Time-Resolved and Equilibrium Measurements of the Effects of Poly(ethylene glycol) on Small Unilamellar Phospholipid Vesicles†

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ABSTRACT: The effects of poly(ethylene glycol) (PEG) on sonicated unilamellar vesicles made of phosphatidylcholine have been examined. Stopped-flow and equilibrium data are presented for vesicle aggregation, vesicle leakage, lipid mixing, and aqueous contents mixing. Vesicle aggregation is detected as a monoexponential increase in light scattering, for PEG concentrations between 5 and 10%. In the region between 10–15% and 23–27% PEG, under our experimental conditions, the increase in light scattering follows a more complex biexponential kinetics, and, under these conditions, vesicle aggregation is accompanied by lipid mixing, a combination of events denoted as "close apposition". Above 23–27% PEG, the increase in light scattering is accompanied by fast lipid mixing, and also mixing of aqueous contents, all this being indicative of vesicle fusion; in addition, leakage occurs under these circumstances. Fusion takes place at high PEG concentrations, as indicated above, without any dilution step. From a methodological point of view, the Tb/DPA assay is shown to be more appropriate than the ANTS/DPX method for leakage and fusion studies in the presence of PEG.

The interaction of poly(ethylene glycol) (PEG)[1] with phospholipid bilayers has received considerable attention in view of the well-known fusogenic effect of PEG on living cells, a phenomenon whose mechanism is not fully understood yet. Investigations on PEG–liposome interaction were started by Boni et al. (1981a,b), who described "fusion", i.e., increase in size, of sonicated phosphatidylcholine (PC) liposomes in the presence of PEG 6000. Sáez et al. (1982) were able to show that, in the process of PEG-induced vesicle fusion, SUV became leaky and that fusion was preceded by vesicle aggregation. The early studies on the effects of PEG on phospholipid vesicles have been reviewed by Boni and Hui (1987).

PEG is known to promote lipid mixing between vesicles (Tilcock & Fisher, 1982; Parente & Lentz, 1986; Burgess et al., 1991; Wu & Lentz, 1991). In addition, PEG has also been shown to induce vesicle contents leakage and mixing (Sáez et al., 1982; Parente & Lentz, 1986). Using the ANTS/DPX method (Ellens et al., 1985), extensive leakage of LUV contents was found at and above 25% PEG (Burgess et al., 1991). With respect to mixing of aqueous contents, the behavior of large and small unilamellar vesicles toward PEG has been recently compared by Lentz et al. (1982) using different vesicle preparation methods. These authors conclude that no fusion occurs for PC vesicles of diameter greater than 770 Å, although lipid transfer can be detected between vesicles of all sizes. With respect to SUV, they see an increase in vesicle diameter when treated with high concentrations of PEG, but observations of mixing of vesicle contents are apparently prevented by concurring phenomena, e.g., leakage.

The present paper attempts to describe in further detail the effects of PEG on PC vesicles, including vesicle aggregation, vesicle leakage, and lipid and aqueous content mixing. Small unilamellar vesicles are used for two main reasons, namely, the variety of effects produced by PEG on these vesicles, as discussed by Lentz et al. (1992), and the relatively low production of light scattering, as compared to LUV, that allows fluorescence measurements to be performed under certain conditions free from scattering artifacts. In addition, it should be remembered that some natural vesicles involved in fusion processes (e.g., synaptic vesicles) are of a comparable size. Novel features in our study include the use of stopped-flow rapid kinetic techniques in order to explore the early stages of PEG/SUV interaction; also vesicle leakage and mixing of vesicle contents have been separately studied in SUV under equilibrium and kinetic conditions; moreover, in contrast with many of the published data, no dilution of PEG-treated vesicles into water or buffer takes place under our experimental conditions, thus performing the spectroscopic measurements in "real time", and without introducing factors other than PEG treatment in the process. The overall data provide a comprehensive, unified description of fast and slow processes taking place in phospholipid vesicles upon PEG treatment.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (PC) was grade I from Lipid Products (South Nutfield, England); it was found to be >98% pure by thin-layer chromatography. Poly(ethylene glycol) (PEG) 1500 was supplied by Merck (Darmstadt, Germany) and used without further purification; occasionally, PEG 1500 purified according to Honda et al. (1981) was used, but no difference between this and the commercial material was observed. In some experiments, PEG 8000 (Sigma, St. Louis, MO) was utilized, as indicated below. Octadecylrhodamine B, chloride salt (R18), *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylylenebis(pyridinium bromide) (DPX), dipicolinic acid (DPA), and $TbCl_3$ were purchased from Molecular Probes (Eugene,

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPA, dipicolinic acid; DPX, *p*-xylylenebis(pyridinium bromide); LUV, large unilamellar vesicle(s); NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PEG, poly(ethylene glycol); R18, octadecylrhodamine B chloride salt; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; SUV, sonicated unilamellar vesicle(s).

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OR). All other reagents were analytical grade. Polystyrene microspheres (9.6 μm in diameter) were purchased from Polysciences (Warrington, PA).

Aliquots of phospholipid in chloroform were evaporated to dryness in a rotary evaporator, and left under vacuum for at least 4 h to remove solvent traces. The lipid was dispersed in buffer (300 mM NaCl, 1 mM EDTA, 2 mM TES, and 2 mM His, pH 7.4, except for vesicle contents mixing measurements, where specific probes were encapsulated as described below) with vortex mixing. For SUV preparation, the resulting suspension was sonicated with an MSE probe sonicator, at 10–12-μm amplitude, for 30 min, at 4 °C. Electron microscopic controls showed that, under these conditions, most if not all of the vesicles are limited by a single bilayer and have an average diameter of 28 nm. The sonicated suspension was then centrifuged (100000*g*, 60 min, 4 °C) to remove lipid and probe debris. SUV for contents mixing and leakage measurements were freed from nonentrapped fluorescent probes by gel filtration chromatography (Wilschut & Papahadjopoulos, 1979; Ellens et al., 1985). Lipid phosphorus was assayed by standard methods in all liposome preparations, and adjusted so that the final PC concentration was 0.7 mM, with the exceptions indicated below.

PEG-induced vesicle aggregation was measured as an increase in light scattering of the vesicle suspension. Lipid mixing was determined as dequenching of R18 (Hoekstra et al., 1984), or by resonance energy transfer between NBD-PE and Rh-PE (Struck et al., 1981). Contents mixing and leakage were quantified using the DPA/Tb method described by Wilschut and Papahadjopoulos (1979); some experiments were performed following the ANTS/DPX method (Ellens et al., 1985).

For equilibrium measurements, 1 volume of vesicle suspension was mixed with 2 volumes of the appropriate PEG soltuion in the same buffer, and the mixture was left to equilibrate for 30 min. Fluorescence and light scattering at 90 °C were measured in a Perkin-Elmer MPF-66 spectrofluorometer. For light-scattering measurements, both monochromators were adjusted at 650 nm. R18 fluorescence was measured with excitation at 560 nm and emission at 590 nm; the 0% and 100% fluoresence levels, 0% and 100% fusion, respectively, were determined as described elsewhere (Nieva et al., 1989). In fluorescence energy transfer assays, with Rh-PE and NBD-PE, the ratio of fluorescence intensities at 530 and 590 nm (F_{530}/F_{590}) was recorded; other details were as in Struck et al. (1981). When vesicle contents mixing and leakage were measured by the ANTS/DPX system, excitation was at 355 nm, and emission at 530 nm; a Schott 06505 cutoff filter (515 nm) was used to avoid scattered light; vesicle preparation and calibration of the assay system were as described by Nieva et al. (1989). The DPA/Tb complex was excited at 276 nm; fluorescent emission was measured through a 545-nm monochromator. The DPA/Tb system was calibrated for quantitative measurements of leakage and contents mixing as described by Wilschut et al. (1980).

Time-resolved measurements were carried out in a modular stopped-flow spectrofluorometer manufactured by Hi-Tech (Salisbury, U.K.). The main components are a SHU-51 sample handling unit, an 8.8-A xenon lamp, an MG-10 grating monochromator, and a PM-60 photomultiplier and filter holder. SUV and PEG suspensions were mixed at a 1:2 v/v ratio; preliminary experiments demonstrated that, under these conditions, good mixing occurred even with the most viscous PEG solutions. For light-scattering measurements, an incident light of 650 nm was used; scattered light was received through a 15-mm variable shutter. R18 fluorescence was excited at 560 nm and received through a Hi-Tech OG 590 cutoff filter (590 nm). For DPA/Tb fluorescence, excitation was at 276 nm, via monochromator, and emission was received through a Hi-Tech OG 530 cutoff filter (530 nm). The filters served the 2-fold purpose of selecting the emission wavelength and preventing scattered light from reaching the photomultiplier. The stopped-flow spectrophotometer was operated through an Apple IIe computer. Data were transferred to an IBM PC computer and analyzed using standard software. Six transients were usually averaged for each measurement.

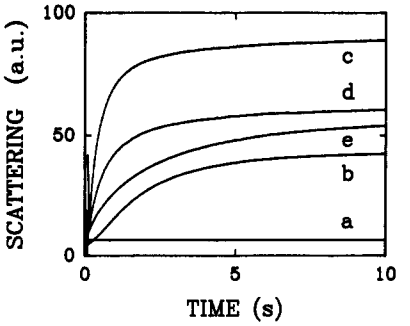

FIGURE 1: PEG-induced changes in light scattering (650 nm) of a suspension of sonicated PC vesicles (0.70 mM): a time-resolved study. PEG concentrations (% w/v): a, 0%; b, 10%; c, 15%; d, 25%; e, 39%.

Osmolalities were measured using a cryoscopic osmometer (Osmomat 030) form Gonotec (Berlin, Germany). Viscosities were determined with an Ubbelohde viscosimeter, at 22 °C. All PEG concentrations are given as percent w/v.

RESULTS

Changes in Light Scattering. Changes in scattering (650 nm) of PC SUV suspensions (final lipid concentration 0.70 mM) in the presence of increasing concentrations of PEG 1500 were followed by stopped-flow rapid kinetics spectroscopy. Representative traces are shown in Figure 1; exponential curves with positive slopes are obtained. Using standard software, the curves were fitted to single or double first-order exponentials, or to a single second-order fit. It was found that, for PEG concentrations up to 10%, the best fit was a single first-order exponential, while at or above 15% PEG, the experimental curves were best described by the sum of two exponentials. This is a clear indication that the nature of the aggregation process is somehow changing when the PEG concentration is increased to 15%. The kinetics of these more complex changes in light scattering, occurring at or above 15% PEG, have been analyzed elsewhere (Viguera and Goñi, manuscript in preparation).

The kinetics of vesicle aggregation in its simpler form, i.e., when it can be described in terms of a single exponential, have been further explored by varying the phospholipid concentration in the presence of 6.6% PEG 1500. The corresponding kinetic parameters are shown in Figure 2. Essentially similar results were found with 10% and 13% PEG (not shown). Both amplitudes and apparent exponential constants (Figure 2A,B) follow straight lines. The fact that the scattering values are proportional to the initial vesicle concentration reflects a linear relationship between scattering intensity (amplitude), average vesicle size, and lipid concentration, up to 0.8 mM.

Lipid Mixing. The extent of PEG-induced vesicle lipid mixing was estimated following either the dequenching of octadecylrhodamine fluorescence or resonance energy transfer between NBD-PE and Rh-PE. Control experiments were performed in order to estimate the direct effects of PEG on

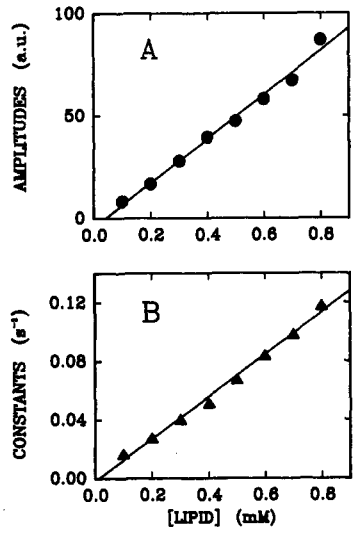

FIGURE 2: Influence of lipid concentration on the kinetic parameters of PEG-induced changes in light scattering (650 nm) of a sonicated PC vesicle suspension. PEG concentration was 6.6% (w/v). (A) Amplitudes of the exponential curves. (B) Apparent exponential constants.

the fluorescence of the various probes. The fluorescence of R18, when incorporated into lipid vesicles, was only slightly perturbed by PEG. Increasing polymer concentrations, up to 40%, produced a gradual decrease in fluorescence emission intensity; the decrease was never larger than 10%, and it was taken into account in all further calculations. RET between NBD-PE and Rh-PE, incorporated into the same vesicles, was also examined in the presence of PEG solutions. Energy transfer between NBD-PE and Rh-PE increases with PEG, its value for vesicles in 35% PEG being about 70% higher than in pure buffer. The increase is independent of the phospholipid: probe ratio between 25:1 and 125:1 mole ratios. In view of these results, this method can only be used for semiquantitative estimations of lipid mixing in the presence of PEG.

Measurements of R18 dequenching under apparent equilibrium conditions, i.e., 30 min after PEG addition, were carried out for SUV (0.70 mM final PC concentration) as a function of PEG 1500 concentration. Lipid mixing starts at about 5% PEG and then increases rapidly, so that 100% mixing occurs 30 min after addition of 15% PEG; higher concentrations of PEG do not produce any additional effects (data not shown). Again, recording the process as a function of time provides new insights on the mechanisms of PEG/SUV interactions; this is particularly interesting in experiments involving R18 since this probe has some tendency for spontaneous exchange; thus, the equilibrium data may contain contributions from both lipid mixing and free probe transfer. Representative time-resolved traces are shown in Figure 3. In general, lipid mixing appears to be a slower process than vesicle aggregation; considering a time scale of 500 s, 12.5% PEG (trace b) hardly induces any lipid mixing, while 20% PEG (trace c) is clearly effective. A large increase in the initial rate is observed going from 20% to 39% PEG (trace d). These changes are more clearly seen when the initial rates are plotted vs PEG concentration (Figure 4). The initial rate of lipid mixing is abruptly modified above two PEG concentrations: 10% (main figure) and 25% (insert). It should be noted that, even below 10% PEG, lipid mixing appears to occur, although at a very low rate, in an apparent zero-order reaction with respect to PEG, perhaps reflecting spontaneous probe exchange.

Measurements of lipid mixing between small unilamellar vesicles were also carried out using the RET method.

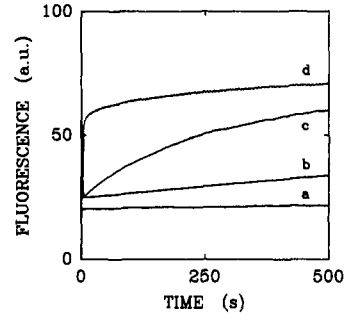

FIGURE 3: PEG-induced lipid mixing: time-resolved measurements. PEG concentrations (% w/v): a, 0%; b, 12.5%; c, 20%; d, 39%. (Note the extended time scale, as compared with the light-scattering measurements.)

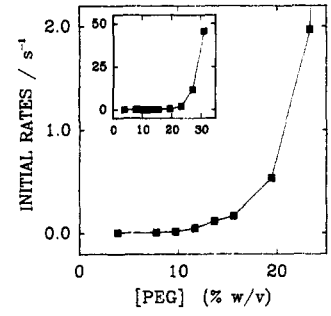

FIGURE 4: PEG-induced lipid mixing: time-resolved measurements. Initial rates estimated from the "increase in fluorescence vs time" curves, as a function of PEG concentration. The main figure shows the result corresponding to PEG concentrations between 3.5 and 23%. The insert shows, at a much reduced ordinate scale, the increase in lipid mixing at ≈30% PEG.

Experiments in which two vesicle populations, one containing Rh-PE and the other NBD-PE, were mixed, as well as measurements in which probe-free vesicles were mixed with liposomes containing both Rh-PE and NBD-PE, gave equivalent results; the results were also consistent with the observations using R18: a low level of lipid mixing exists independently of the presence or absence of PEG, but mixing is considerably enhanced at PEG 1500 concentrations above 10%, and increases again at concentrations at or above 25% (not shown).

Lipid mixing measurements were also carried out by the R18 dequenching method in the presence of PEG 8000. The results were superimposable with those obtained in the presence of PEG 1500 (data not shown).

Vesicle Leakage. Measurements of PEG-induced vesicle leakage were attempted both with the ANTS/DPX and with the DPA/Tb systems. For that purpose, control experiments were performed in order to observe any direct effects of PEG on their fluorescence properties. ANTS and ANTS/DPX mixtures were found to be very sensitive to PEG: the λ_{max} of their fluorescence emission bands was gradually blue-shifted up to 20 nm when the PEG concentration varied from 0 to 35% (not shown). In addition, the maximum fluorescence intensity of ANTS increases over 4-fold along the same range of PEG concentrations; also, very significantly, when DPX was added until the ANTS fluorescence in water was quenched by about 50%, it was found that the change in fluorescence of the ANTS/DPX system with PEG did not parallel the variation of pure ANTS, i.e., F/F_0 ratios varied with PEG concentration for the same ANTS/DPX mixture. In general, for a given ANTS/DPX mixture, quenching by DPX appears to be less effective at higher PEG concentrations; this is reasonable taking into account the essentially collisional nature

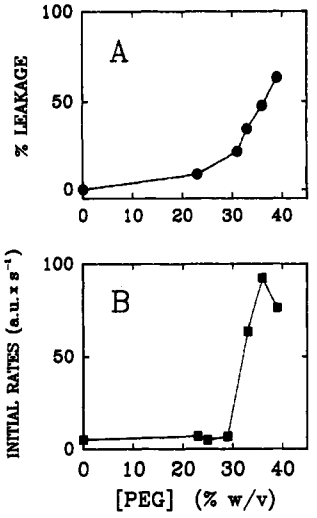

FIGURE 5: PEG-induced leakage of sonicated PC vesicles. (A) Equilibrium values. (B) Initial rates. PC final concentration was 0.70 mM.

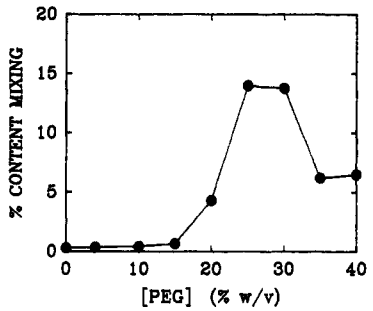

FIGURE 6: PEG-induced mixing of aqueous contents of sonicated PC vesicles measured by the Tb/DPA method. Equilibrium values. Final PC concentration was 0.70 mM.

of ANTS fluorescence quenching by DPX, and the large increase in viscosity (from 1 to 15 cP) produced by increasing PEG concentrations from 0 to 35% (data not shown). In light of these results, ANTS/DPX was not deemed appropriate for vesicle leakage measurements under our conditions. In contrast, both fluorescence intensities and positions of DPA/Tb mixtures were insensitive to PEG concentrations, in the 0–35% range. The fluorescence of DPA/Tb in solution did not vary either when "empty" PC SUV (0.70 mM) were added. Consequently, all further experiments of vesicle leakage and mixing of aqueous contents were carried out using the DPA/Tb system.

PEG-induced release of aqueous contents from SUV (0.70 mM) loaded with both DPA and Tb was studied under equilibrium conditions and as a function of time. The percent leakage 30 min after PEG 1500 addition is presented as a function of PEG concentration in Figure 5A. Some leakage occurs at low polymer concentrations, but it is at or above 30% when the process becomes significant. Kinetic studies also reveal that the initial rates increase markedly above ≈30% PEG (Figure 5B).

Mixing of Aqueous Contents. Mixing of aqueous contents is an important phenomenon in order to assess vesicle fusion. The DPA/Tb system allows the detection of contents mixing without perturbation arising from contents leakage. Equilibrium values for percent fusion (contents mixing) are given in Figure 6. Significant mixing occurs only at or above 25% PEG, and 25–30% is the region of PEG concentration at which maximum contents mixing is detected, because above 30% PEG fusion is accompanied by extensive leakage (Figure 5).

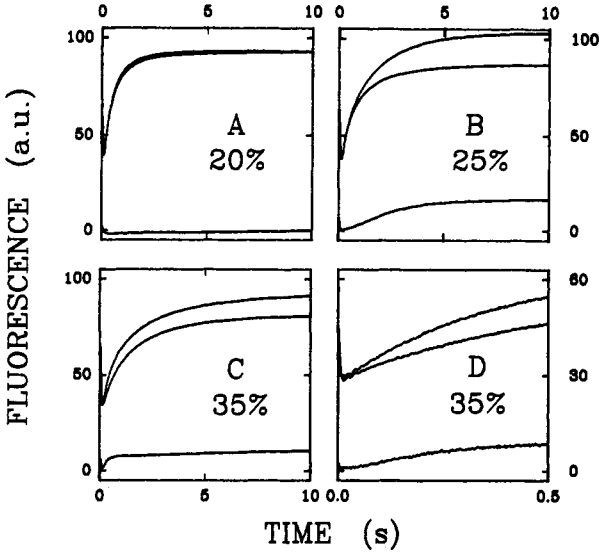

FIGURE 7: PEG-induced mixing of aqueous contents of sonicated PC vesicles: time-resolved measurements. Two curves are shown in the upper part of each panel for each PEG concentration: "control", corresponding to liposomes devoid of fluorescent probes, and "fusion", obtained with vesicles loaded with DPA and $TbCl_3$. Mixing of aqueous contents (curve in the lower part of the panel) is estimated by subtracting the "control" from the "fusion" curve. (A) An example of nonfusogenic PEG 1500 concentration. (B–D) Examples of fusogenic PEG concentrations. Note the different time scales in panels C and D.

The same measurements were also performed with PEG 8000, with equivalent results. It should be noted, however, that the experimentally observed fusion may be an underestimation, because of the fluorescence inner-filter effect of the vesicle aggregates.

Mixing of vesicle contents occurs in a time scale of seconds in our system; thus, dynamic measurements can only be performed with the stopped-flow spectrofluorometer. One additional problem is that, in this instrument, the fluorescence emission signal (at >530 nm) is contaminated with scattered excitation light, even when a cutoff filter is used, as indicated under Materials and Methods. This problem was circumvented by performing parallel control experiments, in which sodium citrate-loaded vesicles were used instead of those containing DPA; for each PEG 1500 concentration, fusion (as mixing of aqueous contents) was quantified as the difference between apparent fusion and the control curve. For both the "empty" and loaded vesicles, the final PC concentration was 0.70 mM. Comparable measurements could not be carried out with LUV because, under our conditions, the scattering effect of these large vesicles could not be subtracted from the change in fluorescence.

Examples of time-resolved observations of vesicle contents mixing in the presence of PEG 1500 for the first 10 s after polymer addition are given in Figure 7. At 20% PEG, the apparent fusion and the control curves are virtually superimposable (Figure 7A), and no significant fusion is observed. Contents mixing is already detectable at 25% PEG (Figure 7B) and is seen to occur at a much faster rate at 35% PEG (Figure 7C,D), when the process reaches an apparent equilibrium in less than 1 s. The amplitudes and initial rates of contents mixing curves are summarized in Figure 8. The amplitude is seen to be maximal at 25% PEG, decreasing afterward, in agreement with the data in Figure 6. Also noteworthy in Figure 8 is the large increase in the rate of contents mixing around 30% PEG, in agreement with the data of lipid mixing (Figure 4) and leakage (Figure 5).

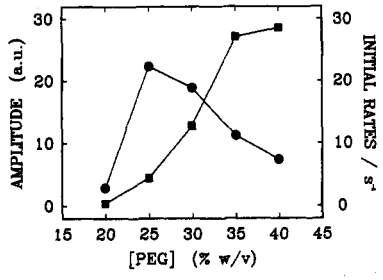

FIGURE 8: PEG-induced mixing of aqueous contents of sonicated PC vesicles: time-resolved measurements. (●) Amplitudes of the "fusion" curves; (■) initial rates. Data were estimated from fusion curves as shown in Figure 7.

DISCUSSION

In this paper, the effects of poly(ethylene glycol) on small unilamellar vesicles made of phosphatidylcholine have been examined in real time, using a combination of light-scattering and fluorescence techniques. Four regions of PEG concentrations can be distinguished from the combined experimental data: (region I) below 5% PEG, *no PEG-induced events* are detected; (region II) between 5 and ≈10% PEG, a monoexponential increase in light scattering occurs (Figure 2) that is interpreted in terms of *vesicle aggregation*; (region III) between 10–15% and 23–27% PEG, the increase in light scattering follows a more complex biexponential kinetics, and, in addition, lipid mixing is detected (Figure 4), this combination of events being characteristic of what is called *close apposition*; (region IV) above 23–27% PEG, the increase in scattering is accompanied by fast lipid mixing (Figure 4) and also mixing of aqueous contents (Figure 6), all this being indicative of vesicle *fusion*; in addition, *leakage* occurs. [Note that, for PEG molecular weights or PC concentrations other than those used in the present study, the threshold PEG concentrations may not be exactly the same as above; see Tilcock and Fisher (1982).]

Bentz et al. (1988) have distinguished between vesicle aggregation and what they call "close approach of the surfaces" ("close apposition" in region III). The latter is a combined phenomenon, consisting of vesicle aggregation and lipid exchange, that has also been observed in other systems (Wilschut et al., 1985; Nieva et al., 1989). It is undoubtedly significant that the start of lipid mixing (>10% PEG, Figure 4) accompanies the change in kinetics of the increase in light scattering (10–15% PEG). The estimation by Tilcock and Fisher (1979) that above ≈13% PEG all water molecules experience the presence of the polymer should also be noted. Yamazaki and Ito (1990) point out that PEG-induced osmophobic association can be stabilized by a tight aggregation of the vesicles, that excludes the solvent from the intervesicular contact area. We propose that the "tight aggregation" mentioned by the above authors corresponds to our "close apposition" stage. "Close apposition" is also probably the state of the aggregated vesicles shown in the electron micrographs published by Sáez et al. (1982), corresponding to 20% PEG 1000.

With PEG concentration above 23–27%, a number of events take place, of which mixing of vesicle lipids and of aqueous contents are taken together as indicative of vesicle fusion (Ellens et al., 1985). These results are in agreement with Yamazaki and Ito (1990) and Boni et al. (1984), who observed liposome fusion (as an irreversible increase in light scattering) only above 22% and 20% PEG, respectively. Our description of an increased rate of lipid mixing above 23% PEG is also in accordance with a similar increase (above 20% PEG) demonstrated by Lentz et al. (1992). However, these authors were unable to detect contents mixing in SUV, with the ANTS/DPX system. Using the PEG-insensitive Tb/DPA, we have measured PEG-induced SUV fusion (contents mixing) under time-resolved and equilibrium conditions (Figures 6–8). Contents mixing is observed only in "region IV", i.e., above 20% PEG, in agreement with the observations of Lentz et al. (1992) for medium-sized (77 nm) vesicles. In addition, our kinetic results indicate an increase in the apparent rate constant of contents mixing above 30% PEG (Figure 8), as expected from the concomitant increase in the lipid mixing rate (Figure 4, insert). The range of PEG concentrations around 25% represents a borderline between what we have called regions III and IV, and contents mixing occurs there at a low rate, probably limited by lipid mixing, but reaches a relatively large amplitude, because of the low leakage.

Parente and Lentz (1986) suggested that close apposition may be sufficient to induce vesicle fusion, whereas in our case we find a range of PEG concentrations (region III) where close interbilayer contact appears to occur without fusion. Ohki and Arnold (1990), and, more recently, Burgess et al. (1992), also support the idea that close adhesion is not sufficient to induce membrane fusion. Several authors have pointed out changes in properties of PEG solutions that could explain the transition from our region III to region IV: above 20% PEG, the dielectric constant of water is drastically reduced (Arnold et al., 1985), and above 25% PEG, the main T_c gel–fluid transition temperature of DPPC multilamellar vesicles increases significantly (Tilcock & Fisher, 1979). Both effects will lead to structural changes in the bilayer which, in turn, may affect the transition from close apposition to bilayer fusion. In addition, Burgess et al. (1992) have pointed out the putative role of high bilayer curvature in the promotion of SUV fusion, in the lines of previous proposals on the metastability of sonicated vesicles (Lawaczeck et al., 1976).

Most previous studies were designed in such a way that measurements were carried out after the PEG/vesicle mixture was diluted with water or buffer. Lentz et al. (1992) explicitly mention the issue of whether dilution is or is not a prerequisite for PEG-induced liposome fusion. Our results provide a direct answer to this question, since lipid and aqueous contents mixing are observed in concentrated PEG solutions, without a dilution step (Figures 6–8).

At and above 30% PEG, release of aqueous contents from the lipid vesicles is observed concomitant with fusion, also in agreement with previous studies (Blow et al., 1978; Aldwincle et al., 1982; Yamazaki & Ito, 1990). Lentz et al. (1992) have suggested that SUV may rupture in the presence of PEG before they fuse. Our results, obtained with the Tb/DPA system, do not support this idea because, if that were the case, no contents mixing would be observed, in contrast with the data in Figure 6.

The present study includes detailed observations of PEG effects as a function of time. It is thus pertinent to comment on the time scales of the various processes under study. A summary of the results is presented in Figure 9, for regions III and IV of the PEG/SUV interaction. At 20% PEG (stage of vesicle close apposition), increase in scattering (S) and vesicle leakage (L) appear to be much faster than lipid mixing (M). It is reasonable that vesicle aggregation (detected as an increase in light scattering) occurs prior to lipid mixing. This was also observed by Nieva et al. (1989) for a different liposome fusion process. Also, Parente and Lentz (1986) find an extremely fast release of vesicle contents. At the fusogenic concentration of 33%, the change in light scattering is still the

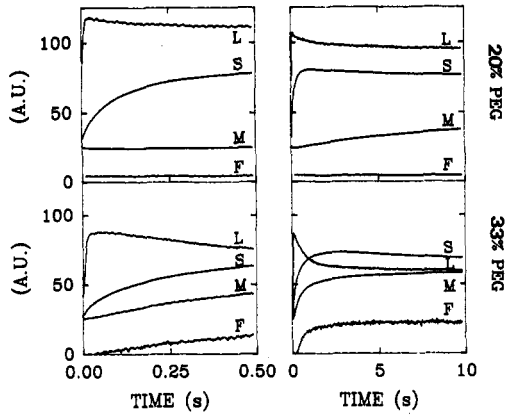

FIGURE 9: Overview of the time course of PEG effects on sonicated PC vesicles. Upper panels, 20% PEG (a nonfusogenic concentration). Lower panels, 33% PEG (a fusogenic concentration). Left, early events. Right, early and delayed events. L, leakage; S, light scattering (350 nm); M, lipid mixing; F, contents mixing.

fastest phenomenon to be observed (particularly visible at the 0.5-s time scale), but the two main facts to be noted are the presence of mixing of aqueous contents (F) and the increase in the rate of lipid mixing (M) which is now strictly simultaneous with contents mixing and leakage (Figure 9).

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