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Published on Web 05/01/2002

Stable Polymeric Nanoballoons: Lyophilization and Rehydration of Cross-linked Liposomes

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Received October 12, 2001

Abstract: The cross-linking of supramolecular assemblies of hydrated lipids is an effective method to stabilize these assemblies to disruption by surfactants or aqueous alcohol. The heterobifunctional lipids, Acryl/DenPC$_{16,18}$ and Sorb/DenPC$_{18,21}$, are examples of a new class of polymerizable lipid designed for the creation of cross-linked lipid structures. The robust nature of cross-linked liposomes was demonstrated by lyophilization of the liposomes followed by their essentially complete redispersion in water. The resulting liposomes were compared to the original sample by quasi-elastic light scattering and transmission electron microscopy. There was no major change in the size or structure of the cross-linked liposomes after rehydration of the freeze-dried powder of liposomes. Moreover, the rehydrated cross-linked liposomes continued to be resistant to surfactant solubilization. Neutral cross-linked liposomes were predominantly redispersed after freeze-drying with the aid of bath sonication. The small amount of residual liposome aggregation observed with neutral liposomes could be prevented by incorporating a surface charge into the liposome or attaching hydrophilic polymers, for example, poly(ethylene glycol), onto the liposome.

Introduction

Amphiphilic molecules such as phospholipids spontaneously form bilayer membranes that can be converted to vesicles, that is, liposomes, by various methods.[1] The liposome is composed of an amphiphilic bilayer that encloses an aqueous interior volume. Liposomes are useful as biological membrane models, drug delivery vehicles, nano-compartments for the formation of biomaterials, among others.[2−5] Water-soluble drugs can be encapsulated into the liposome interior, and hydrophobic compounds may also be incorporated into the hydrophobic core of the lipid bilayer membrane. However, conventional liposomes are inherently unstable in the presence of surfactants or organic solvents. An effective solution for this problem utilizes polymerizable lipids to stabilize preformed liposomes.[6,7] The polymerization of supramolecular assemblies of hydrated lipids has proven to be a practical way to modify the chemical and physical properties of the assembly. Previous results show that linear and cross-linked polymeric assemblies have significantly different physical properties, for example, permeability, chemical stability, and solubility.[8−12] In general, linear polymers provide some stabilization of the bilayers, but not as great as cross-linking polymerization. Thus, cross-linked liposomes are not solubilized even in the presence of more than 10 equiv of surfactant per lipid molecule.[10]

Recent progress in understanding the cross-linking polymerization of lipid bilayers raises the interesting possibility that cross-linked liposomes should properly be considered as nano-sized spherical molecular objects. If this is the case, it should be possible to prepare cross-linked polymeric liposomes in water, then remove the supporting water without disruption of the polymeric spherical shell. To demonstrate this capability, we used the heterobifunctional lipids Acryl/DenPC$_{16,18}$ and Sorb/DenPC$_{18,21}$ (Figure 1). Previous polymerization studies of these two lipids showed that redox-initiated radical polymerization affords cross-linked polymeric liposomes in water which were stable in the presence of surfactants.[13] This paper reports experimental data that demonstrate that the cross-linked liposomes are not destroyed upon complete drying and can be redispersed in water with no apparent change in microstructure. These observations suggest that the range of applicability of cross-linked liposomes and other cross-linked lipid bilayer structures, such as bicontinuous cubic phases[14,15] and inverted hexagonal phases,[16,17] may be greater than previously recognized. Because of the strength and the flexibility of the hollow

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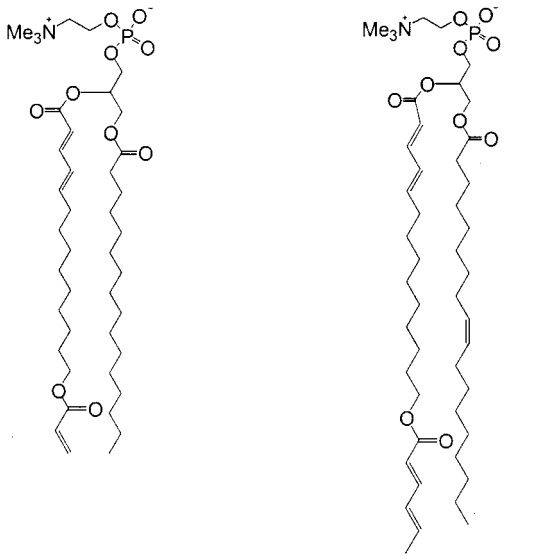

Acryl/DenPC$_{16,18}$ Sorb/DenPC$_{18,21}$

Figure 1. Structures of heterobifunctional lipids.

polymeric nanostructures derived from liposomes, we have termed them polymeric nanoballoons.

Results and Discussion

Large unilamellar bilayer liposomes (LUV) composed of either Acryl/DenPC$_{16,18}$ or Sorb/DenPC$_{18,21}$ were prepared by the freeze−thaw extrusion method.[18] The LUV had an average diameter of ca. 115 ± 10 nm. They were polymerized with a redox initiator at 60 °C to stabilize the lipid bilayer. Examination of the liposomes by negative staining TEM showed that the liposomes were generally smooth and spherical. Typical electron micrographs of liposomes are shown in Figure 2a and indicate the liposomes are predominantly unilamellar. No significant change in the liposome appearance was caused by the cross-linking polymerization (Figure 2b), although the average of the calculated mean diameters obtained by quasi-elastic light scattering (QELS) was slightly smaller after polymerization.[19]

We examined the effect of sample lyophilization and redispersion in water on the liposomes' size and appearance. Freeze-drying of the cross-linked liposomes produced a white powder. Simple hydration of the dried liposome powder did not completely redisperse them in water, but produced a mixture of suspended liposomes and larger aggregates. To reduce the size and proportion of the aggregates, the lipid samples were simply sonicated with a bench type bath sonicator for 5−15 min to obtain a homogeneous clear suspension.

Table 1 shows the results of redispersion of the polymerized liposomes measured by QELS. The percent LUV redispersion is defined as the measured percent of redispersed particles that has a diameter of approximately 115 nm, which is taken to be the population of un-aggregated liposomes. The balance of the observed light scattering particles was usually considerably greater than 200 nm in diameter. It is well known that larger particles scatter light more than smaller particles. Consequently, this analysis tends to understate the fraction of ca. 115 nm diameter liposomes that was successfully redispersed. More than 80% redispersion was observed for cross-linked liposomes prepared from either Acryl/DenPC$_{16,18}$ or Sorb/DenPC$_{18,21}$. Examination of these redispersed liposomes by TEM indicated they had a similar size distribution to that determined prior to freeze-drying (Figure 2c and d). We found that the addition of the surfactant Triton X-100 (TX-100) to the system increased the efficiency of redispersal of the freeze-dried liposomes. The measured redispersion increased to nearly 100% with retention of liposome size and shape.

To test whether membrane surface charge might effect the ease of redispersion of freeze-dried liposomes, the liposomes were prepared with 5 mol % of the anionic lipid dioleoyl phosphatidic acid (DOPA). We observed a small increase in percent redispersion of Sorb/DenPC$_{18,21}$ LUV (from 90 to 100% measured by QELS). However, a decrease of the percent redispersion was observed for Acryl/DenPC$_{16,18}$ LUV. The amount of redispersion of DOPA-containing liposomes was dependent on the pH of the system. When the pH was less than 5 before freeze-drying, the amount of redispersion was only 55%, but when the pH was basic before freeze-drying, the redispersion increased to 75%. The redispersion of Sorb/DenPC$_{18,21}$−DOPA liposomes was 80% at pH less than 5 and nearly 100% under basic conditions. The dependence of the efficiency of redispersion on pH may be due to the amount of ionized DOPA in the liposomes. Certainly at basic pH values all of the DOPA was ionized. On the basis of the known solution pK_a of phosphates, most of the phosphatidic acid should be ionized at a pH of about 5. However, the effective pK_a of the DOPA phosphate group at the membrane surface may be greater than that expected from solution values. When the DOPA was clearly deprotonated, the electrostatic repulsion between the liposomes by the introduction of negative charge on the surface reduced the extent of liposome aggregation during redispersion. A possible reason for the lower amount of redispersion of the Acryl/DenPC$_{16,18}$−DOPA system may be the large difference in the phase transition temperature (T_m) between Acryl/DenPC$_{16,18}$ (27.8 °C) and DOPA (−8 °C). These circumstances will favor phase separation of any un-ionized DOPA from the polymerizable lipid, which suggests these polymerized liposomes may have domains of unpolymerized DOPA that would respond differently to freeze-drying and rehydration than the cross-linked domains of the liposomes.

Addition of 5 mol % of a pegylated lipid, DOPE-PEG$_{2000}$, to the composition of polymerized liposomes resulted in a significant increase in percent and ease of redispersion (Figure 2e and f). Liposomes from either of the polymerizable lipids were 100% redispersed within 5 min of bath sonication. The redispersed liposome suspensions were also more stable than the unpolymerized Acryl/DenPC$_{16,18}$ and Sorb/DenPC$_{18,21}$ liposomes.

Surfactant solubilization is a useful method to evaluate liposome stability in solution. Figure 3 shows the average

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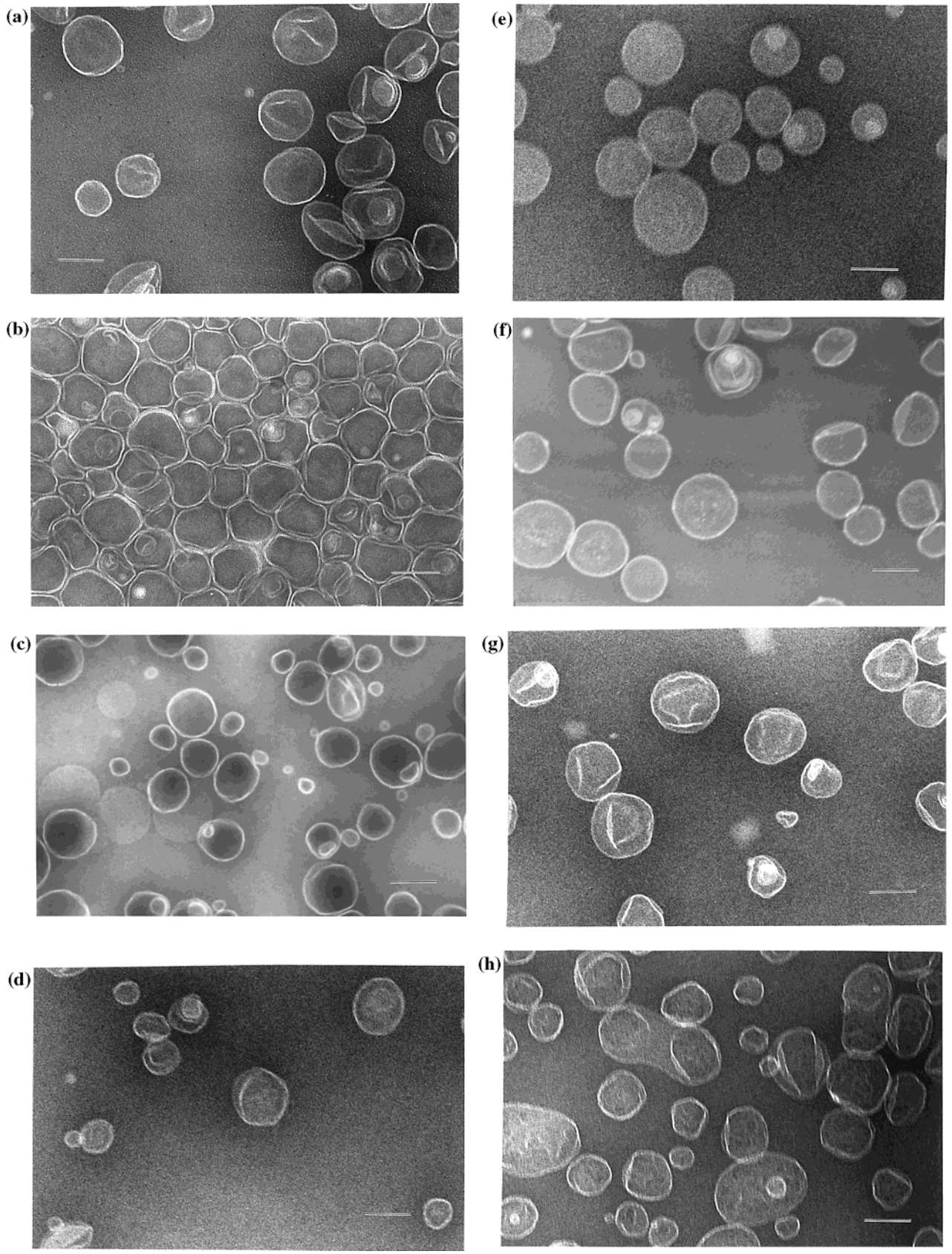

Figure 2. Transmission electron micrographs of liposomes prepared from heterobifunctional lipids (see Experimental Section for details). The scale bar is 100 nm in length. (a) Acryl/DenPC, before polymerization. (b) Acryl/DenPC, after polymerization, followed by addition of 12 equiv of TX-100. (c) Acryl/DenPC, after polymerization, followed by freeze-drying and rehydration. (d) Sorb/DenPC, after polymerization, followed by freeze-drying and rehydration. (e) Sorb/DenPC + 5% PEG, after polymerization. (f) Sorb/DenPC + 5% PEG, after polymerization, followed by freeze-drying and rehydration. (g) Acryl/DenPC, after polymerization, followed by addition of an equal volume of methanol. (h) Sorb/DenPC, after polymerization, followed by addition of an equal volume of methanol.

Table 1. Average of the Mean Diameter (nm) of Liposomes Determined by QELS[a]

				freeze-dried sonication		freeze-dried TX-100	
lipid	before pzn	after pzn	pzn + TX-100	size	recovery	size	recovery
A/DPC	112	108	105	124	86%	122	100%
A/DPC+DOPA	118	102	105	128	55−75%	105	100%
A/DPC + PEG	112	102	103	122	100%	101	100%
S/DPC	122	121	117	117	90%	103	100%
S/DPC + DOPA	123	118	110	118	80−100%	99	100%
S/DPC + PEG	118	112	105	115	100%	113	100%

[a] The standard deviation was ±10 nm. The percent recovery is the percent of the redispersed liposomes that is approximately 115 nm in diameter. A/DPC, Acryl/DenPC$_{16,18}$; S/DPC, Sorb/DenPC$_{18,21}$; PEG, DOPE-PEG$_{2000}$.

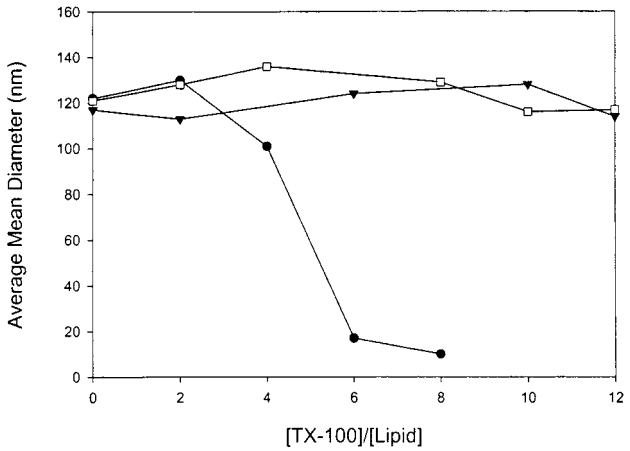

Figure 3. Average of the mean diameter of lyophilized and redispersed polymerized liposomes (▼) of Sorb/DenPC$_{18,21}$ as a function of added equivalents of TX-100 at 25 °C. Both (●) monomeric liposomes and (□) polymerized liposomes of the same lipid before lyophilization and redispersion are shown for comparison.

diameter of Sorb/DenPC$_{18,21}$ LUV as measured by QELS in comparison to the ratio of the surfactant TX-100 to lipid. When 4−6 equiv of TX-100 was added to unpolymerized liposomes composed of Sorb/DenPC$_{18,21}$, a sharp decrease in the average of the mean diameter of the suspended particles was observed. On the other hand, the liposomes cross-linked by a redox initiator were essentially unchanged in size by the addition of up to 12 equiv of TX-100. In the case of lyophilized and redispersed polymerized liposomes, no significant change in the size was observed, indicating that they have comparable resistance to surfactant action as the original polymerized liposomes (Figure 3). Moreover, the addition of surfactant is another effective method to redisperse the freeze-dried liposomes. The results in Table 1 show that after the addition of 12 equiv of TX-100 to freeze-dried liposomes, 100% liposome recovery was obtained for all systems studied.

These results indicate that appropriately cross-linked liposomes can be successfully lyophilized for storage, then rehydrated to recover the liposome. The observed stability of the cross-linked liposomes prompted an initial evaluation of their stability to organic solvents. When cross-linked liposomes in water were diluted with an equal volume of methanol, the liposomes remained in suspension. TEM images showed spherical structures similar to the original liposomes in water (Figure 2g and 2h).

Finally, the electron micrographs reveal that cross-linked liposomes can be quite flexible. Several of the micrographs show collapsed structures, much like a partially deflated volleyball. It is not unusual to find collapsed structures in the images of liposomes that are obtained by negative staining, because the drying of the stained sample exerts an osmotic pressure on the outside that exceeds that on the inside of the liposomes. The micrograph shown in Figure 2b is particularly interesting, because the liposomes are packed together in a manner that permits a cursory examination of the effect of liposome contact on liposome morphology. The image indicates that these cross-linked liposomes conform to the neighboring bilayers. Therefore, these liposomes after cross-linking do not become hard spheres, but rather flexible nanostructures bounded by an elastomeric polymer. A direct examination of the elasticity of these cross-linked liposomes must await future experiments.

Both QELS and TEM data indicate that cross-linked liposomes from either Acryl/DenPC or Sorb/DenPC exhibit remarkable stability and can be freeze-dried and redispersed in water. Although it is well known that cross-linked liposomes are more stable to surfactant than are unpolymerized or linearly polymerized liposomes, it is unprecedented to observe the redispersal and recovery of cross-linked liposomes from the lyophilized poly(lipid) powder. It is fair to ask how the water escapes and later re-enters the polymeric nanoballoons. It must be remembered that the water permeability of conventional lipid membranes is very high, nearly 10 orders of magnitude greater than that of sodium ion.[20,21] Consequently, we could infer from the present results that the formation of polymer chains in the lipid bilayer does not substantially reduce the passive transport of water across the bilayer membrane of the cross-linked liposomes. Another possibility is that a small fraction of the lipids in the original liposome is either not polymerized or not connected to the cross-linked polymeric matrix that makes up the elastomeric shell of the nanoballoon. The percent conversion of monomeric lipid is quite high, 97% or more.[13] If 3% of the lipids in a liposome did not react, then about 2×10^3 lipids in a 100 nm liposome could still be monomeric. These lipids are likely to be trapped in a random manner during the growth of the polymer, but they are unlikely to be lost from the liposome during the freeze-drying and rehydration processes. To the extent that these lipids are lost, the polymeric nanoballoons would incur small defects in the cross-linked elastomeric shell. The importance of these putative defects will depend on the intended use of the polymeric nanoballoons. Preliminary measurements of the permeability of entrapped pyrene tetrasulfonic acid (PTSA) in cross-linked liposomes showed that the dye permeability was reduced by polymerization of the bilayer and that freeze-drying did not substantially increase the loss of the PTSA from the cross-linked liposomes. These initial data suggest that the freeze-drying can be performed in a manner that does not cause

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significant defects in the polymeric shell of the nanoballoons. However, it is important to not overinterpret these observations, because the permeability of the cross-linked liposomes depends on the properties of the polymeric bilayer as well as the size and nature of the solute of interest.

The lyophilization of conventional liposomes returns the lipids to a dry state. Hydration of these lipids normally yields the thermodynamically preferred lamellar phase. The reformation of unilamellar liposomes from the lamellar phases requires the application of energy, via sonication or extrusion. However, spontaneous liposome formation is known to occur upon hydration of dried lipid films, if a sufficient amount of PEG-lipid is included in the lipid mixture.[22] The inclusion of 12 mol % of PEG-lipid having a PEG molecular weight of 2000 was reported to be sufficient for spontaneous liposome formation. Our liposome preparations either used 5 mol % PEG_{2000}-lipid or none. In either case, the thermodynamically stable phase is lamellar.

In recent years, the storage of liposomes by lyophilization has been examined both from a pharmaceutical and biological perspective. If rehydration and recovery of the liposomes after freeze-drying is possible, this increases the usefulness of liposomes as drug carriers, since freeze-drying can increase the shelf life of the liposomes. However, the conventional liposome structure is lost during the freeze-drying process. To protect the liposomes against the stress of drying (lyoprotection), the use of carbohydrates was introduced.[23,24] Sugars, such as trehalose, form an amorphous matrix on the surface of the liposomes during freezing.[25] The sugar coating on the surface of the liposomes imparts a low molecular mobility, which minimizes damage caused by fusion processes or crystal formation after drying. In addition, the carbohydrate suppresses the possible phase transition of the lipid bilayer, which could lead to the leakage of the encapsulated compounds. Van Winden and Crommelin studied the stability of freeze-dried, lyoprotected liposomes.[24] Freeze-drying and rehydration of the liposomes below the lipid phase transition temperature resulted in a small increase of the average liposome size for most lipid compositions. Heating the dried liposomes above their phase transition temperature during rehydration resulted in an increase in the size of the liposomes. Lyoprotection is an important method to preserve the essential liposome structure during lyophilization. Of course, it is fundamentally different from the formation of stable three-dimensional objects by cross-linking the lipid bilayer shell of liposomes. It is this difference that will guide the utilization of polymeric nanoballoons in the future.

In 1990, Tsuchida and co-workers reported that linearly polymerized liposomes could be freeze-dried and then rehydrated.[26] The resulting dispersion was then extruded to re-form unilamellar liposomes from the linear polymeric lipids. If the filter pore size was too small, some of the polymers could not be extruded. The liposomes formed by the extrusion could then be cross-linked in a subsequent second polymerization step. To our knowledge, there are not any reports on the lyophilization and redispersion of cross-linked liposomes.

In this paper, we have emphasized the efficiency of redispersion of lyophilized cross-linked liposomes. The aggregation of liposomes during freeze-drying was found to depend on the liposome surface properties, that is, the electrostatic or steric effects of incorporated molecules. To increase the redispersion efficiency of the freeze-dried liposomes, two surface modifications were introduced, that is, the incorporation of either a negatively charged lipid, for example, DOPA, or a hydrophilic polymer, for example, PEG. In the future, it will be important to determine the fraction of unpolymerized lipids that can be tolerated and still retain cross-linked molecular objects similar to those observed in this study.

Finally, it is important to note that these studies open the way to the exciting possibility of combining the unusual physical characteristics of these new nanoballoons with the broad range of chemical characteristics that have come to be associated with liposomes, that is, resistance to nonspecific adsorption, chemical and biosurface modification, encapsulation of chemical and biological agents, and the reconstitution of functional proteins. Moreover, the design of similar amphiphiles for the formation and cross-linking stabilization of bicontinuous cubic as well inverted hexagonal phases suggests that molecular objects with interpenetrating channels can be prepared as well.

Experimental Section

Materials. The polymerizable lipids, Acryl/DenPC and Sorb/DenPC, were synthesized as described previously.[13] 1,2-Dioleoyl-*sn*-glycero-3-phosphate (sodium salt) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) [MW 2000] (DOPE-PEG_{2000}) were purchased from Avanti Polar Lipids Co. Lipid purity was evaluated by thin-layer chromatography (TLC) with chloroform/methanol/water (65/25/4 by volume) and visualized with an UV lamp. Potassium bromate, L-cysteine hydrochloride hydrate, and Triton X-100 were purchased from Aldrich Chemicals and used as received. The lipids were hydrated in Milli-Q water from Millipore Corp. Compounds and liposomes containing UV-sensitive groups were handled under yellow light.

Liposome Polymerization. Large unilamellar liposomes (LUV) of polymerizable lipid were prepared as follows. Approximately 5 mg of polymerizable lipid from a benzene stock solution (10 mg/mL) was freeze-dried under high vacuum for at least 4 h. The dried lipid was then hydrated with deoxygenated Milli-Q water to a concentration of 1 mM. Samples were vortexed to uniformity and subjected to 10 freeze−thaw−vortex cycles (−77 → 45 °C). The LUV with a diameter of ca. 115 nm were prepared by extrusion 10 times (4 × 0.2 μm + 6 × 0.1 μm) through two stacked Nuclepore polycarbonate filters at 45 °C using a stainless steel extruder from Lipex Biomembranes.

The redox initiator was prepared from potassium bromate (33.4 mg, 0.2 mmol) and L-cysteine hydrochloride hydrate (31.5 mg, 0.2 mmol), which were weighed into a 10 mL volumetric flask and dissolved in water. An aliquot of potassium bromate/L-cysteine (1/1 mol/mol) solution was added to a liposome suspension, giving a [M]/[I] ratio of 1. The sample was sealed in an ampule with a septum, and flushed with argon for 0.5 h. Polymerization was performed at 60 ± 2 °C in a water-circulating bath under a positive argon pressure for 18 h. Polymerization was monitored by UV absorption spectroscopy of aliquots diluted with Milli-Q water to ca. 60 μM. The UV absorption at 260 nm, attributed to the diene group and/or sorbyl group, was analyzed with a UV spectrometer (Varian DMS 200) to determine the polymerization conversion of Acryl/DenPC or Sorb/DenPC.

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Surfactant Dissolution of Liposomes. The LUV were prepared as described above. After polymerization, the LUV were characterized by QELS (BI8000 autocorrelator from Brookhaven Instrument Corp.) for a 2 mL sample with a lipid concentration of 150−300 μM. Aliquots of 50 mM TX-100 solution, each 2 equiv with respect to lipid, were added until the liposomes dissolved or 12 equiv was added. The light-scattering intensities were determined at least three times at 90° angle and room temperature by QELS, at each concentration of TX-100. The average mean diameter of liposomes was calculated by the nonnegatively constrained least squares program.

Redispersion of Lyophilized Liposomes. Only samples with greater than 90% monomer conversion, as determined by UV/vis spectroscopy, were used in these studies. Samples were lyophilized after polymerization. The residual solid was weighed and Milli-Q water added to a concentration of 1 mM. The samples were sonicated for 5−15 min in a bath (Branson 1200, 150 W, from Branson Ultrasonics Corp.) or mixed with 12 equiv of TX-100 and allowed to stand for at least 30 min before the size of the redispersed LUV was measured by QELS.

Transmission Electron Microscopy (TEM). A 30 μL aliquot of ammonium molybdate solution (11% w/w, pH = 7.2) was added to 100 μL of liposome suspension (150 μM). The solution was incubated for 5 h at room temperature. A small piece of carbon-coated mica sheet having one end pointed and the other flat was then immersed in the negative-stained sample. The carbon film was separated from the mica surface and floated on the surface of the sample solution. A freshly washed grid was placed underneath the floating specimen film and then raised with a pair of forceps to collect the sample and carbon film. The excess liquid was carefully removed with a pointed filter paper. The liposome suspensions were sandwiched between the copper grid and the carbon film. The sample was observed with a JEM-100CX II (JEOL) electron microscope operated at 80 kV.

Acknowledgment. The authors thank the National Science Foundation (DMR) for support of this research. We thank David L. Bentley for assistance with electron microscopy and preparation of the micrographs.

JA0123507