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THE INTERACTION OF PHOSPHOLIPID MEMBRANES WITH POLY(ETHYLENE GLYCOL)

VESICLE AGGREGATION AND LIPID EXCHANGE

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(1) The water soluble polymer, poly(ethylene glycol), causes aggregation of sonicated vesicles of dimyristoylphosphatidylcholine in a manner consistent with a steric exclusion mechanism. (2) Poly(ethylene glycol) promotes the exchange of lipids between multilamellar vesicles of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine when the lipids are in the liquid-crystalline state. (3) ^{31}P-NMR studies demonstrate that the bilayer configuration of smectic mesophases of dipalmitoylphosphatidylcholine is substantially maintained in the presence of poly(ethylene glycol).

Introduction

Poly(ethylene glycol)s have been used for the fractional precipitation of proteins from solution [1–3] and it has been considered that the mechanism by which this precipitation occurs is, in part, due to excluded volume effects, whereby proteins are sterically excluded from regions of the aqueous solution occupied by PEG [4–6].

We have observed that sonicated dispersions of dimyristoylphosphatidylcholine are aggregated by dilute solutions of PEG and consider that this aggregation occurs by means of a steric exclusion mechanism, analogous to that for proteins. Our findings indicate that for PEGs of number average molecular weight 1500, 4000 and 6000, there is a good correspondence between the concentrations of PEG required to induce the complete aggregation of a vesicle suspension and calculated values of the concentration of PEG at which the total volume of the solution would be excluded to a second macromolecular species.

It would be expected that the structuring of water at a membrane-water interface would result in a decrease in the strength of hydrophobic interaction which stabilize lipid bilayers. It is therefore possible that if PEG were to induce the close apposition of two lipid bilayers, thereby entrapping a layer of highly structured water between adjacent membrane surfaces, then either lipid exchange and/or membrane fusion would be facilitated. PEG, at high aqueous concentrations, promotes cell fusion (Ref. 7 and references therein) and we have investigated whether PEG might also promote the fusion of lipid vesicles. We find that PEG induces the exchange of lipid between mutilamellar phosphatidylcholine vesicles, the kinetics of the exchange being consistent with a vesicle fusion event.

Abbreviations: PEG, poly(ethylene glycol); DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid.

Materials and Methods

Materials. PEG 200, 400, 600, 1500, 4000 and 6000 were supplied by BDH (Poole, Dorset) and were used unpurified.

Dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were obtained from Sigma (London) Chemical Company and were shown to be homogeneous by thin-layer chromatography, lyso and free fatty acid contaminants comprising less than 0.5% of total lipids. Water was doubly distilled and deionized. All other reagents were of analytical grade.

Free water contents. The free water contents of aqueous solutions of PEG were determined by differential scanning calorimetry as previously described [8] and are expressed as g free water per 100 g of solution (%w/w).

Density measurements. The densities of aqueous solutions of PEG 200,...,PEG 6000 at room temperature (18–22°C) were determined by preparing solutions of known w/w composition and weighing the solutions in tared volumetric flasks.

Volume of hydrated poly(ethylene glycol) molecules. The volume occupied by single hydrated molecules of polymer was calculated as described in Refs. 9 and values are listed in Table I.

Vesicle aggregation. Dimyristoylphosphatidylcholine (40 mg) was dispersed in 15 ml of water by extensive vortex mixing at 50°C. The suspension was sonicated using a Branson Model 150 sonifier for 1 h (50% duty cycle, temperature 30°C) until optically translucent and then centrifuged (200000 × *g* for 90 min) at 10°C. The supernatant was removed and used immediately. Samples were removed for quantitative phosophorus analysis [10] and thin-layer chromatography. No degradation products were detectable.

We have shown by dynamic light scattering measurements (results not included), that vesicles prepared by this protocol are of weight average molecular weight $2.54 \cdot 10^6$ (range$(2.26–2.89) \cdot 10^6$) with diameter 25–29 nm.

To 2.5 ml of the sonicated lipid, or dilution thereof, was added with stirring either (a) 0.02 ml aliquots of neat PEG 200, 400 or 600 or (b) 0.02 ml aliquots of PEG 1500, 4000 or 6000, each prepared as a 40%w/w aqueous solutions. The absorbance of the sonicate at 450 nm was monitored after the addition of each aliquot. Polymer was added until no further increment in sample absorbance could be detected. Absorbance plots were normalized by expressing the absorbance obtained after the addition of each aliquot of polymer as a percentage of the maximum absorbance obtainable. All measurements were made at 32°C. Aqueous solutions of PEG exhibit no absorbance at 450 nm.

Virtually complete (> 95%) reversibility of vesicle aggregation could be demonstrated for PEGs 600, 1500, 4000 and 6000 by diluting samples with water following aggregation by PEG. For the other polymers, PEG 200 and 400, reversibility was not complete (< 80%). For PEG 200 we have observed by freeze-fracture (results not included) that larger (> 100 nm diameter) structures are formed when sonicated vesicles are dispersed in this PEG at polymer concentrations required to induce aggregation.

Lipid exchange. The technique used to monitor lipid exchange between lipid vesicles was a modification of that employed by Papahadjopoulos et al. [11]. Multilamellar vesicles composed of either dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine were prepared by dispersing each lipid, at a concentration of 0.34 M in 5 mM EDTA, by heating to 50°C and vortex mixing repeatedly. Equimolar ratios of the two lipids were combined with neat PEG 400 (final polymer concentration 45% w/w) and the mixture incubated at 60°C.

After various periods, a sample was transferred to a specially constructed 100 μl capacity DSC pan, based on the design of Steim [12]. DSC traces were recorded using a Perkin-Elmer DSC-1B calorimeter in the low temperature mode with liquid nitrogen as coolant. Samples were scanned at 4 K/min in triplicate over the temperature range 275 K–330 K–275 K. Lipid purity was checked by thin-layer chromatography both prior to and after scanning. Lyso and free fatty acid contaminants were not detectable in amounts greater than 0.5% of total lipids at any stage of analysis.

Nuclear magnetic resonance. ^{31}P-NMR spectra of dipalmitoylphosphatidylcholine multilamellar vesicles, dispersed in $H_2O/^2H_2O$ (85:15, v/v), both in the absence and presence of PEG 400, were recorded using a Bruker WH-90 Fourier

Transform NMR spectrometer operating at 36.4 MHz for ^{31}P. All spectra were obtained in the presence of high power (18 W) broadband proton decoupling and at temperatures above the gel-liquid crystal transition of dipalmitoylphosphatidylcholine in the presence of PEG [13]. Accumulated free induction decays were collected for up to 20000 transients, employing a 0.17 s interpulse time and a 45° radio frequency pulse.

Results

Densities and free water contents of poly(ethylene glycol) solutions

The quantity of unbound water in solutions of PEGs that was detectable by differential scanning calorimetry, decreased linearly with increasing concentrations of the polymer (Table I). The lowest concentration of polymer at which all the water in solution was bound, and hence not detectable by DSC, ranged from 59.5% w/w for PEG 200 to 48.1% w/w for PEG 6000. If it is assumed that each of the terminal hydroxyl moieties of the polymers binds one molecule of water, then the number of water molecules bound by each of the ether-linked oxygen atoms in the polymers is as shown in Table I and ranges from 1.8 molecules for PEG 200 to 2.7 molecules for PEG 6000.

It was noted that the polymers of higher molecular weight bound more water than those of lower molecular weight; PEGs 1500–6000 apparently binding more than two molecules of water per monomer unit. Such additional water-binding probably does not occur because PEGs of higher molecular weight actually bind more water per monomer unit, but because of the bulk properties of the polymer in that for PEGs of molecular weight 1500 and above, folding and random coiling in aqueous solution provides sites for additional water entrapment in excess of that already bound on a 2:1 molar ratio at each ether oxygen.

Vesicle aggregation

The effect of PEG 6000 on the absorbance of sonicated preparations of dimyristoylphosphatidylcholine at six concentrations of lipid is shown in Fig, 1. For each lipid concentration the absorbance increased in a sigmoidal manner until a maximum value was obtained at about 6% w/w PEG. The aggregation of the vesicles was dependent upon the lipid concentration, in disagreement with the results of Boni et al. [14], the higher the lipid concentration, the lower the concentration of

TABLE I

SOME PROPERTIES OF AQUEOUS SOLUTIONS OF POLY(ETHYLENE GLYCOL)S

Densities are expressed as functions of the concentration of polymers (X % w/w) which were derived from the linear regression lines based on eight density values determined for solutions of 5–40% w/w (correlation coefficient >0.99). Free water contents are expressed as functions of the concentration of polymers (X % w/w) which were derived from the linear regression lines based on at least nine data points (correlation coefficient >0.98). Value as given as the mean ± S.D. ($n = 8$) of the volumes calculated for eight solutions of 5–40%w/w).

PEG	Density (g/cm^3)	Free water content (% w/w)	Lowest polymer concn. at which all water is bound (% w/w)	Molecules of water bound per monomer unit	Volume of hydrated molecule (nm^3)
200	$0.998+0.00148\ X$	$100-1.68\ X$	59.5	1.8	0.511 ± 0.002
400	$0.998+0.00175\ X$	$100-1.72\ X$	58.1	1.8	1.030 ± 0.007
600	$0.999+0.00163\ X$	$100-1.78\ X$	56.2	2.1	1.600 ± 0.012
1500	$0.998+0.00175\ X$	$100-1.89\ X$	52.9	2.2	4.308 ± 0.013
4000	$0.996+0.00185\ X$	$100-2.00\ X$	50.0	2.5	12.150 ± 0.037
6000	$0.996+0.00186\ X$	$100-2.08\ X$	48.1	2.7	19.052 ± 0.022

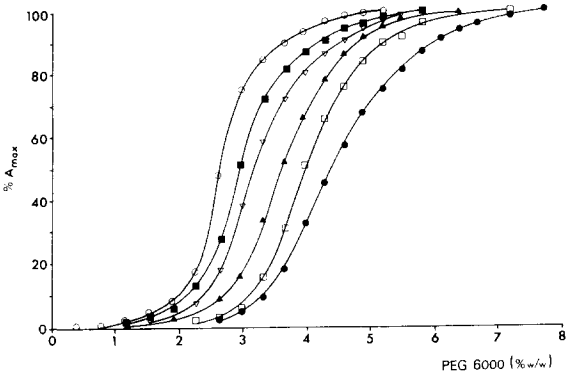

Fig. 1. The aggregation of dimyristoylphosphatidylcholine vesicles by PEG 6000. Values for the normalized absorbance (% A_{max}) of sonicated dispersions of dimyristoylphosphatidylcholine in the presence of PEG 6000 are shown for six concentrations of lipid: 4.75 mg/ml (○), 1.58 mg/ml (■), 0.810 mg/ml (▽), 0.372 mg/ml (▲), 0.352 mg/ml (□) and 0.176 mg/ml (●). Each point is the mean ± S.D. of at least four determinations; the values of S.D. are within the spread of the symbols.

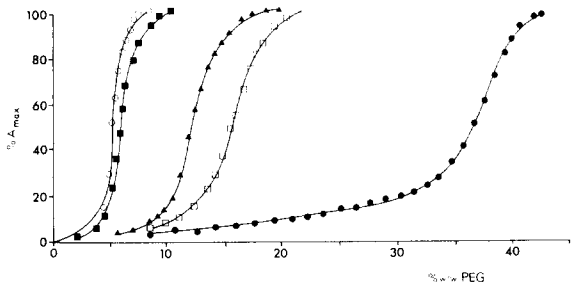

Fig. 3. The aggregation of dimyristoylphosphatidylcholine vesicles by PEGs 200–4000. Values for the normalized absorbance (% A_{max}) of sonicated dispersions of dimyristoylphosphatidylcholine in the presence of PEG are shown for PEG 200 (●), PEG 400 (□), PEG 600 (▲), PEG 1500 (■), PEG 4000 (○), the lipid concentrations varied between preparations and for PEG 200 through to PEG 4000 were 3.07, 1.94, 2.80, 1.81 and 1.54 mg/ml, respectively. Each point is the mean ± S.D. of at least four determinations; the values of S.D. are within the spread of the symbols.

PEG required to induce a half-maximal change in absorbance. This feature is further illustrated in Fig. 2 which shows the concentration of PEG 6000 required to produce an increase in absorbance to 50% of the maximum absorbance attainable for each of 17 concentrations of lipid.

Similar aggregation effects were produced by polymers of different molecular weights. In Fig. 3 the changes in absorbance of sonicated preparations of dimyristoylphosphatidylcholine by PEG 200, 400, 600, 1500 and 4000 are shown. A strict comparison between these results cannot be made since varying concentrations of lipid were employed. However, it is clear that the relative effectiveness of poly(ethylene glycol)s to induce the aggregation of dimyristoylphosphatidylcholine vesicles, increases with the molecular weight of the polymers.

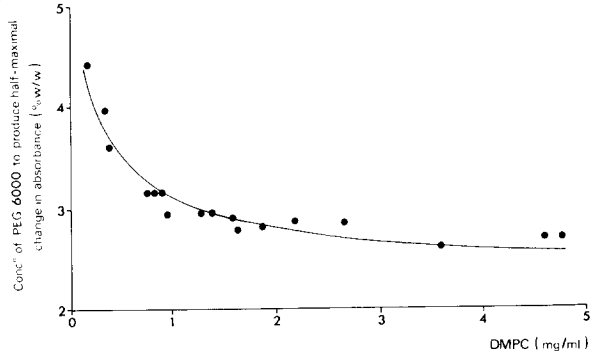

Fig. 2. The effect of lipid concentration on the aggregation of dimyristoylphosphatidylcholine vesicles by PEG 6000. The concentration of PEG 6000 (% w/w) required to produce a half-maximal change in the normalized absorbance is plotted against the concentration of dimyristoylphosphatidylcholine (DMPC). Each point is the mean ± S.D. of at least four determinations; the values of S.D. are within the spread of the symbols.

Lipid exchange

Fig. 4 shows the DSC traces from the combined dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine multilamellar vesicles in 5 mM EDTA, in the absence and presence of 45% w/w PEG 400. The gel-liquid crystal transition temperatures of both lipids were elevated by approx. 4°C in the presence of PEG, consistent with previous findings [13]. Incubation of the vesicles together in the absence of PEG at either 40°C or 60°C for 1 h, or repeated scanning over the temperature range 275 K–330 K–275 K, did not result in any detectable mixing between the vesicle populations.

From Fig. 4 it is evident that upon incubation of the mixed vesicle populations in PEG 400 at 60°C for periods longer than 7 min, a transition endotherm corresponding to the equimolar ratio of the two lipids was detected. The area of this central endotherm, relative to the total transition area,

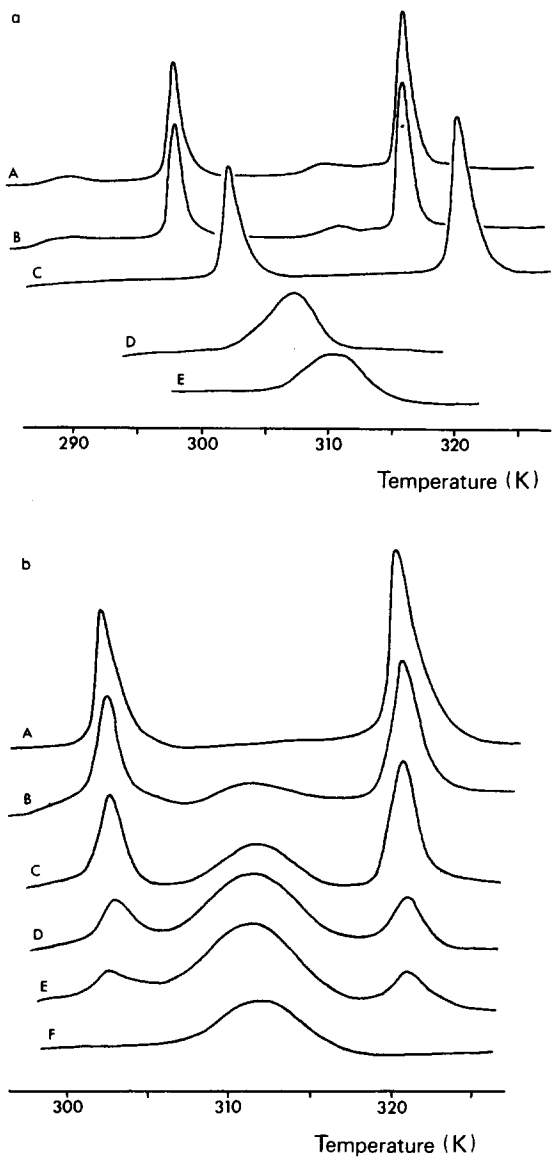

Fig. 4. Poly(ethylene glycol)-induced lipid exchange. (a) Control experiments. A, Differential scanning calorimetric heating scans of equimolar mixtures of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine multilamellar vesicles in 5 mM EDTA; B, as in A, incubated at 40°C or 60°C for 1 h or repeatedly scanned over the temperature ranges 275 K–330 K or 250 K–230 K; C, as in A, but mixed with PEG 400 (45% w/w) at room temperature; D, vesicles formed from equimolar ratio of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine in 5 mM EDTA; E, as in D, but mixed with PEG 400 (45% w/w) at room temperature. (b) Equimolar mixtures of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine multilamellar vesicles in 5 mM EDTA, were mixed at room temperature (A) and incubated at 60°C for B, 7 min; C, 10 min; D, 16 min; E, 20 min; F, 25 min.

increased with incubation time until after approx. 25 min, only the central endotherm was detectable. The transition temperatures of the two parent vesicle populations was not significantly altered upon prolonged incubation in PEG, although there was some degree of broadening of the transition endotherms. Such broadening may simply be a result of the presence of PEG, which has been previously shown to decrease the cooperativity of the gel-liquid crystal phase transition of dipalmitoylphosphatidylcholine [13].

Fig. 5 shows the ^{31}P-NMR spectra of dipalmitoylphosphatidylcholine multilamellar vesicles in the absence and presence of PEG 400. In the absence of polymer, the vesicles exhibited a spectral lineshape characteristic of lipids in an extended lamellar configuration [15]. The isotopic component superimposed on certain of the spectra probably reflects the presence of small vesicles

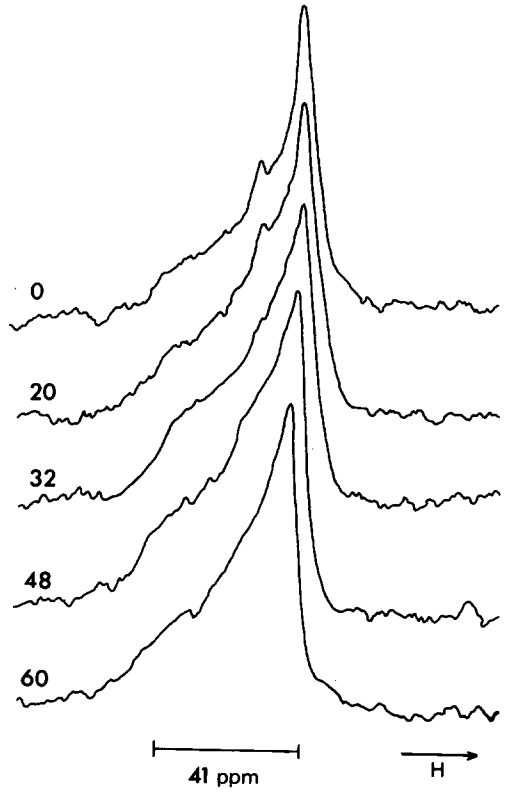

Fig. 5. ^{31}P-NMR spectra of multilamellar vesicles of dipalmitoylphosphatidylcholine in the presence of PEG 400. The final concentration of polymer (% w/w) is shown adjacent to each spectrum.

within the dispersion. In the presence of increasing concentrations of PEG 400 there was a slight broadening of the lineshape, which probably reflects a PEG-induced reduction in the freedom of motion of the headgroups, possibly as a consequence of the increased viscosity of the medium and/or due to the structuring effect of PEG on water. There was no significant change in the value of the effective chemical shift anisotropy, indicating that PEG did not induce any gross changes in the conformation of the phosphatidyl choline headgroup, as was suggested might be the case on the basis of monolayer studies [16]. In disagreement with the findings of Boni et al. [17], there was no evidence of a PEG-induced bilayer to non-bilayer phase transformation.

Discussion

Vesicle aggregation

By using values for the PEG molecular volumes (Table I), it is possible to calculate the maximum excluded volumes of the PEG molecules and hence the minimum concentration of PEG at which the entire volume of the PEG solution would be excluded to a second macromolecular species, i.e. the concentration of PEG at which vesicle aggregation would be complete. Calculation of the excluded volumes for PEG may assume, as two extremes, that the molecules approximate to either a spherical or cyclindrical shape in aqueous solution.

The concentration PEG at which the total volume of the solution would be excluded to a second macromolecular species is given by the equation:

$$\%\text{w/v PEG} = (10^{23} - E_L) \cdot M/(E_P \cdot N_{Av}) \qquad (1)$$

where M is the molecular weight of the PEG, E_P is the excluded volume of one PEG molecule (nm^3) and N_{Av} is Avogadro's number. E_L is the excluded volume of a dimyristoylphosphatidylcholine sonicate of concentration I mg · ml^{-1} and is given by the equation:

$$E_L(nm^3) = 1.8825 \cdot 10^{19} \cdot I$$

E_L is calculated assuming (1) a homogeneous vesicle population with respect to size, (2) vesicles of diameter 25 nm and (3) a complement of 3000 lipid diameter per vesicle (see Materials and Methods). Derivations of these relationships are given in Ref. 9.

Values for the concentration of PEG (corrected to %w/w) for total exclusion for PEGs 200–6000 are listed in Table II.

The results presented in Fig. 1 showed that the aggregation of dimyristoylphosphatidylcholine vesicles by PEG 6000 was complete in all instances, except for the lowest concentration of lipid, by approx. 6% w/w PEG. This agrees reasonably well, considering the simplifications and assumptions made, with the lower calculated value of 5.8%w/w given for PEG 6000 in the presence of lipid (Table II). This suggests that PEG 6000 exists in aqueous solution as a random coil approximating to a globular configuration. For PEG 4000 (Fig. 2), aggregation was complete by about 8%w/w PEG, a value closer to that calculated on the basis of a spherical structure (6.6% w/w) than that or a linear chain (10.7% w/w). For PEG 1500 (Fig. 2), aggregation was complete by approx. 10% w/w PEG, a value mid-way between calculated values for either a linear chain or a spherical model, consistent with studies which suggest that a molecular weight of 1000 represents the transition region for random coil formation of poly(ethylene glycol)s in aqueous solution [18].

TABLE II

CALCULATED VALUES FOR THE CONCENTRATION OF POLY(ETHYLENE GLYCOL)S THAT TOTALLY EXCLUDE A SECOND MACROMOLECULAR SPECIES

For PEG 200–4000, lipid concentrations are the same as given in Fig. 3. For PEG 6000, the lipid concentration was 4.75 mg/ml. Upper and lower concentration limits are given due to the heterogeneous molecular weight distributions within commercially available PEGs [20].

PEG	Poly(ethylene glycol) (% w/w)	
	Sphere model	Cylinder model
200	7.2–8.0	14.2–15.7
400	7.3–8.1	14.4–15.9
600	6.9–7.6	13.7–15.1
1500	6.6–7.2	13.0–14.3
4000	5.4–6.6	10.7–13.0
6000	5.8–7.3	11.7–14.6

For PEG 200, 400 and 600, the correspondence between experimentally determined and calculated values for the concentration of PEG at which aggregation is complete, becomes progressively worse the lower the molecular weight of the PEG. The reason for this disparity is unknown, although there are possible explanations. Firstly, it is possible that PEGs 200, 400 and 600 undergo self-association, however this would be inconsistent with their known physical properties [19]. Secondly, low molecular weight PEGs may be able to permeate into dimyristoylphosphatidylcholine vesicles. Thirdly, calculation of the excluded volumes for PEG 200, 400 and 600 as linear chains assumed parallel orientations which is undoubtedly an over-simplification. Non-parallel orientations of adjacent PEG chains would serve to decrease the excluded volume and hence increase the concentration of PEG for completion of vesicle aggregation.

Lipid exchange

The results shown in Fig. 4a demonstrated that multilamellar vesicle preparations of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were stable (with respect to lipid exchange), in the absence of PEG. Lipid exchange between the two vesicle populations was greatly enhanced by PEG when both lipids were in the liquid-crystal state. Such a lipid exchange process may occur as a result of aqueous diffusional exchange, contact-mediate exchange, vesicle fusion or breakage and resealment of the vesicles.

Because PEG induces the aggregation of phosphatidylcholine vesicles it is improbable that aqueous diffusion was the predominent mechanism by which lipid exchange occurred.

If invoking either contact-mediated exchange or vesicle fusion to explain the observed results it is necessary to postulate that PEG, in addition to inducing inter-vesicular aggregation, must also induce intra-vesicular bilayer-bilayer contacts (or close apposition). If PEGs do not permeate into multilamellar vesicle they will induce an osmotic gradient across the outermost bilayer of multilamellar vesicle, thereby causing shrinkage of the vesicles and a reduction in the interlamellar spacing. This has been demonstrated by Boni et al. [17].

Without further evidence it is not possible to differentiate between the mechanisms of vesicle fusion, contact-mediated exchange or breakage and resealment. The freeze-fracture studies of Boni et al. [14] and the DSC results presented here are both consistent with a vesicle fusion process, however neither study provides unequivocal proof that that is the case.

Irrespective of the precise mechanism by which PEG acts, it is clear that PEG can promote the exchange of lipids between multilamellar phosphatidylcholine vesicles under conditions where, in the absence of polymer, no mixing occurs. For exchange to occur, the lipids must be in the liquid-crystal state, consistent with previous findings [11].

There was no evidence (Fig. 5) to suggest that PEG could induce bilayer to non-bilayer phase transformation in multilamellar dipalmitoylphosphatidylcholine vesicles. We have also examined by ^{31}P-NMR the effect of PEG on mixed lipid systems composed of soya phosphatidylethanolamine and soya phosphatidylserine and have found that PEG does not destabilize bilayer structure for these mixtures (unpublished observations). Cullis and Hope have similarly found that the bilayer structure for model systems comprised of erythrocyte plasma membrane lipids, is maintained in the presence of high ($>50\%$) aqueous concentrations of PEG (Cullis, P.R., personal communication). These observations contradict those of Boni et al. [17] who reported the occurrence of isotropic resonances for various lipids in the presence of PEG. We have no explanation for this disparity, but would offer the following comments upon their studies.

It has been pointed out [15] that a variety of lipid structures can give rise to isotropic motional averaging on the NMR time scale inducing small bilayer vesicles, micelles, inverted micelles, 'honeycomb' structures as well as other lipid phases such as cubic or rhombic. We suggest that the isotropic resonances observed by Boni et al. [17] represent the occurrence of small bilayer structures either entrapped within or between larger aggregated multilamellar structures. Boni et al. [17] suggest that the absence of broadening or shifting of the isotropic resonance upon addition of shift reagent argues against the occurrence of small

vesicles. Since shift reagent was added to pre-aggregated multilamellar vesicles [17], it is conceivable that their inability to affect the isotropic resonance may reflect reduced permeability of the lipid membranes to shift reagent in the presence of PEG.

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