Physical Conjugation of (Tri-) Block Copolymers to Liposomes toward the Construction of Sterically Stabilized Vesicle Systems

K. Kostarelos,[†,‡] Th. F. Tadros,*[,†,‡] and P. F. Luckham[‡]

ZENECA Agrochemicals, Jealotts Hill Research Station, Bracknell, Berkshire RG12 6EY, U.K., and Department of Chemical Engineering and Chemical Technology, Imperial College of Science, Technology and Medicine, London SW7 2BY, U.K.

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The physical conjugation of (tri-) block copolymer molecules to phospholipid vesicle bilayers in order to construct sterically stabilized vesicles can be carried out in two different ways: by allowing the copolymer molecules to freely participate in the small unilamellar vesicle (SUV) formation process along with the lipids or by adding the copolymer molecules to pre-formed small unilamellar liposomes. Structurally and morphologically different copolymer coated vesicle systems occur. The effect on the mean vesicle diameter and the vesicle surface characteristics is monitored by dynamic light scattering and laser Doppler electrophoresis techniques for a wide variety of block copolymer molecules of the PEO−PPO−PEO type (PEO is poly(ethylene oxide); PPO poly(propylene oxide)). Systematic investigations as a function of copolymer added concentration and molecular structure were undertaken throughout. The results indicate a dramatic increase in mean vesicle diameter when the polymer molecules are present during vesiculation, while in the case of copolymer addition to already formed liposomes the mean vesicle size follows a classic Langmuirian-type adsorption curve as a function of copolymer concentration. The ζ-potential values obtained decrease in a very similar pattern irrespective of the way of addition for the large PF127 ($PEO_{99}-PPO_{65}-PEO_{99}$) molecule, illustrating the presence of polymer chains at the vesicle surface. For the small, more hydrophobic L61 ($PEO_{10}-PPO_{16}-PEO_{10}$) molecule, the reduced ζ-potential value is maintained only when the copolymer molecules participate in bilayer formation, indicating absence of interaction between the polymer and the lipids when added to preformed liposomes, due to the preferred copolymer tendency to aggregate into micelles separate from the lipid bilayer particles (that eventually leads to phase separation). According to the molecular models proposed to describe the occurring lipid−copolymer interactions, addition of copolymer molecules after liposomes have been formed leads to their adsorption onto the outer liposome surface, its effectiveness being dependent on the influence that the hydrophilic (PEO) and hydrophobic (PPO) blocks exert on the copolymer molecular behaviour. Copolymer−lipid coparticipation toward bilayer formation, at low added polymer concentrations, leads to PPO block protection by arranging along with the lipids as integral parts of the vesicle bilayer, hence anchoring the PEO chains that dangle in the aqueous solution onto the vesicles. Simple geometrical considerations are also included, reinforcing the theoretical feasibility of the described models. The latter type of physically conjugating polymer chains onto vesicle surfaces is proposed as an improved alternative to the weak adsorption of amphiphilic molecules and the cumbersome chemical modification of the lipid polar headgroups to confer steric protection to liposomal surfaces.

Introduction

Liposomes are biodegradable, biocompatible membrane models that could be applied in protecting "encapsulated" active ingredients from any hostile external environment and they can also be used for sustained release of the active. However, the limited stability of liposomes, both in vitro and in vivo, limited their widespread application and realization of their potential advantages. A great deal of research has been carried out to improve the stability of liposomes and this work resulted in novel vesicle systems that have better physical stability both in vitro and in vivo.

Crommlin and van Bommel[1] used a freeze−thaw technique to increase the long-term physical stability of vesicles. Phospholipids with high transition temperature[2] have also been used to provide more "rigid" bilayer vesicles. The "gel-packing" of the lipid chains at low temperatures (10−40 °C) enhances the stiffening of the bilayer. A similar effect can be produced by incorporation of cholesterol, which reduces the mobility of the phospholipid molecules.[3,4] Another method of vesicle stabilization was obtained by polymerization of modified lipids that were incorporated in the bilayer[5] or by polymerization of molecules that were "adsorbed" on the vesicle outer phase (liposome "in a net").[6] Surface modification of the vesicles was also applied to prevent their flocculation, fusion, or binding. The liposome surface was also coated with specific chemical groups (e.g., antibodies, lectins) that can recognize and bind to specific target sites.[7−9]

In principle, two stabilization mechanisms may be considered for phospholipid vesicles. The first mechanism

[†] ZENECA Agrochemicals.

[‡] Imperial College of Science, Technology and Medicine.

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is based on electrostatic stabilization as described by Deryaguin, Landau, Verwey, and Overbeek (DLVO) theory.[10] Indeed such a mechanism has been previously reported by Yoshikoda et al.[11] An alternative mechanism of repulsion can be provided by polymer chains attached to the vesicle surfaces, usually referred to as steric stabilization.[12] This repulsion arises from the unfavorable mixing of the chains (when these are in good solvent conditions) and reduction in configurational entropy on overlap of the chains. This mechanism has been described in detail by Napper,[12] who highlighted its effectiveness in stabilization of colloidal particles such as latices, suspensions and emulsions. Steric stabilization of vesicles was achieved by attaching large hydrophilic groups on the surface either by adsorption of macromolecules (such as glycolipids, lipids or proteins) from aqueous solution[13−15] or by covalent bonding of poly(ethylene glycol) (PEO) chains to the phospholipid headgroup.[16] The vesicles were prepared using these modified lipids ("sheet" liposomes).[16]

In this paper, we propose an alternative procedure for providing steric stabilization to the vesicles, simply by conjugation of a triblock copolymer (an ABA block, with A being poly(ethylene oxide), PEO and B being poly(propylene oxide), PPO) into the vesicles. The physical conjugation of these polymer molecules onto the vesicle surfaces was carried out in two different ways. When the copolymer is allowed to participate in the vesicle formation process, the hydrophobic chain, namely the PPO, becomes an integral part of the bilayer, leaving the hydrophilic chains (the PEO chains) dangling in the aqueous medium. This process provides strong "anchoring" of the polymer molecule onto the vesicle, thus preventing any desorption during particle collision. An alternative way to conjugate the copolymer onto the vesicle surface is by simple physical adsorption, whereby the polymer is added to an aqueous dispersion of preformed vesicles. This method has been previously attempted[17,18] with the objective of increasing the in vivo stability of the vesicles (blood circulation half-lives). Following the above two methods of block copolymer incorporation, two types of vesicle systems could be provided and it was interesting to compare and contrast their structure, morphology and stability. For this purpose, we have used dynamic light scattering and electrophoresis measurements which allowed us to obtain information on the conformation of these block copolymers at the vesicle/solution interface. Simple geometric calculations were used to throw some light on the bilayer structure.

Experimental Section

Materials. All liposome systems were prepared by using a mixture of soybean lecithin lipids (approximately 50% D-α-dimyristroylphosphatidylcholine, DMPC) purchased from Sigma. The aqueous dispersions were prepared using doubly distilled water, which was previously deionized. The ABA type (tri-) block copolymers (with A being PEO and B being PPO) were of the Synperonic PE family supplied by ICI surfactants (Belgium). Some of their molecular structure and characteristics are shown in Table 1. These block copolymer molecules are commonly used for steric stabilization of dispersions. The concentration of the block copolymer was expressed as percent by weight of then total sample weight, unless otherwise stated.

Table 1. Some Molecular Characteristics of the A−B−A Block Copolymers

synperonic	structure PEO−PPO−PEO	tot. PEO mol wt content	tot. PPO mol wt content	tot. mol wt
L35	10−16−10	950	950	1900
L61	2−32−2	209	1881	2090
L64	13−30−13	1160	1740	2900
F38	44−16−44	3840	960	4800
P75	24−36−24	2075	2075	4150
P105	37−56−37	3250	3250	6500
F68	76−29−76	6680	1670	8350
F127	99−65−99	8330	3570	11900
F108	127−48−127	11200	2800	1400

Methods. Preparation of Vesicle Systems. The liposomes were prepared by dispersion of the lipids in the aqueous buffer solution. Alternatively, they were also prepared using the solvent ($CHCl_3$:CH_3OH = 4:1) dispersion−evaporation−rehydration method. Preliminary studies did not show any significant difference between the resulting vesicle systems. The small unilamellar vesicles were prepared by the sonication method[19] using a Kerry ultrasonic bath (50 Hz). Sonication was shown to produce small size vesicles of the order of 40−50 nm diameter. The temperature of the ultrasonic bath was kept at a constant range between 25 and 30 °C. After preparation, the vesicle systems were filtered through 0.2 μm pore size filters (Millipore) to reduce the polydispersity of the samples. The pH of all the dispersions fluctuated between 5 and 6. The standard phospholipid concentration used to produce the vesicles was 2% w/w.

Two methods were used to incorporate the ABA copolymers into the vesicle systems. In the first method, the vesicles were prepared using the hydration and sonication steps in the presence of the copolymer molecules at the desired concentration. In the second method, the liposomes were first formed, and after sonication, they were diluted with copolymer solutions to reach the required final concentrations. These systems were left to stand for 245 h before any measurement was carried out. For ease of presentation, the samples denoted I refer to vesicle systems prepared according to the first method, i.e., where the block copolymer was added initially. The samples denoted A refer to systems prepared using the second method (where the polymer was added after the vesicles had been formed). In all the systems studied, the vesicle dispersion was diluted with the appropriate copolymer solution to give a final dispersion concentration of 0.02%. This was necessary for dynamic light scattering measurements (see below).

Dynamic Light Scattering (PCS Measurements). In a dynamic light scattering experiment, the *z*-average diameter is determined from the diffusion coefficient, D, of the vesicles as they randomly move due to Brownian motion. The diffusion coefficient of the vesicles is calculated from the intensity fluctuation of scattered light, expressed by the rapid decaying correlation function

$$g(\tau) = 1 + \exp(-2Dq^2\tau) \quad (1)$$

where τ is the sample time and q the scattering vector of light. The scattered light intensity is modulated by the Brownian motion of the diffusing vesicles resulting in laser line width broadening. By examination of the spectral breadth of the scattered light, the vesicle size can be calculated.

From the diffusion coefficient D, the mean hydrodynamic radius R_H is calculated using the Stokes−Einstein equation

$$D = \frac{kT}{6\pi\eta R_H} \quad (2)$$

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where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium.

To obtain R_H using PCS, one has to make sure that there is no interparticle interaction. For that reason all samples were diluted 100 times using buffer or polymer solution prior to the PCS measurements. This ensured absence of any multiple scattering due to interparticle interaction.

The PCS instrument was a Malvern 4700 apparatus (Malvern Instruments, U.K.). The scattered intensity fluctuations were recorded at an angle of 90° and at a temperature of 25 °C. At least three series of 10 measurements were performed for each sample. The pinhole of the photomultiplier was normally adjusted to 100 μm, to obtain optimum photon counts. The viscosity value (0.8905) and the refractive index (1.337 at 488 nm) of water were used for all the measurements. The z-average diameter and the polydispersity index of the vesicles were automatically provided by the instrument using cumulants analysis. All dynamic light scattering experiments were carried out three times to ensure consistency of the results. The experimental error of the dynamic light scattering results was between 1 and 2%.

Electrophoresis Measurements. The electrophoretic mobility was measured using laser velocimetry. For that purpose, a Malvern Zetasizer 4 (Malvern Instruments, U.K.) operating with a helium−neon laser beam (5 mW)) was used. The principle of operation is that two laser beams of equal intensity are allowed to cross at a particular point within the cell containing the vesicles. At the intersection of the two beams, which is focused at the stationary layer, interferences of known spacing are formed. The particles moving through the fringes under the influence of electric field scatter light whose intensity fluctuates with a frequency that is related to the mobility of the vesicles. From the electrophoretic mobility, u_E, of the vesicles the zeta potential, ζ, can be calculated using the Huckel equation

$$\zeta = 1.5\frac{\eta u_E}{\epsilon\epsilon_o} \tag{3}$$

where η is the viscosity of the medium, ϵ is the relative permittivity of the medium, and ϵ_o is the permittivity of free space.

Results and Discussion

Dynamic Light Scattering (PCS) Results. Addition of Copolymer after (A) Formation of Vesicles. Figure 1 shows the variation of the z-average diameter (in nm) of 0.02% (w/w) liposome dispersions vs copolymer concentration in percent by weight. The results in Figure 1 are those obtained with block copolymers containing high PEO content relative to PPO. For comparison, the results obtained using block copolymers with a high PPO:PEO ratio are shown in Figure 2.

The results of Figure 1 show an initial increase in hydrodynamic diameter of the vesicles with increase in percent by weight of copolymer, and eventually a plateau is reached above a certain copolymer concentration. With the smallest molecular weight copolymer F38 (M = 4800), the increase in hydrodynamic diameter is relatively small (~1−2 nm) and the plateau is reached at low block copolymer concentration (<0.005%). With the higher molecular weight block copolymers (F127, M = 11 900; F108, M = 14 000), the increase in hydrodynamic diameter is significant (~5−7 nm) and the plateau is reached at high concentration (~0.03%). The intermediate molecular weight copolymer F68 (M = 8350) shows an increase of ~2 nm and the plateau is reached at ~0.01% (w/w) copolymer concentration.

The products for the high PPO:PEO ratio block copolymers (Figure 2), namely L61, L35, L64, P75, and P105, all show a much smaller increase in hydrodynamic diameter (of the order of 1−2 nm) when compared with the products obtained using the high PEO:PPO ratio.

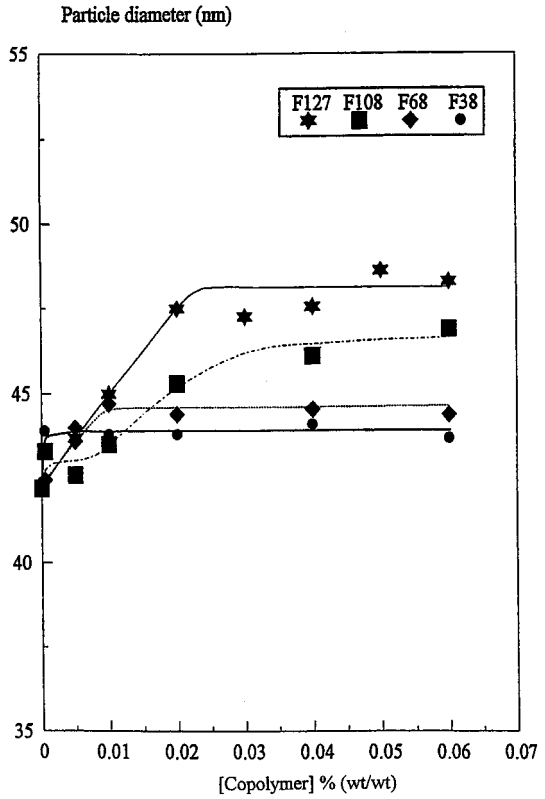

Figure 1. Vesicle mean diameter as a function of added copolymer concentration. Copolymers of high PEO content were added after A vesicle formation. Note that for all the PCS results following, the (%) concentrations refer to the total sample weight, the experimental error is ±1.5−3%, and the bulk lipid concentration is 0.02% (w/w).

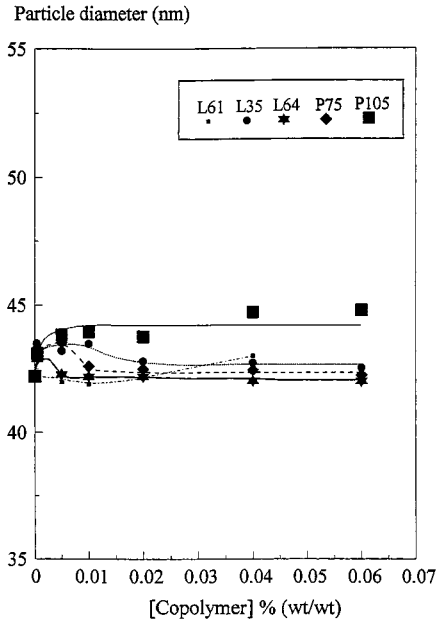

Figure 2. Vesicle mean diameter as a function of added copolymer concentration. Copolymers of low PEO content were added after A vesicle formation.

The above results indicate that the block copolymers are physically adsorbed at the vesicle surface. Evidence for this is obtained from a comparison between the results obtained using the high PEO:PPO ratio and those obtained using the high PPO:PEO ratio. If the assumption is made that the block copolymer adsorbs with the PPO chain in close contact with the surface and the PEO chains dangling

in solution, then one would expect that the block copolymers with the high PEO:PPO ratio will cause a greater increase in the hydrodynamic diameter of the vesicles, when compared with the copolymers containing a high PPO:PEO ratio which have shorter PEO chains. One can obtain a rough estimate of the adsorbed layer thickness δ from the increase in hydrodynamic diameter on addition of the block copolymer. This gives the following values for δ: 3.5, 2.8, 1.3, and 0.8 nm for F127, F108, F68, and F38, respectively. These results are consistent with the increase in PEO chain length in the order F108 > F127 > F68 > F38 (see Table 1). The only discrepancy is that between F108 and F127. The F108 contains 127 PEO units in each of the A chains (in the ABA block copolymer), whereas the F127 contains 99 EO units in each of the A chains. One would expect a larger adsorbed layer thickness for the F108 when compared with F127 (which was not found experimentally). Two suggestions may be made for this discrepancy. First is the accuracy of the results. As discussed in the Experimental Section, the accuracy of the dynamic light scattering results was between 1 and 2% of the vesicle diameter. Taking the upper limit of the error, the accuracy of the diameter is $\pm$0.8 nm, which will account in part for the discrepancy. The second possible reason for this discrepancy may be due to the different conformations that can be produced with the two block copolymers. It is possible that with F108, which contains a larger PEO chain and shorter PPO chain when compared with F127, some coiling of the PEO chains may occur resulting in a smaller adsorbed layer thickness.

The results for the second series of block copolymers (Figure 2) are consistent with the much shorter PEO chains. With L61, that contains only 2 EO units per A chain, there is no increase in the vesicle diameter (within experimental error) and hence if even adsorption occurs, the very short PEO chains will not give an appreciable adsorbed layer thickness. With P105 containing the longest PEO chains (37 units per A chain), the increase in vesicle diameter is $\sim$2 nm, giving an adsorbed layer thickness of $\sim$1 nm, which is comparable to that of F38, which contains 44 EO units per A chain. Clearly such small thicknesses are very difficult to estimate since they are within the experimental error of the measurements. Thus, with copolymers containing short PEO chains, the light scattering results do not provide conclusive evidence of adsorption of these block copolymers.

A comparison may be made between our data and those previously obtained by other investigators. Using polystyrene latex particles, Kayes and Rawlins[20] obtained thicknesses for the high PEO content block copolymer molecules, namely F38, F68, F88, and F108, and these were found to range between 5.4 and 13.4 nm, which are considerably higher than our results using vesicles. Results obtained using a block copolymer with lower PEO chain length, namely L64 and P75, on polystyrene latex were obtained by Illum et al.[21] and these gave a minimum thickness of 2.4 and 3.5 nm respectively, which is also considerably larger than our results using similar block copolymers. Several investigations[22−24] were carried out to study the effect of latex size on the adsorbed layer thickness of these block copolymers. These results showed a decrease in adsorbed layer thickness with increase in particle size, and hence this cannot explain our smaller thicknesses. The only possible reason for these lower values could be due to the hydrophilic nature of the vesicles. Indeed results obtained using hydrophilic silica surfaces[25,26] showed adsorbed layer thicknesses that are almost half those obtained on the hydrophobic polystyrene latex particles. Previous results using vesicles are scarce and most of these are conflicting and hence of limited scope for comparison with our data. For example, Jamsaid et al.[27] obtained much higher adsorbed layer thickness (2−10.2 nm) for a series of high PEO content (> 70% of the molecular weight) block copolymers. Moghimi et al.[28] could not achieve any adsorption of PF127 onto preformed vesicles. The only results that are in agreement with our data are those obtained by Woodle et al.[29] who obtained a layer thickness for PF127 of $\sim$3.5 nm.

The above limited studies on the adsorption of block copolymer onto preformed phospholipid vesicles indicate the complexity of the interaction of the polymer with the hydrophilic surface. The adsorbed copolymer may adopt some "flat" configuration of the PPO chains, leaving the PEO chains "dangling" in solution. This seems to be the case for the block copolymers with high PEO content. For copolymers with short PEO chains (which have limited water solubility), adsorption of the block copolymer occurs at low concentrations, and it is possible that the polymer molecules may aggregate in solution. From the total surface area of the liposome and the concentration at the plateau, one can roughly estimate the amount of polymer adsorbed per unit area. This is illustrated in Appendix I.

Initial (I) Addition of Block Copolymer (Formation of Vesicles in the Presence of the ABA Block Copolymer). In this case, two sets of experiments were carried out, whereby the PEO:PPO ratio was kept constant at 80:20 or 50:50, while the total molecular weight was systematically increased. The results for the first set (F38, F68, F127, and F108) are shown in Figure 3, whereas Figure 4 shows the results for the second set (L35, P75, and P105). Both sets of results show a striking effect, namely an increase in the vesicle diameter with increase in percent by weight of copolymer, reaching a maximum at a given copolymer concentration and this is followed by a sharp decrease reaching diameters compared to those of the bare vesicles or even lower. The results for the first set (F38, F68, F127, and F108) show that the increase in vesicle diameter obtained at the maximum decreases in the order F108 $\sim$ F127 > F68 > F38. This at a first sight seems to suggest that the increase in vesicle diameter is related to the length of the PEO chain. It is difficult, however, to explain the results ion a qualitative manner. First, the increase in vesicle diameter seems to be much larger than expected from the length of the PEO chains. For example, if one considers the F108 and F127 with 127 and 99 EO units respectively, the increase in diameter is of the order of 36 nm, corresponding to a PEO layer thickness of the order of 18 nm. Two explanations may be given for such large apparent diameters. The first is "multilayer" adsorption, although this is not common with polymers. Alternatively, one can consider that by incorporation of the ABA block copolymer in the vesicle

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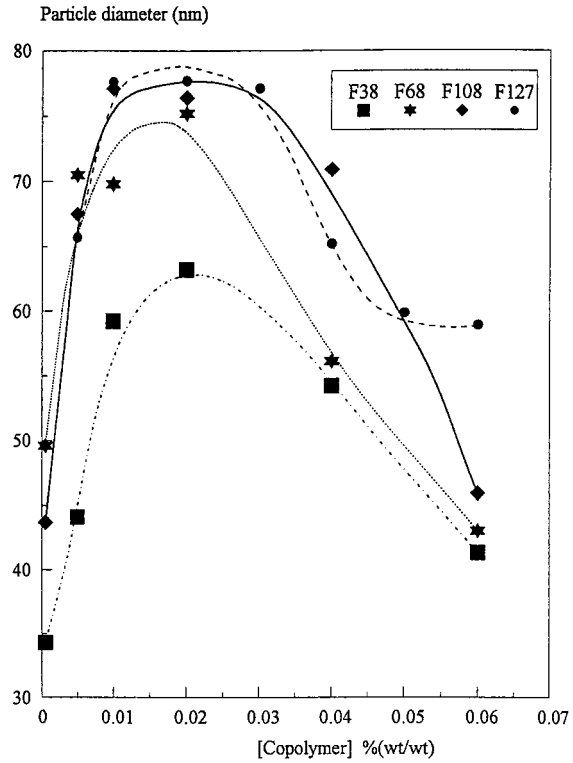

Figure 3. Mean vesicle diameter as a function of copolymer concentration added initially (I) before vesiculation. The copolymer PEO content 80% of total polymer molecular weight, for all molecules shown.

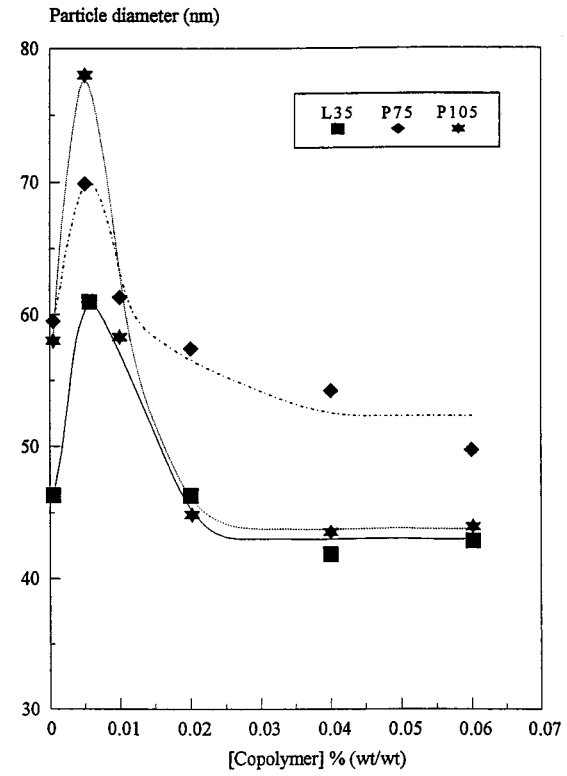

Figure 4. Mean vesicle diameter as a function of copolymer concentration added initially (I) before vesiculation. The copolymer PEO content was kept constant at 50% of the total polymer molecular weight.

structure, larger vesicles are produced as a result of packing constraints. The second problem with the results of Figure 3 is the sharp reduction in vesicle diameter above the maximum. It is highly likely that excess block

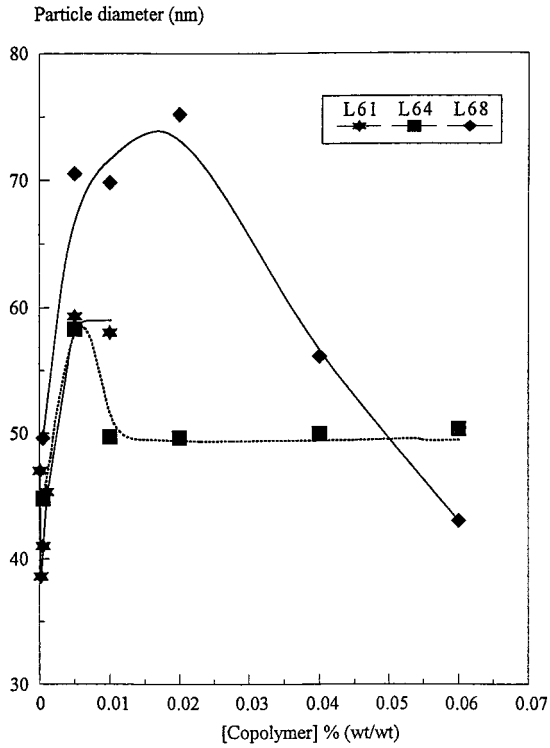

Figure 5. Mean vesicle diameter as a function of copolymer concentration added initially (I) before vesiculation. The copolymer PPO block was kept constant at approximately 30 units.

copolymer remains in the bulk solution, and these "free" polymers have much smaller diameters than the vesicles. Thus the results obtained are the consequence of the PCS measurements which give a *z*-average value for all the units in the dispersion.

The results of Figure 4 (L35, P75, and P105 with 10, 24, and 27 EO units per A chain respectively) are also difficult to explain. The sharp increase in vesicle diameter (>10 nm) at low block copolymer concentration is also inconsistent with the length of the PEO chains. As with the above results, one can consider "multilayer" adsorption or increase in vesicle diameter on incorporation of the ABA block copolymer. Again the reduction in diameter at high polymer concentrations may also be due to the presence of "free" block copolymer molecules.

Whatever the explanation, it seems that the increase in vesicle diameter on addition of the block copolymer is determined by the PEO chain length of the molecule. This is clear if one compares the results of Figure 3 with those of Figure 4; the higher PEO chain length in the first series leads to a much higher increase in vesicle diameter when compared with the results of the second series with lower PEO chain length. Further evidence for this behavior was obtained by using three block copolymers with the same PPO block, but with an increase in the number of EO units (L61, L64 and F68). The results are shown in Figure 5 which shows that F68 with the highest EO units gives the largest increase in vesicle diameter (~25 nm). However, it is difficult to account for the increase in vesicle diameter when using L61, which contains only 2 EO units per A chain, unless the assumption is made that incorporation of the block copolymer during the preparation of the vesicle resulted in an intrinsically larger vesicle. Similar results were obtained using two molecules with shorter PPO (16 PO units) and increasing the number of EO units (L35 and F38) (Figure 6). The maximum increase in vesicle diameter seems to be larger for F38 (from ~35

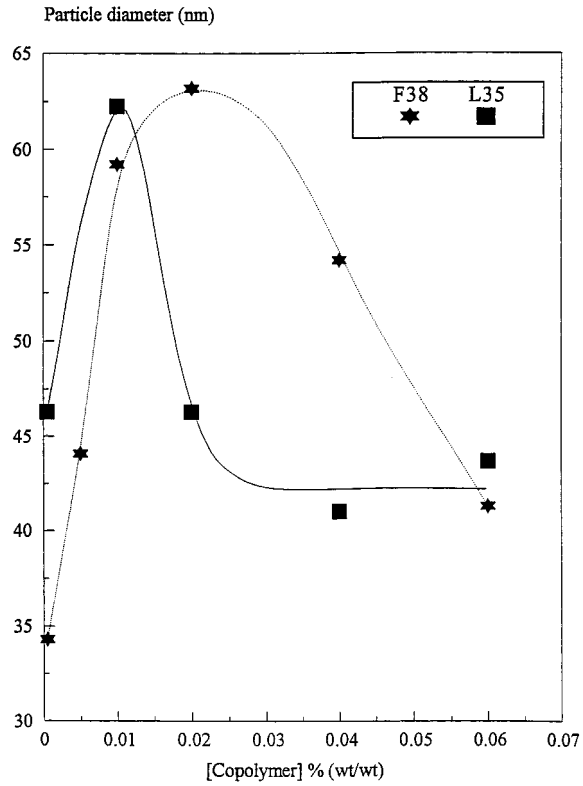

Figure 6. Mean vesicle diameter as a function of copolymer concentration added initially (I) before vesiculation. The copolymer PPO block was kept constant at approximately 16 units.

to ~63 nm) when compared with L35 (increase from ~46 to ~63 nm).

It seems from the above discussion that the increase in vesicle diameter when forming the vesicles in the presence of the ABA block copolymer (initial addition of the copolymer) is determined by other factors than simply the increase in the PEO chain length of the molecule. As discussed above, it is quite possible that the whole geometry of the vesicle may change when the block copolymer is incorporated in the vesicle structure. This can be visualized if one considers a model for the bare vesicle, that containing physically adsorbed block copolymer (A) and that with initial addition of the block copolymer (I). This is illustrated in Figure 7. As can be seen, incorporation of the block copolymer in the bilayer will cause a change in vesicle diameter when compared with the situation with a physically adsorbed polymer.

The incorporation of surfactant molecules, other than the ABA block copolymers studied here, inside the vesicle bilayer has bee reported by several authors. For example, Kronberg et al.[30] attempted to incorporate Tween 80 inside the vesicle bilayer, whereas Virden and Berg incorporated Synperonic NP10 and NP50. Co-sonication of lipids with various biological macromolecules in order to mimic the cell membrane surface, has resulted in bilayer incorporation of glycolipids,[31] protein segments,[32] and ganglocides.[33] Indication of incorporation of simple surfactant molecules such as Triton X-100 and $C_{12}E_8$ has also been reported,[34,35] although these studies referred to incorporation of the

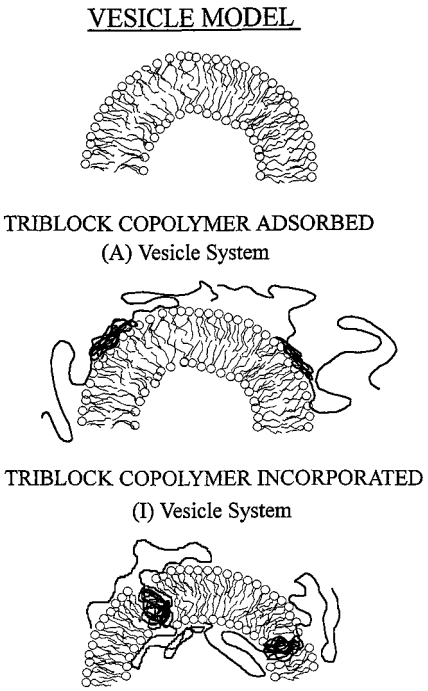

Figure 7. Models of lipid−copolymer vesicle systems.

amphiphiles by penetration of the molecules from aqueous medium rather than during formation of the vesicles.

Electrophoresis Measurements. Figure 8 shows the variation of zeta potential (ζ) with added F127 concentration for the two methods of vesicle preparation, namely A (polymer added after formation of the vesicles) and I (polymer initially added before formation of vesicle). The ζ potential value of the vesicles in the absence of copolymer, namely −51.4 mV, agrees well with the values reported in the literature.[36,37] In both cases, there is a reduction in the ζ potential with increase in block copolymer concentration and eventually a plateau is reached at $c > 0.03\%$ w/w. This reduction in ζ potential with increase in block copolymer concentration is consistent with the presence of the copolymer at the vesicle/solution interface. Adsorption or incorporation of the ABA block copolymer results in a shift in the shear plane outward from the surface and this causes a reduction in ζ potential. This shift is comparable for the two systems investigated, which implies that the conformation of the PEO chains at the vesicle/solution interface is very similar for the two systems as illustrated in Figure 7. The shift in shear plane is proportional to the thickness of the PEO layer which is nearly the same whether the block copolymer is added initially or postadded. Clearly the results of ζ potential measurements do not reflect any change in vesicle size which is different for the two systems. Addition of the block copolymer, after formation of the vesicle results in its physical adsorption and the reduction in ζ potential is the result of the shift in shear plane produced by the PEO

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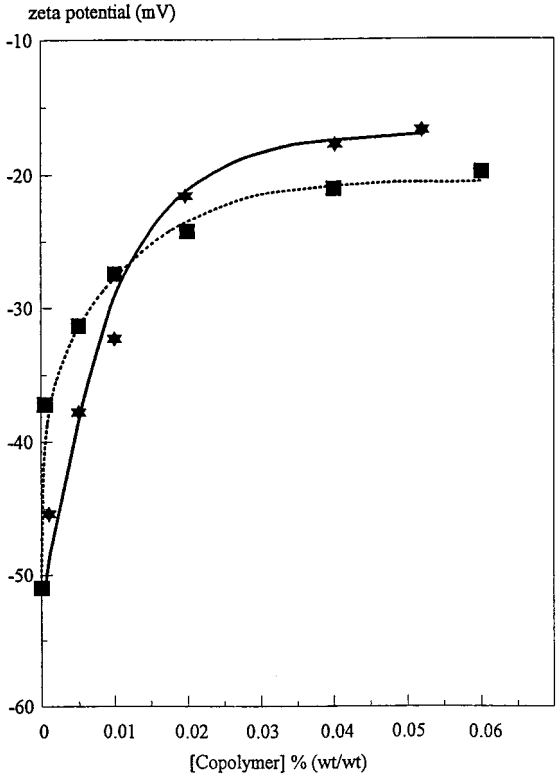

Figure 8. Vesicle *z*-potential (mV) as a function of added PF1 concentration: A vesicle systems (■) and I vesicle systems (★). The high copolymer concentration points for the I vesicles are not included, because vesicle destruction is thought to occur. Bulk lipid concentration: 0.02% (w/w).

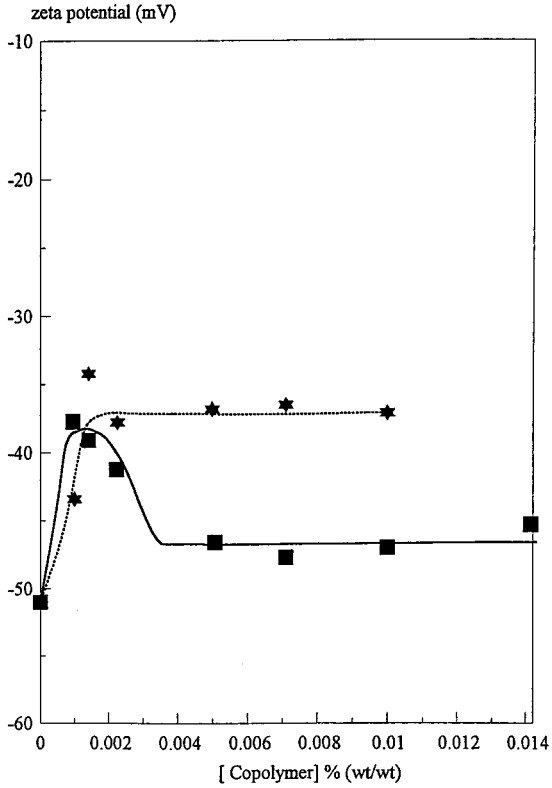

Figure 9. Vesicle *z*-potential (mV) as a function of added L61 concentration: A vesicle systems (■) and I vesicle systems (★).

chains. In contrast, initial addition of the block copolymer before formation of the vesicle results in a change in the vesicle size as a result of the incorporation of the block copolymer in the lipid bilayer. The reduction in ζ potential is also the result of shift in shear plane by the PEO chains which have the same conformation as with the A type vesicles.

The results for a block copolymer with a high PPO:PEO ratio and a much shorter PEO chain (2 EO units per A chain), namely L61, are shown in Figure 9. As expected, the reduction in ζ potential in this case is much smaller (on the order of 10 mV) when compared with the results obtained using F127. In addition, there seems to be a significant difference between the results obtained when the copolymer was added after formation of the vesicles (A) and those obtained when the polymer was added initially (I). However, due to the scatter of the data and the small reduction in ζ potential on addition of the copolymer, it is difficult to quantify the difference between the two systems.

Appendix I. Calculation of Total Liposome Surface Area and Copolymer Adsorbed Amount

The total surface area that will be available to block copolymer molecules, when added to preformed liposomes, was calculated in two different ways.

According to the first method, the bilayer volume per liposome will be

$$\text{volume}_{\text{lip}} = 4/3\pi(a_{\text{outer}}{}^3 - a_{\text{inner}}{}^3) \qquad (1A)$$

and the weight per liposome will accordingly be

$$\text{weight}_{\text{lip}} = {}^4/_3\rho\pi(a_{\text{outer}}{}^3 - a_{\text{inner}}{}^3) \qquad (2A)$$

Table 2

block copolymer molecules	Γ (mg/m^2)
PF38	0.86
PF68	1.72
PF108	3.19
PF127	2.52
L35	1.2
P75	0.79
P105	2.93
L64	0.34

where, ρ is the density of the lipids and $a_{\text{outer/inner}}$ is the vesicle inner and outer radii. From eq 2, the total number of particles in 2 g of lipid material used can be calculated assuming that all lipid molecules aggregate into liposomes. The total liposome surface area can then be calculated multiplying by $4\pi a_{\text{outer}}{}^2$, the area per liposome. Using the experimentally determined values: mean liposome outer radius, aouter = 21.25 nm (PCS); bilayer thickness d_b = 3.6 nm (cryo-TEM), and the lipid density from literature then, total liposome surface area = 275.5 m^2/g (of lipid used).

According to the second method, the outer surface area per liposome is

$$\text{S.A.}_{\text{outer}} = 4\pi a_{\text{outer}}{}^2 = 5674 \text{ nm}^2/\text{liposome}$$

and the inner surface area per liposome is

$$\text{S.A.}_{\text{inner}} = 4\pi(a_{\text{outer}} - d_b)^2 = 3914.7 \text{ nm}^2/\text{liposome}$$

Assuming that the area/lipid molecule is the same for the phospholipids at the inner and outer monolayers of the bilayer, and $A_L = 0.635$ nm^2, the number of phospholipid molecules was calculated at the outer and inner monolayers of the liposome bilayer: $n_{\text{outer}} = 8936.22$ molecules and $n_{\text{inner}} = 6165$ molecules. The number of liposomes in

every gram of lipid material used will therefore be

$$n_{Liposomes} = 0.51 \times 10^{17},$$

The total liposome surface area = 290.15 m^2/g (of lipid used).

Hence, both ways of calculating the total liposome surface area available to the block copolymer molecules for adsorption yield similar values. It can be assumed that full coverage of the liposome surface occurs at copolymer concentrations where the plateau in the mean vesicle diameter was reached (Figure 1) or where the peak vesicle diameter is observed (Figure 2). Using these concentrations, the adsorbed amount of the triblock copolymer molecules added to the preformed liposome system can be calculated (Table 2). Previously reported values of PF127 adsorbed amounts onto flat phosphatidyl choline surfaces are lower (0.9 mg m^{-2}) than the ones obtained above, based on the dynamic light scattering results. Of course some differences with the present calculations were expected due to the high curvature of the liposomes.

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