the effective polymer chain length will be reduced, resulting in decreased sensitivity of *D* on the measured X_n.

Each of the three methods used to characterize cross-linking of reactive lipids in bilayers indicated that a substantial mole fraction (0.25−0.35) of the bis-substituted lipid was necessary to cause cross-linking of the bilayer. The general agreement between the methods provides confidence that these originally suprising results accurately indicate the inefficiency of the cross-linking process in bilayers composed of lipids having a reactive group at the hydrophobic terminus of the lipid tail(s). A few mole percent (or even less) of a suitably designed bifunctional monomer is generally sufficient to yield cross-linked polymers in isotropic polymerizations. There are at least two possible explanations for the inefficient nature of the bilayer cross-linking with the lipids studied here. The high fraction of bis-PC required to reach the gel point could be a consequence of the bilayer conformation of mono- and bis-PC phospholipids.[46,47] Structural studies of phospholipids in the the L_β phase, i.e. at temperatures below the T_m, show the glycerol backbone to be perpendicular to the plane of the bilayer. This conformation causes the *sn*-1 chain to penetrate further into the the bilayer than the *sn*-2 chain. This places the two polymerizable groups of bis-PC at nonequivalent depths within the bilayer. On the other hand, molecular simulations of PC bilayers in the L_α phase reveal a dynamic mixture of lipid tail conformations, especially for the portion of the tail distal from the lipid−water interface.[48,49] The motion of the lipid tails coupled with the lateral diffusion of the lipids provides the circumstances that permit radical chain growth, but probably not in the sense that is implied by static drawing of lipids in an assembly.

Scheme 1



Consider an explanation of the cross-linking in the bilayer based on a relatively static picture. A drawing such as shown in Scheme 1 implies that the two reactive groups in a bis-PC are positionally inequivalent. This suggests an AB type cross-linking with the *sn*-1 tail of bis-PC and mono-PC preferentially reacting with each other rather than with the *sn*-2 tail of bis-PC. In this case, a cross-link between polymer chains occurs when a reactive group on the *sn*-2 tail of a bis-PC lipid in one polymer chain reacts with a similar group in a neighboring polymer chain. Cross-linking appears to require reactive *sn*-2 tails to form dimeric links between poly(lipid) chains. A competitive process is the reaction of reactive groups in *sn*-2 tails with others in the same polymer chain. Such an intrachain reaction would modulate the polymer motion without linking polymer chains together and therefore require a larger mole fraction of the bis-PC to cause gelation. In contrast, a dynamic picture of the polymerization process would provide a more favorable environment for the reaction of a reactive group in *sn*-1 tails with each other as well as with reactive groups in *sn*-2 tails. Such a process would lead to an irregular polymer backbone with an occasional bis-PC bridge between two polymer chains via an *sn*-1 group in one polymer chain and an *sn*-2 group in the second chain. A further inefficiency in cross-linking would result from intramolecular macrocyclization of the *sn*-1 and *sn*-2 groups in the same lipid. Although the formation of such a large macrocycle is statistically unfavored in solution, the constrained nature of the bilayer greatly reduces the number of conformations available to the lipid tails. To the extent that the degrees of freedom of the lipid are reduced, the

possibility of macrocyclization will be increased. At this time it is not possible to choose between these possibilities, except to note that the polymerizations were all performed in the more dynamic L_α phase.

These studies provide a new insight into the behavior of polymerizations in organized assemblies. Since lipid assemblies frequently are composed of more than a single lipid component, it is especially useful to know the fraction of bis-substituted lipid necessary for cross-linking polymerization. The present study provides that information for lipids with a reactive group at the end of the lipid tails and offers a reasonable starting point for the consideration of the behavior of reactive groups at other sites along the lipid tail. The cross-linking of lipids through reactive groups associated with the hydrophilic head group may be quite different. Until additional studies are completed, we can only speculate on the effect of placing the reactive group at these lipid sites. The determination of the critical compostions for cross-linking of supramolecular assemblies will aid in the design of new materials based on bilayers or other types of assemblies, e.g. inverted hexagonal or bicontinuous cubic phases.[39,50] The effective stabilization of nonlamellar phases appears to require cross-linking rather than linear polymer formation. Cross-linking lipid polymerizations in bilayers composed of reactive and nonreactive lipids can be used to lock-in preexisiting lipid domains or cause the phase separation of the lipids into enriched domains.[51] The latter process has been usefully employed to reorganize lipid bilayers[52] and to destabilize vesicles.[53−55]

Experimental Section

Methods and Materials. Compounds containing UV-sensitive groups were handled under yellow lights. UV−vis absorption spectra were recorded on a Varian DMS 200 spectrophotometer. QELS was performed with a BI8000-autocorrelator from Brookhaven Instruments Corp., and particle sizes were calculated with the accompanying software. The polymerizable lipids, i.e. mono-SorbPC, bis-SorbPC, mono-AcrylPC, and bis-AcrylPC, were synthesized as described previously.[8,10] Lipid purity was evaluated by thin-layer chromatography (TLC) with chloroform/methanol/water (65:25:4 by volume) and visualized with a UV lamp. Azobis(isobutyronitrile), AIBN (Eastman Kodak), was purified by recrystallization three times from methanol. Potassium persulfate, sodium bisulfite, Triton X-100 (TX-100), and HFIP were purchased from Aldrich Chemicals and used as received. The lipids were hydrated in Milli-Q water, Millipore Inc. Benzene was distilled from sodium benzophenone ketyl.

Thermal AIBN Polymerizations. Large multilamellar vesicles of polymerizable lipid were prepared as follows: approximately 2.5−15 mg of lipid was evaporated from stock solutions (10 mg/mL in benzene) by passing a stream of argon over the sample and drying under high vacuum (0.4 mmHg) for a minimum of 4 h. Each lipid was dried and weighted separately for mixtures of mono- and bis-PC. The lipid weight was determined and each lipid film redissolved in 2 mL of benzene, then the two lipids were combined. The appropriate amount of initiator from a freshly prepared AIBN stock solution (1−1.5 mg/mL in benzene) was added to yield a monomer to initiator ratio ([M]/[I]) of 5, where [M] represents the concentration of polymerizable groups. The solvent was evaporated as above and dried under high vacuum for 2 h. All steps involving lipid and AIBN were performed in subdued light. The dried lipid/AIBN film was hydrated with deoxygenated MilliQ water to a final concentration of 7 mM. Samples were vortexed to uniformity and subjected to ten freeze−thaw−vortex cycles (−77 °C → T_m + 5 °C) cycles. Samples were placed in an ampule, which was sealed with a septum and flushed with argon for 0.5 h. Polymerizations were performed at 60 ± 2 °C in a water-circulating bath under a positive argon pressure. All polymerizations were carried out in the absence of light. Polymerizations were monitored by UV absorption spectroscopy of aliquots diluted with Milli-Q water to ca. 80 μM.

Redox Polymerizations. LUV of SorbPC were prepared as follows: approximately 2.5−15 mg of lipid was evaporated from stock solutions and dried separately as with AIBN polymerizations. The two lipids were combined. The dried lipid film was hydrated with deoxygenated Milli-Q water to a final concentration of 7 mM. Samples were vortexed to uniformity and subjected to ten freeze−thaw−vortex cycles (−77 °C → 37 °C). The lipid suspension was extruded 10 times (2 × 0.6 μm−2 × 0.4 μm−6 × 0.1 μm) through two stacked Nuclepore polycarbonate filters at 37 °C using a stainless steel extruder from Lipex Biomembranes.

The redox initiator was prepared from $K_2S_2O_8$ (300 mg, 1.1 mmol) and $NaHSO_3$ (115 mg, 1.1 mmol) weighed into a 10 mL volumetric flask and diluted. An aliquot (200−300 μL) was pipetted into the vesicle suspension. The sample was placed into an ampule, which was sealed with a septum and flushed with argon for 0.5 h. Polymerizations were performed at 60 ± 2 °C in a water-circulating bath under a positve argon pressure for 18 h. All polymerizations were carried out in the absence of light. Polymerizations were monitored by UV absorption spectroscopy of aliquots diluted with Milli-Q water to ca. 80 μM.

Direct Photoirradiation. LUV, prepared as above, were placed into a 3 mL quartz cuvette equipped with a magnetic stirbar and placed 1 cm from a low pressure Hg vapor Pen Lamp. Samples with a lipid concentration of 100−300 μM were irradiated for 45 min at a temperature of 40 °C. Polymerizations were monitored by UV absorption spectroscopy as above.

Weight Percent Solubility. Only samples with greater than 85% monomer conversion as determined by UV−vis spectroscopy were used in the solubilty studies. Samples were lyophilized after polymerization. The lipid was weighed and HFIP added until the concentration was 5 mg/mL. The samples were shaken for 2 min and allowed to stand for 5 min. The solution was filtered into a dry test tube through a pipet with glass wool to remove any solid particles. The pipet was then rinsed with 0.5 mL of HFIP. The solvent was removed by passing a stream of argon over the sample then drying under high vacuum for a minimum of 4 h, leaving the soluble polymer, which was weighed and used to calculate the percent solubilty.

Surfactant Dissolution of Vesicles. LUV were prepared as described above. After polymerization the LUV were characterized by QELS for a 2 mL sample with a lipid concentration of 100−300 μM. An aliquot of 45 mM TX-100 solution was added. Each aliquot was equal to 2 equiv of lipid. The light-scattering intensities were determined again by QELS. TX-100 was added in 2 equivalent increments up to a total of 15 equiv. Measurements at each concentration of TX-100 were performed a minimum of three times. Light scattered (in photons/second) varied for each sample and was normalized as described by the following expression.

$$\text{normalized light scattered} = (I_{\text{Triton X}-100} - I_\infty)/(I_o - I_\infty)$$

The term $I_{\text{Triton X}-100}$ is the intensity of photons scattered after the addition of each equivalent of Triton X-100, I_∞ the intensity of photons scattered by a micellar dispersion of TX-100 at a similar concentration, and I_o the intensity of photons scattered by vesicles in the abscence of TX-100. The average mean diameter of vesicles/particles was calculated by multiple mathematical procedures, i.e. CUMULANT, exponential sampling, non-negatively constrained least square, and CONTIN. With the exception of exponential sampling, consistent results were obtained.

Acknowledgment. This research was supported by a grant from the Division of Materials Research of the National Science Foundation.

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MA961104Q