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THE USE OF FLUORESCENCE ENERGY TRANSFER TO DISTINGUISH BETWEEN POLY(ETHYLENE GLYCOL)-INDUCED AGGREGATION AND FUSION OF PHOSPHOLIPID VESICLES

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Two fluorescence energy transfer assays for phospholipid vesicle-vesicle fusion have been developed, one of which is also sensitive to vesicle aggregation. Using a combination of these assays it was possible to distinguish between vesicle aggregation and fusion as induced by poly(ethylene glycol) PEG 8000. The chromophores used were 1-(4′-carboxyethyl)-6-diphenyl-*trans*-1,3,5-hexatriene as fluorescent 'donor' and 1-(4′-carboxyethyl)-6-(4″-nitro)diphenyl-*trans*-1,3,5-hexatriene as 'acceptor'. These acids were appropriately esterified giving fluorescent phospholipid and triacylglycerol analogues. At 20°C poly(ethylene glycol) 8000 (PEG 8000) caused aggregation of L-α-dipalmitoylphosphatidylcholine (DPPC) vesicles without extensive fusion up to a concentration of about 35% (w/w). Fusion occurred above this poly(ethylene glycol) concentration. The triacylglycerol probes showed different behaviour from the phospholipids: while not exchangeable through solution in the absence of fusogen, they appeared to redistribute between bilayers under aggregating conditions. DPPC vesicles aggregated with < 35% poly(ethylene glycol) could not be disaggregated by dilution, as monitored by the phospholipid probes. However, DPPC vesicles containing approx. 5% phosphatidylserine which had been aggregated by poly(ethylene glycol) could be disaggregated by either dilution or sonication. Phospholipid vesicles aggregated by low concentrations of poly(ethylene glycol) appear to fuse to multilamellar structures on heating above the lipid phase transition temperature.

Introduction

Membrane fusion is a complex phenomenon of both fundamental and practical importance [1], yet convenient methods for its study are not available. An ability to differentiate between aggregation and fusion of vesicles or cells is critical for many applications. Fluorescence energy transfer offers several advantages for this purpose [2,3].

In this study of phospholipid aggregation and fusion, donor and acceptor chromophores are incorporated into the systems to be fused. Two methods are used; the first involves donor and acceptor labelled vesicles mixed separately and then fused. The second method involves donor and acceptor chromophores being premixed in the same sets of vesicles and fusion is then induced in the presence of an excess of unlabelled vesicles. The first method is sensitive both to aggregation and to fusion, whilst the second method is sensitive only to fusion. These procedures thus provide a tool with which vesicle aggregation and fusion can be differentiated clearly. Whilst studies similar to the above have been reported [4–7] there have been no

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reports on the use of both the separately mixed and premixed systems in a single study. Poly(ethylene glycol)-induced vesicle and cell aggregation and fusion have been widely studied recently [8–12]. In this study the aggregation and fusion of unilamellar phospholipid vesicles as induced by PEG 8000 is investigated.

Materials and Methods

1-(4′-(2-Carboxyethyl))-6-DPH was synthesised as described [13]. 1-(4′-(2-Carboxyethyl))-6-(4″-nitro)-DPH was prepared in an exactly similar fashion, but using 4′-nitrophenylpentadienal in the Wittig condensation. A 65% yield of 1-(4′-(2-carboxyethyl))-6-(4″-nitro)-DPH methyl ester was obtained, m.p. 188–190°C (uncorr.), recrystallised from dimethylformamide/methanol; λ_{max} (chloroform) 410 ($\epsilon = 34.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$); M^+ 363. Alkaline hydrolysis gave the acid, m.p. 228°C (uncorr.) recrystallised from acetone.

Acylation of lysophosphatidylcholine and dipalmitin by 1-(4′-(2-carboxyethyl))-DPH and 1-(4′-(2-carboxyethyl))-6-(4″-nitro)-DPH was performed as described [13] using the mixed anhydrides generated with pivaloyl chloride, and using dimethylaminopyridine as acylation catalyst. Both phospholipid derivatives were isolated in 5–10% yield by column chromatography over silica gel (Merck, Kieselgel 60) using a chloroform/methanol/acetic acid/water (75 : 28 : 7.5 : 2.5, v/v) mixture as eluent. The dipalmitin derivatives were isolated in 50–60% yield by column chromatography over silica gel using chloroform as eluent, followed by crystallisation from ethanol. The 1-(4′-(2-carboxyethyl))-DPH derivative of dipalmitin had m.p. 90–92°C (uncorr.), the corresponding 4″-nitro analogue had m.p. 81°C (uncorr.). All four derivatives proved homogeneous on TLC examination in a range of solvents.

Poly(ethylene glycol), number average molecular weight 8000 (previously sold as PEG 6000) was obtained from Sigma. It was either used without purification, or was recrystallised five times from absolute ethanol. Alternatively, the poly(ethylene glycol) was purified as described [14]. Poly(ethylene glycol) (10 g) was dissolved in chloroform (80 ml) and precipitated by the addition of diethyl ether (2000 ml). The final step used in our purification was ultrafiltration using an Amicon UM membrane (mol. wt. cut off of 5000). The concentrated sample was repeatedly diluted with distilled water and ultrafiltered in order to remove low molecular weight contaminants, and finally evaporated to dryness under vacuum. The above attempts at purification resulted in a significant decrease in the ultraviolet absorption at 290 nm of the commercial grade poly(ethylene glycol).

L-α-DPPC was obtained from Sigma and used without further purification. All samples were stored as solutions in absolute ethanol at −4°C.

Vesicle dispersions were prepared in fresh unbuffered KCl (pH 6, 50 mM) containing 0.02% sodium azide as preservative. Typically the lipid and probe (or mixture of probes) were mixed in absolute ethanol (with approx. 5% THF to aid solubility in the case of the triacylglycerol probes) and injected into rapidly vortexing KCl at 55°C [15]. Vesicle dispersions typically contained a total of 0.08 mg/ml phospholipid and approx. 1% ethanol. The samples were incubated for at least 30 min at 42°C after injection and used within 6 h.

Fluorescence spectra were obtained using a Schoeffel RRS 1000 spectrofluorimeter interfaced to a microcomputer as previously described [13]. Any background Raman and fluorescence contributions from the poly(ethylene glycol) were subtracted from all fluorescence spectra.

The efficiency of energy transfer for both sets of probes was calculated according to the relationship [16]: $E = 1 - (F/F_0)$ where F_0 is the intensity of the donor in the absence of acceptor and F is the intensity of donor in the presence of the respective acceptor.

Aggregation and fusion by poly(ethylene glycol) were initiated by addition of vesicle dispersions to the poly(ethylene glycol) (solid) followed by rapid stirring of the solution at room temperature.

The electron microscopy study was performed using a Corinth 500 transmission electron microscope. The samples were negatively stained with uranyl acetate (1%) on Formvar-carbon coated grids and examined at appropriate magnifications.

Results

Uncorrected fluorescence excitation and emission spectra for the phospholipid probes in DPPC vesicles are shown in Fig. 1. The spectra of the triacylglycerols proved very similar. The emission-excitation overlap between the DPH and nitro-DPH probes suggested that they could form efficient donor-acceptor pairs for Förster resonance energy transfer. That energy transfer can indeed occur is shown in Fig. 2, where the efficiency of quenching of donor fluorescence (energy transfer efficiency) is plotted as a function of the surface density of acceptor for both the phospholipid and triacylglycerol donor-acceptor pairs. The measurements were made in DPPC vesicles at constant

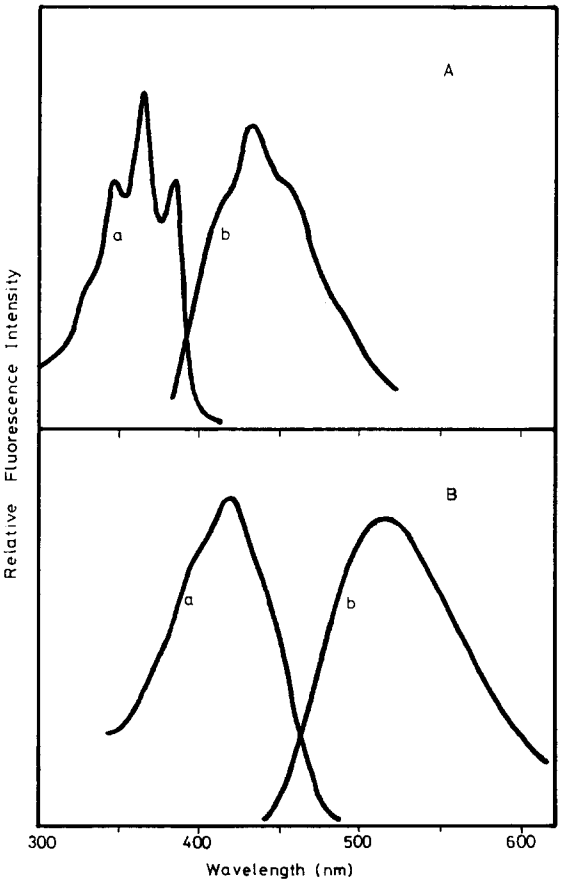

Fig. 1. Excitation (a) and emission (b) spectra of the phospholipid donor (Fig. 1A) and acceptor (Fig. 1B) in DPPC injection vesicles containing 0.5 mol% fluorescent phospholipids.

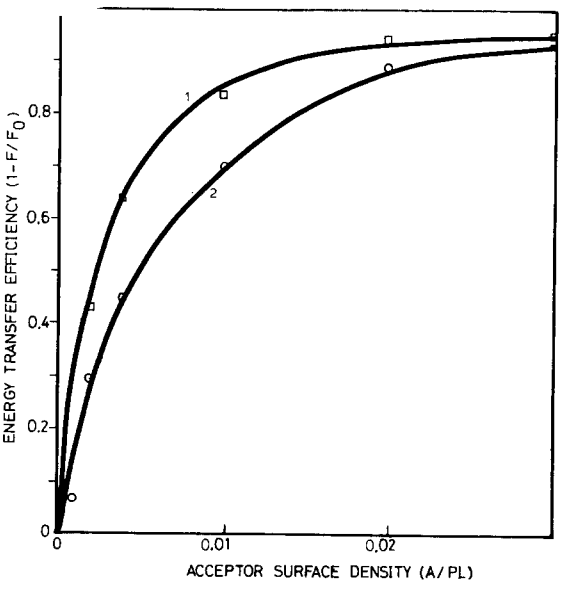

Fig. 2. Efficiency of energy transfer as a function of the surface density of energy acceptor (acceptors/phospholipid) for the two sets of donor-acceptor pairs in DPPC vesicles: triacylglycerol donor to triacylglycerol acceptor (□, 1); phospholipid donor to phospholipid acceptor (○, 2). The molar ratio of donor to total phospholipids was kept constant (0.1 mol%) for both experiments while the amount of acceptor was varied. For each measurement donor and acceptor were premixed in the same set of vesicles.

total lipid concentration. The results show that the triacylglycerol probes exhibit a higher transfer efficiency than the phospholipids in the range of acceptor surface densities used. This suggests that the triacylglycerol probes may exhibit lateral phase separation.

The effect of PEG 8000, a known fusogen, on DPPC vesicles was investigated as a function of both poly(ethylene glycol) concentration and temperature, using the 'premixed' and 'separately labelled' donor-acceptor pairs described above. For each poly(ethylene glycol) concentration, experiments were carried out using the triacylglycerol-based energy transfer probes and repeated using the phospholipid analogues. No spontaneous probe transfer was observed on the time scale of these experiments.

Poly(ethylene glycol) effects as reported using triacylglycerol probes

The fluorescence spectrum of a sample containing both 'premixed' donor/acceptor in DPPC

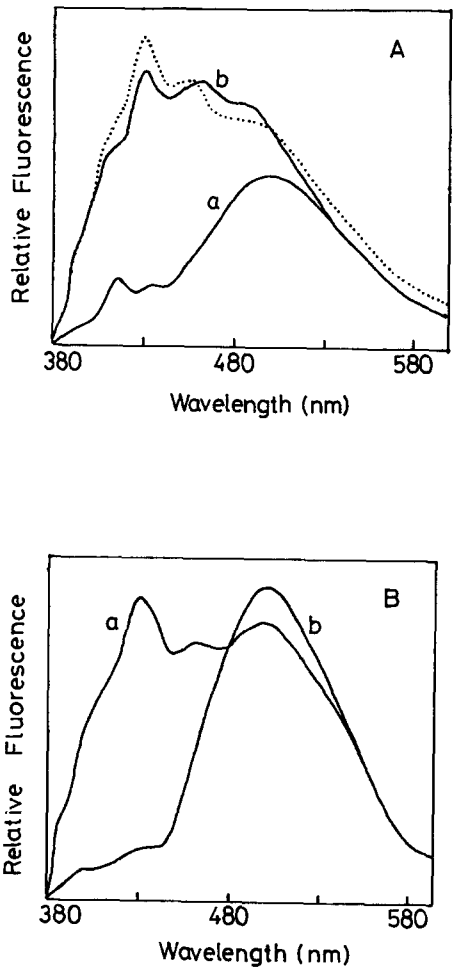

Fig. 3. Effect of poly(ethylene glycol) on energy transfer for vesicles containing the triacylglycerol donor and acceptor (A). Effect of 5% poly(ethylene glycol) on fluorescence of a vesicle dispersion containing premixed donor and acceptor (0.3 mol% donor and 0.6 mol% acceptor in DPPC) with 5-fold excess of unlabelled vesicles (total phospholipid concentration of 0.08 mg/ml). Curve (a) shows vesicles before addition of poly(ethylene glycol) while curve (b) shows the equilibrium situation after 45 min. The dotted line represents the spectrum obtained from vesicles containing donor and acceptor premixed in a total of 0.08 mg/ml DPPC. (B) Effect of 5% poly(ethylene glycol) added to a vesicle dispersion containing donor and acceptor (0.5 mol% donor and 2 mol% acceptor in DPPC) in separate vesicles which were subsequently mixed in a ratio of 1:2 (donor to acceptor vesicles, respectively) giving a final DPPC concentration of 0.08 mg/ml. (a) before addition of poly(ethylene glycol); (b) equilibrium situation approx. 30 min after poly(ethylene glycol) addition.

vesicles and a 5-fold excess of unlabelled DPPC vesicles is shown in Fig. 3A(a). Addition of 5% poly(ethylene glycol) to this mixture caused a relief of energy transfer which increased progressively with time at room temperature, equilibrium being reached in approx. 45 min under these conditions (Fig. 3A(b)). Increasing the poly(ethylene glycol) concentration decreased the time to reach equilibrium, as did heating above the phase transition temperature of DPPC. In the absence of the 5-fold excess of unlabelled DPPC, no effect of PEG 8000 on the fluorescence spectrum of the 'premixed' vesicles was noted. The expected spectrum indicating complete probe redistribution was obtained from a vesicle sample prepared by injection of a donor/acceptor/DPPC mixture in ethanol to give the appropriate total probe and DPPC concentrations (Fig. 3A(dotted curve)). Again, addition of PEG 8000 had no effect on the fluorescence spectrum of this control sample.

In an analogous series of experiments using separately labllled DPPC vesicles, addition of poly(ethylene glycol) caused a decrease in donor fluorescence due to energy transfer from donor to acceptor as the separately labelled vesicles aggregated. These effects were apparent at poly(ethylene glycol) concentrations as low as approx. 5% (w/w), equilibrium being reached more rapidly as the poly(ethylene glycol) concentration was raised up to 35%. Fig. 3B shows the initial (a) and equilibrium (b) situation for this particular system which is the same regardless of how much poly(ethylene glycol) is added (above 5%). On addition of 5% poly(ethylene glycol) equilibrium is reached after about 30 min. As the poly(ethylene glycol) concentration is increased the time taken to reach equilibrium decreases until at approx. 35% poly(ethylene glycol) equilibrium is reached in approx. 5 min.

Poly(ethylene glycol) effects as reported using the phospholipid probes

The above experiments were repeated using the phospholipid donor and acceptor and incorporating similar controls. Dramatically different results were obtained: an immediate partial quenching of donor fluorescence was seen on adding low concentrations of poly(ethylene glycol) (approx. 35%) to separately labelled vesicles (Fig. 4A), this degree

of quenching being invariant with time. However, heating above the lipid phase transition caused further quenching of donor fluorescence. Using ≥ 35% poly(ethylene glycol) (the PEG concentration required to induce fusion), a much greater degree of quenching of donor fluorescence was observed (Fig. 4B). Further, addition of 5% poly(ethylene glycol) to a mixture of 'premixed' vesicles and excess unlabelled vesicles produced no immediate change in donor fluorescence (Fig. 5A). Substantial relief was only achieved with ≥ 35% poly(ethylene glycol) (Fig. 5B): this effect occurred within 5 min of mixing with poly(ethylene glycol). Heating either the 5% or 35% poly(ethylene glycol)-treated sample to 55°C for 15 min gave total relief of donor quenching. Figs. 5A and 5B, curves b and c, respectively.

Both these results are in direct contrast to those obtained with the triacylglycerol probes, where the equilibrium situation corresponding to complete

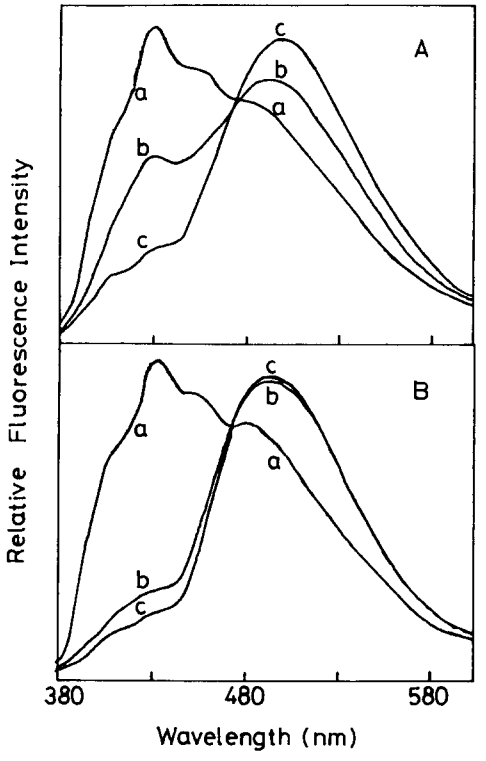

Fig. 4. Effect of poly(ethylene glycol) on the emission spectrum of vesicles separately labelled with the phospholipid donor and acceptor probes (0.5 mol% donor and 2 mol% acceptor). Donor and acceptor vesicles were subsequently mixed in a ratio of 1 : 1 giving a final DPPC concentration of 0.08 mg/ml. (A) Effect of 5% poly(ethylene glycol) on the separately labelled vesicles. Curve (a) shows the emission spectrum before poly(ethylene glycol) addition while curve (b) is the spectrum after incubation with 5% poly(ethylene glycol) at room temperature for 10 min. Subsequent heating to 55°C for 15 min caused further quenching of donor fluorescence (curve (c)). (B) Effect of 35% poly(ethylene glycol) on the separately labelled vesicles. Curve (a) emission spectrum before poly(ethylene glycol) addition; curve (b) equilibrium situation after fusion with 35% poly(ethylene glycol) at room temperature for 10 min; curve (c) subsequent heating to 55°C for 15 min has only a small effect on donor fluorescence.

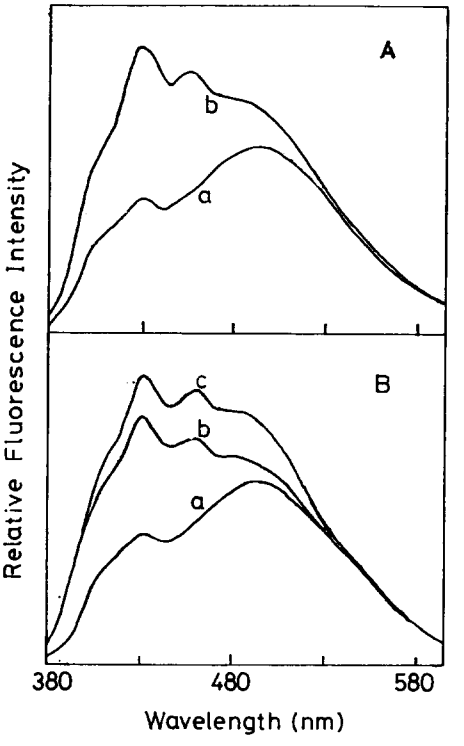

Fig. 5. Effect of poly(ethylene glycol) on the emission spectrum of vesicles containing mixed phospholipid donor and acceptor (0.2 mol% donor and 1 mol% acceptor in DPPC) with a 5-fold excess of unlabelled vesicles (total DPPC concentration of 0.08 mg/ml) (A) Effect of 5% poly(ethylene glycol). Curve (a) before and after aggregation with 5% poly(ethylene glycol). There is no change in the spectrum. Subsequent heating to 55°C for 15 min (curve (b)) fuses the vesicles with relief of quenching of the donor fluorescence. (B) Effect of 35% poly(ethylene glycol). Curve (a) before addition of poly(ethylene glycol); curve (b) after fusion of vesicles using 35% poly(ethylene glycol) at room temperature for 5 min. Subsequent heating to 55°C for 15 min caused a further relief of donor quenching (curve (c)).

probe randomisation could be achieved with relatively low (approx. 5% w/w) poly(ethylene glycol) concentrations. On standing at room temperature for 1–2 h a small amount of relief of quenching was noted for samples containing low poly(ethylene glycol) concentrations suggesting limited fusion after this period of time.

Electron microscopy studies

Electron micrographs of DPPC vesicles (negatively stained with uranyl acetate) were obtained, examples of which are shown in Fig. 6. Samples were incubated with varying concentrations of poly(ethylene glycol) for 60 min and some were subsequently heated to 55°C for a further 20 min. The micrographs show highly aggregated vesicles in samples which had been incubated at room temperature at the lower concentrations of poly(ethylene glycol) (Fig. 6A). At higher ($\geqslant$ 35%) poly(ethylene glycol) concentrations multilamellar structures were evident (Fig. 6B). Micrographs of samples containing as low as 5% poly(ethylene glycol) showed multilamellar structures similar to those shown in Fig. 6B after heating to 55°C for 15 min. The micrographs did not suggest extensive fusion at the lower poly(ethylene glycol) concentration for vesicles incubated at room temperature. Attempts at purification of poly(ethylene glycol) did not affect the results obtained. These data would not be expected on the basis of the fluorescence experiments so far described unless poly(ethylene glycol)-induced aggregation were able to facilitate triacylglycerol, but not phospholipid probe exchange between vesicles.

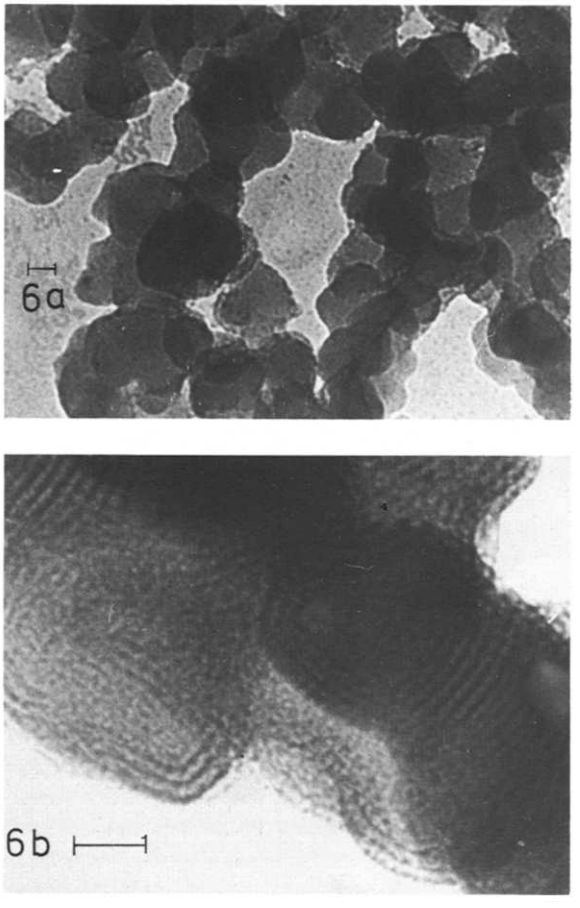

Fig. 6. (A) DPPC vesicles treated with 5% PEG 8000 (w/w) incubated at 20°C for 60 min prior to staining. Similar results were found up to approx. 35% poly(ethylene glycol). Bar = 200 Å. Instrument magnification was 80000×. (B) DPPC vesicles incubated with > 35% poly(ethylene glycol) (w/w) at 20°C for 60 min prior to staining. Bar = 200 Å. Instrument magnification was 120000×.

Discussion

Commonly used methods such as light scattering and gel filtration [17–20] do not in general distinguish lipid mixing with the formation of large aggregates from slowly reversible aggregation.

In this study the effect of poly(ethylene glycol) on dispersions of unilamellar DPPC vesicles has been monitored by fluorescence energy transfer. Poly(ethylene glycol) has been widely used as a fusogen for both cells [8,21] and vesicles [9–12]: our electron microscope studies confirm previous work [10,22] indicating that poly(ethylene glycol) induces vesicle aggregation at concentrations as low as 5%, concentrations of $\geqslant$ 35% being necessary to induce fusion at room temperature. The fluorescence results with the phospholipid donor and acceptor in separate vesicles are in line with these observations.

Vesicles prepared by premixing donor and acceptor phospholipid probes show no change in fluorescence spectrum at 5% poly(ethylene glycol) in the presence of excess unlabelled vesicles. This shows that the phospholipid probes are unable to exchange across apposed bilayers under these con-

ditions. Addition of $\geqslant 35\%$ poly(ethylene glycol) causes vesicle fusion. Comparison of the results of experiments with the probes premixed in a vesicle subset with those found using mixtures of vesicles separately labelled allows aggregation to be clearly distinguished from vesicle fusion. The effect of poly(ethylene glycol) on DPPC vesicles is thus a rapid aggregation, fusion being promoted by increasing concentrations of poly(ethylene glycol) and by rapid heating above the lipid phase transition temperature. Low poly(ethylene glycol) concentrations can induce (albeit slowly) complete triacylglycerol probe randomisation. Evidently the neutral triacylglycerols can exchange fairly rapidly between apposed lipid bilayers within aggregated vesicles, this process being undetected in the non-aggregated state. Rapid exchange of other lipid soluble probes in similar systems has been reported previously [6]. This type of exchange process between apposed bilayers would be expected to have a much higher activation energy for the phospholipid probes, because of their structural similarity to the bilayer phospholipid.

Under our conditions, attempts to disaggregate DPPC vesicles by dilution (to lower the poly(ethylene glycol) concentration) were unsuccessful. DPPC vesicles containing 5% phosphatidylserine could apparently be reversibly aggregated by addition of poly(ethylene glycol) and subsequent dilution. Sonication of these aggregated vesicles in a bath sonicator for 3 min also promoted disaggregation.

It has been suggested previously that purification of commercial grade poly(ethylene glycol) (mol. wt. 6000) removes the fusogenic component. Our attempts at purification of poly(ethylene glycol) did not prevent fusion. Since the authors [14] do not state their sources of poly(ethylene glycol) no further comment is possible.

The phospholipid probes described emerge as useful tools for the study of vesicle aggregation and fusion phenomena. The triacylglycerol probes exchange too quickly on aggregation to be of use on their own in such aggregation/fusion studies. The rapid exchange of triacylglycerol probes on vesicle aggregation suggests that the poly(ethylene glycol) might act to disorder the head group region of the lipid. This would considerably reduce the activation energy for exchange of neutral lipid and could well facilitate bilayer fusion at increased temperature.

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