Effect of Water-Soluble Polymers on the State of Aggregation, Vesicle Size, and Phase Transformations in Mixtures of Phosphatidylcholine and Sodium Cholate

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ABSTRACT The state of aggregation and the steady-state size of mixed aggregates made of phospholipids and surfactants are both determined by the surfactant/lipid ratio in the mixed aggregates (R_e). Water-soluble polymers, such as dextrans and polyethylene glycols (PEGs) of different molecular weights, induce reversible aggregation of phospholipid vesicles, mostly due to dehydration of the vesicle surface and depletion forces, and only at much higher concentrations, PEGs (but not dextran) also induce irreversible size growth of the vesicles. Here we show that the water-soluble polymers dextrans and PEGs do not affect the vesicle-micelle phase boundaries in mixtures of phosphatidylcholine and the anionic surfactant sodium cholate. By contrast, these polymers affect markedly the steady-state size of cholate-containing vesicles. As compared with pure phosphatidylcholine vesicles, the cholate-containing vesicles have a lower tendency to undergo polymer-induced aggregation, probably due to the electrostatic repulsion between the negatively charged vesicles, but a higher tendency to undergo irreversible size growth at relatively low polymer concentrations. Such irreversible size growth was observed not only for PEG but also for dextran, which in the absence of cholate is incapable of inducing vesicle size growth. These findings are consistent with the prevailing concept that the polymer-induced size growth is due to the effect of large structural fluctuations in the bilayers of deformed aggregated vesicles, the surface of which is dehydrated by the polymer. The presence of cholate in the bilayers at sufficiently high concentrations induces such fluctuations, yielding irreversible size growth within the clusters of dehydrated vesicles formed upon mixing with polymers.

INTRODUCTION

Water-soluble polymers induce reversible aggregation (flocculation) of phospholipid vesicles. This well established phenomenon has been systematically studied in the companion article in this issue (Meyuhas et al., 1996) for dextrans and polyethylene glycols (PEGs) of various molecular weights. These studies demonstrated the central role of depletion forces. Specifically, at polymer concentration above the overlapping concentration C^* (De Gennes, 1979), the polymer is excluded from volume elements between vesicles. This results in reversible flocculation of the vesicles, eventually leading to separation of the system into a polymer-rich phase and a lipid-rich phase of lower density. Separation of the vesicles from the polymer by a dialysis membrane inhibited the aggregation, indicating the essential role of depletion forces, although other forces may still play a significant role (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; Yamazaki et al., 1989).

In most cases the aggregation was reversible; a 10-fold dilution was sufficient to yield vesicles of the original size, as determined by quasi-elastic light scattering (QLS). Irreversible size growth, through a fusion mechanism or lipid transfer (collisional or noncollisional), required much higher concentrations of PEG and was not observed at any concentration of dextran (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982). As an example, 3.5 wt % of PEG 6 kDa were sufficient to induce reversible aggregation of the vesicles whereas irreversible size growth occurred only at 25 wt % of this polymer. The most straightforward interpretation of this finding is that size-growth processes require destabilization of the bilayer structure (Hafeti and Hanstein, 1974). Such destabilization is the likely cause of the alteration of packing of phospholipids within the bilayers (Lehtonen and Kinnunen, 1994), increased rate of lipid transfer between bilayers (Massenburg and Lentz, 1993), and formation of membrane defects leading to leakage of water-soluble entrapped solutes (Wu and Lentz, 1991). All of these effects require PEG concentrations much higher than C_A and are not observed at all with dextrans even at much higher concentrations, probably due to the binding of dextran to the vesicle surface (Minetti et al., 1979).

In comparison with the effects leading to reversible aggregation, polymer-induced membrane perturbations probably require much larger stripping of the membrane surface from water and/or larger shape deformations. Nevertheless, if membrane perturbations are induced by impurities, polymer-induced aggregation of vesicle may lead to their fusion (Lentz et al., 1987). Detergents and particularly bile salts, such as sodium cholate, are known to destabilize lipid bilayers (Otten et al., 1995). We therefore found it of interest to investigate whether in the presence of cholate in the bilayer size growth occurs at polymer concentrations

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that in the absence of detergent cause only aggregation of the vesicles. The results of the present study show that vesicles made of egg phosphatidylcholine (PC) and sufficiently high cholate content indeed undergo rapid irreversible size growth at polymer concentrations that in the absence of cholate are merely sufficient to cause reversible aggregation.

MATERIALS AND METHODS

Materials

Egg PC, cholate, and dextrans of molecular weights of 10,000 (lot 35F0867), 20,000 (lot 20B0940), 40,000 (lot 52H0358), and 2,000,000 (lot 115F0236 and 92H0688) were purchased from Sigma Chemical Co. (St. Louis, MO). Dextran 70,000 (lot QF 11947) was purchased from Pharmacia (Uppsala, Sweden). PEGs of molecular weights of 6,000 (lot 283915 7) and 20,000 (lot 327272 1193) and Tris buffer were purchased from Fluka (Buchs, Switzerland). NaCl and EDTA were analytical grade of Merck (Darmstadt, Germany). Praseodymium chloride ($PrCl_3 \cdot 8H_2O$) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of egg PC small unilamellar vesicles (SUVs)

Egg PC dissolved in $CHCl_3$ was evaporated to dryness under a stream of nitrogen. The resultant PC film was suspended in buffer A (140 mM NaCl, 0.5 mM EDTA, 0.02% NaN_3, and 10 mM Tris, pH 7.4) to form multilamellar vesicles (Bangham et al., 1967). This suspension was sonicated for 20 min using a probe sonicator (XL-2020, Heat Systems Inc., Sarmingdale, NY) as previously described (Huang, 1969).

Preparation of mixed micelles and vesicles

Mixed micellar dispersions of PC and cholate were prepared from mixed solutions of the appropriate amounts of each lipid in a chloroform/methanol mixture (1:1). The mixed solutions were dried under a stream of nitrogen and the lipid film hydrated in buffer A to form mixed micellar solutions. PC-cholate mixed dispersions of different compositions were prepared by dilution of the mixed micelles with buffer A containing different sodium cholate concentrations, with or without a polymer.

Turbidity measurements

Turbidity was measured at 360 nm, using a Shimadzu UV-VIS (UV-160) spectrophotometer. Turbidities are given as measured. Note that in many systems containing aggregated vesicles, a 10-fold dilution resulted in a turbidity decrease greater than a factor of 10, due to aggregation. By contrast, when irreversible size growth occurred, the turbidity of the undiluted samples was less than 10-fold higher than that of the 10-fold dilution samples due to multiple scattering in the undiluted samples.

Viscosity measurements

The viscosity was measured on a size 50 Cannon-Fenske routine viscometer (Induchem Lab Glass Co., State College, PA).

QLS measurements

Particle size was measured on a Malvern photon correlation spectrometer model 4700, equipped with an argon laser (wavelength of 488 nm) at 25°C as previously described (Almog et al., 1990). Vesicle dispersions were measured after 3- to 100-fold dilution to avoid multiple scattering. Such dilution has been previously shown to have only a slight effect (if any) on the size determination (Lichtenberg, 1993). The viscosity of all measured samples differed from that of the medium by no more than 1%.

^{31}P nuclear magnetic resonance (NMR) measurements

SUVs (20 mM PC) were mixed with 9.5 mM cholate and 3.5 wt % 70-kDa dextran. The size of the resultant vesicles as measured by QLS was 190 nm. Cholate was removed by dialysis against four 200-ml changes of buffer A for 24 h. Praseodymium chloride ($PrCl_3 \cdot 8H_2O$) was added to the solution to a final concentration of 10 mM and the NMR spectrum was measured at 202.46 MHz using a Bruker ARX-500 NMR spectrometer. The relaxation delay time (D_1) was 2 s and acquisition time was 0.1966 s.

Microscopic observations

The large vesicles made by mixing 20 mM SUVs with 9.5 mM cholate and 2.5 wt % 20-kDa PEG or 3.5 wt % 70-kDa dextran were dialyzed against four changes of 200 ml of buffer A for 24 h to remove the cholate and subsequently observed by a Nikon (model Optiphot) microscope using interference (Nomarski) optics.

Dialysis experiments

Dialysis bags (Spectra/Por membrane molecular weight cut-off (MWCO): 6000–8000) containing SUV dispersions (2 mM) were immersed in a solution of a fivefold larger volume containing 5.4 mM cholate. After 24 h of incubation, the external medium was supplemented with different concentrations of 6-kDa PEG and the bags were immersed in the medium for an additional 24 h. After this incubation, 1.5 ml of the vesicle dispersions contained in the dialysis bags were transferred into cuvettes and the turbidity was measured at 360 nm. The diameter of the vesicles was measured by QLS.

RESULTS

Dextran and PEG do not affect the phase boundaries in PC-cholate mixtures but induce vesicle size growth

The state of aggregation in mixtures of phospholipids and surfactants is governed by R_e, the ratio of nonmonomeric detergent to PC in the mixed aggregates, and is independent of whether the mixtures are formed by addition of increasing concentrations of detergent to SUVs or by dilution of mixed PC/detergent micelles (Lichtenberg, 1985, 1993). The turbidity of mixed PC/cholate systems shows a bell-shaped dependence on cholate concentration (Almog et al., 1986). As long as $R_e < 0.3$, the lipid is aggregated in the forms of vesicles the size (and turbidity) of which is an increasing function of R_e; in the range of $R_e = 0.3–0.7$, vesicles and micelles co-exist, whereas at $R_e > 0.7$, all of the lipid is solubilized in mixed micelles of decreasing sizes (Almog et al., 1986; Fig. 1, *A* and *B*, open symbols).

The solid symbols in Fig. 1 *A* depict results of an experiment carried out in the presence of dextran. In this experiment, the media contained 10 wt % dextran of a molecular weight of 70 kDa. The turbidity of the resultant dispersions was again a bell-shaped function of cholate concentration,

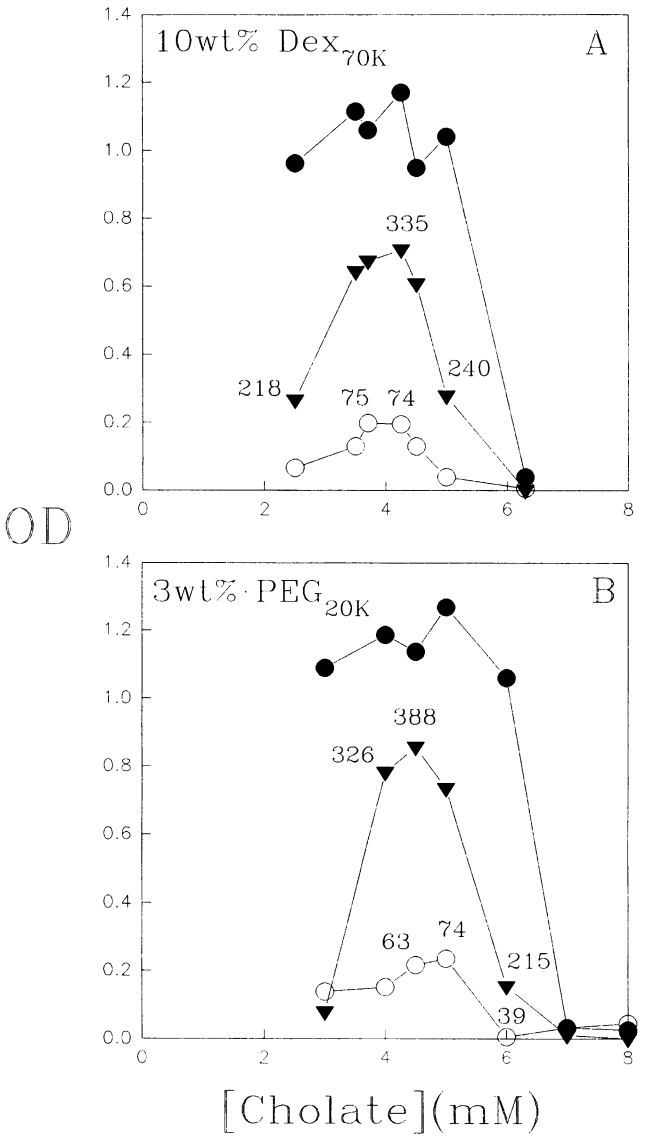

FIGURE 1 The dependence of the steady-state turbidity of PC/cholate mixed aggregates on the cholate concentration in the absence and presence of polymer. Mixed PC/cholate vesicle dispersions were made by a dilution of a mixed micellar system of 100 mM PC and 150 mM cholate in buffer A (to a final PC concentration of 1.845 mM in *A* and 2 mM in *B*). Subsequently, these mixed vesicles were mixed with equal volumes of solutions of buffer A of varying cholate concentrations without any polymer (○), with 10 wt % 70-kDa dextran (Dex_{70K}; *A*, ●) or with 3 wt % 20-kDa PEG (PEG_{20K}; *B*, ●). The turbidity of the samples was measured after 24 h. The turbidity was also measured after a 10-fold dilution (▼). The turbidity is presented as measured. Note that a 10-fold dilution resulted in a relatively small decrease of turbidity due to multiple scattering in the undiluted samples (Viguera et al., 1995). The number on the graph refer to the hydrodynamic diameter of the particles, as measured by QLS.

and given the experimental error of the turbidity measurements, the cholate concentrations required for the onset and completion of solubilization were not significantly different from those obtained in the absence of dextran. However, samples containing less than 6 mM cholate (lower than D_t^{SOL}, the total detergent concentration at the completion of solubilization) were much more turbid than the corresponding dispersions made in the absence of dextran. Furthermore, when the dispersions made in the presence of dextran were first equilibrated and subsequently diluted 10-fold in buffer A containing no dextran, the dispersions were still very turbid (triangles in Fig. 1 *A*). The size of vesicles in these turbid dispersions, as measured by QLS after an additional 3-fold dilution step, was 3- to 5-fold higher than the maximal size obtained in the absence of dextran (Fig. 1 *A*) and the vesicle diameter, as measured by QLS, was not altered by heating of the dispersions for 30 min at 80°C (not shown). These results indicate that the increased turbidity is not a result of reversible cluster formation of smaller vesicles but rather due to size growth of the vesicles.

Similar results were obtained in the presence of PEG. As an example, addition of 20-kDa PEG at a concentration of 3 wt % to mixtures containing 2 mM PC and less than 7 mM cholate resulted is increased turbidity (Fig. 1 *B*). Furthermore, the turbidity of the PEG-containing mixtures with cholate concentrations between 3.5 and 6 mM cholate remained high even after a 10-fold dilution (the turbidity of the diluted dispersions was in fact much higher than one-tenth of the undiluted samples, which can be attributed to multiple scattering in the undiluted samples). Only at 7 mM cholate did the mixture became transparent, due to complete micellization. Notably, the system with 6 mM cholate was significantly more turbid than that obtained in the absence of PEG. To explain this difference, note that, within the range of co-existence, vesicles of R_e^{SAT}, the size of which is maximal for a given system, co-exist with micelles of a composition given by R_e^{SOL}. In the experiment described in Fig. 1 *B*, residual vesicles of a diameter of 74 nm probably exist in the solution at 6 mM cholate even in the absence of PEG. The contribution of these vesicles to the observed turbidity is small. Yet they make a significant contribution to the hydrodynamic diameter, as measured by QLS, yielding an apparent mean diameter of 39 nm. In the presence of PEG, vesicles of a mean diameter of 388 nm that co-exist with micelles contribute to the turbidity and have a more pronounced contribution to the apparent size, which in this case is 215 nm.

Aggregation and fusion of vesicles of R_e = 0.3

The large vesicles formed in a sample containing 20 mM PC, 9.5 mM cholate (R_e = 0.3) and either 2.5 wt % 20-kDa PEG or 3.5 wt % 70-kDa dextran were studied by light microscopy. Micrographs of undiluted samples (Fig. 2, *A* and *B*) revealed aggregates of varying sizes and only few separated large vesicles. A 10-fold dilution caused essentially complete decomposition of the clusters. Yet the vesicles were much larger than those made in the absence of polymer (Fig. 2, *C* and *D*). The mean diameter of these vesicles, as measured by QLS, was 400 nm, as compared with 70 nm in the absence of polymer. The individual size, as estimated from enlarged micrographs, was larger, indi-

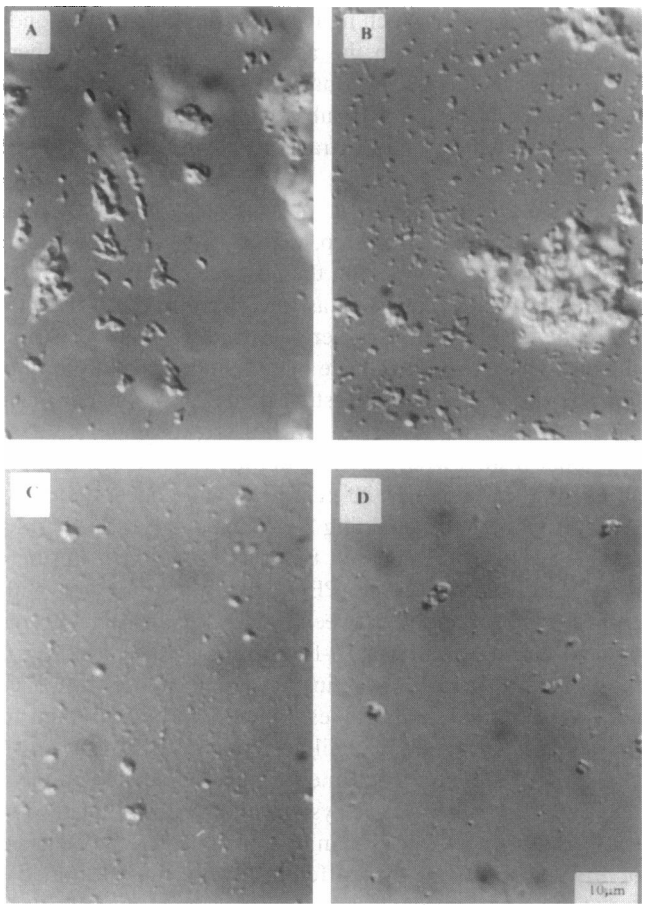

FIGURE 2 Micrographs of PC vesicles. The vesicles were prepared by mixing PC SUVs (20 mM) with cholate (9.5 mM) in the presence of 20-kDa PEG (2.5 wt %; *A* and *C*) or 70-kDa dextran (3.5 wt %; *B* and *D*) and subsequently dialyzing the cholate as described in Materials and Methods. The bar represents 10 μm. (*A* and *B*) Micrographs of undiluted sample. (*C* and *D*) Micrographs of a diluted sample (10-fold in buffer A).

cating that the vesicles are heterogeneous with respect to their sizes as the smaller vesicles cannot be observed microscopically. As dynamic light scattering is always biased toward the larger particles present in the dispersions, it must be concluded that only a minor fraction of the lipid is contained in particles that are sufficiently large to be observed by light microscopy. Given the limit of detection and the qualitative nature of the optical microscopy, it can only be concluded that most of the lipid is contained in vesicles of diameters of approximately 400 nm, as determined by QLS (which is also somewhat biased toward the large particles).

Two resonances appeared in the ^{31}P-NMR spectrum of the vesicles after the addition of $PrCl_3$ to the dispersion, due to downshift of the signal of the head groups of the outer monolayers (not shown). The out/in ratio of integrated intensities was 0.665, indicating that the average number of lamellae in the vesicles (n) is 1.25 (out/in = $1/[2(n - 1) + 1]$). Hence, most but not all of the phospholipid molecules are aggregated as unilamellar vesicles.

The large cholate-containing vesicles present in a system of R_e = 0.3 (1.6 mM PC and 4 mM cholate) made by dilution of a mixed micellar solution in buffer A containing 20-kDa PEG (2.5–8 wt %) became solubilized by increasing the cholate concentration to 6 mM. However, the rate of solubilization depended on the PEG concentration; in a mixture containing 2.5 wt % PEG, the solubilization appeared to be instantaneous as in less than 30 s the turbidity decreased from 1.218 to 0.082 OD units (as given in Table 1 for undiluted samples). Similar results were obtained in the presence of 3 wt % PEG. By contrast, at higher PEG concentration, the solubilization was much slower and its rate appeared to decrease upon increasing the concentration of PEG (from 4 to 8 wt %; Table 1, undiluted). Previous reports have demonstrated that solubilization of unilamellar

TABLE 1 Time dependence of the solubilization of cholate-containing vesicles

	Turbidity (OD units at 360nm)									
	Undiluted					Diluted				
% PEG	0	30 s	30 min	150 min	48 h	0	30 s	30 min	150 min	48 h
2.5	1.218	0.082 (6.7)	0.101 (8.2)	0.101 (8.2)	0.063 (5.1)	0.663	0.015 (2.3)	0.006 (0.9)	0 (0)	0.014 (2.1)
3	1.171	0.140 (11.9)	0.138 (11.8)	0.156 (13.3)	0.043 (3.6)	0.798	0.055 (6.9)	0.048 (6)	0.025 (3.1)	0.027 (3.4)
4	1.44	0.75 (52.1)	0.605 (42)	0.510 (35.4)	0.092 (6.3)	0.788	0.171 (21.7)	0.163 (20.6)	0.145 (18.4)	0.037 (4.7)
5	1.4	1.37 (97.8)	1.27 (90.7)	1.059 (75.6)	0.386 (27.6)	0.764	0.203 (26.6)	0.177 (23.1)	0.143 (18.7)	0.070 (9.1)
8	1.3	1.2 (92.3)	1.2 (92.3)	1.182 (90.9)	1.003 (77.1)	0.717	0.42 (58.6)	0.415 (57.8)	0.391 (54.5)	0.074 (10.3)

Cholate-containing vesicles of R_e = 0.3 (1.6 mM PC and 4 mM cholate) were incubated for 24 h in the presence of 2.5-8 wt % 20-kDa PEG. Subsequently, part of each sample was diluted 10-fold (which results in deaggregation of vesicle clusters). The vesicles present in each of the samples were solubilized by cholate addition to R_e = 1.1. As $R_e = (D_t - D_w^\circ)/L$ (where D_w°, the concentration of monomeric cholate at R_e = 1.1, equals 4.22 mM), it follows that, to obtain the required R_e, 2 mM cholate had to be added to the undiluted samples (R_e = (6 − 4.22)/1.6 = 1.1), whereas for the diluted samples, 4 mM cholate was used (R_e = (4.4 − 4.22)/0.16 = 1.1). The turbidity at time zero is that of the studied samples, as measured before cholate addition. Note that the 10-fold diluted samples have higher molar turbidities than the undiluted samples, reflecting multiple scattering in the latter samples. Numbers in parentheses depict the turbidity at the various times after cholate addition as the percentage of the corresponding initial (time zero) turbidity.

vesicles (at $R_e = R_e^{SOL}$) is very rapid whereas solubilization of multilamellar vesicles involves sequential peeling off of bilayers and is consequently much slower (Lichtenberg et al., 1979; Parteraroyo et al., 1992). It therefore appears that at PEG concentrations below 3 wt % the vesicles formed were predominantly, if not exclusively, unilamellar, whereas at higher PEG concentrations, multilamellar vesicles were formed, similar to these obtained for PEG-induced vesicle size growth observed at much higher PEG concentrations in the absence of cholate (Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982; MacDonald, 1985).

As solubilization of large unilamellar vesicles by externally added cholate may be slowed by their clustering, which depends on the concentration of PEG, we have also studied vesicle solubilization after 10-fold dilution of the turbid dispersions made at R_e^{SAT}. Such dilution is sufficient to cause deaggregation of vesicle clusters but is not likely to affect the lamellarity of the vesicle. Upon exposure of the diluted sample to a solubilizing concentration of cholate (4 mM), solubilization of the diluted system containing 4 wt % PEG or more was relatively slow (Table 1; diluted samples), supporting the conclusion that vesicles formed at high PEG concentration were indeed multilamellar.

As for any other cholate/PC molar ratio within the vesicular range, increasing the polymer concentration in the diluting medium resulted in increased turbidity only when the concentration of the polymer exceeded a threshold value C_A. For 20-kDa PEG, C_A was merely 1 wt % (Fig. 3 *A*) as compared with 4.5 wt % of the isoosmotically equivalent 40-kDa dextran (MacDonald, 1985) (Fig. 3 *B*). Upon 10-fold dilution, the turbidity remained relatively high, indicating that vesicle size growth (as opposed to merely cluster formation) contributed to the observed increase of turbidity. Fig. 3 *C* presents the diameter of vesicles of $R_e = 0.3$ in the presence of 40-kDa dextran and 70-kDa dextran, as measured by QLS after a 30-fold dilution.

As for dextran-induced aggregation in the absence of cholate, the value of C_A observed in the cholate-containing mixtures was a decreasing function of the molecular weight of the polymer. As an example, for dextran of molecular weight of 70 kDa, C_A was equal to 3 wt %, as compared with the value of C_A = 4.5 wt % observed for 40-kDa dextran. Notably, in the presence of cholate in the vesicles ($R_e = 0.3$), vesicle aggregation and size growth began at the same polymer threshold concentration, as obvious from the turbidities of the diluted and undiluted mixtures (Fig. 3).

To determine whether polymer-induced increase in viscosity plays a role in the vesicle size growth, we have measured the size of vesicles of $R_e = 0.3$ as a function of the viscosity of dextran solutions of varying molecular weights and concentrations. The results of these experiments show that in solutions of viscosity lower than 1.5 cp the vesicles were of an average diameter of 100 ± 20 nm, whereas in most solutions of viscosities higher than 1.5 cp the resultant vesicles were of an apparent diameter of 500 ± 150 nm (not shown). However, in the viscous solution (2 cp) made with 1.5 wt % dextran (2 M), vesicles of merely 50-nm diameter were obtained, indicating that the viscosity cannot be regarded as the sole determinant of vesicle size.

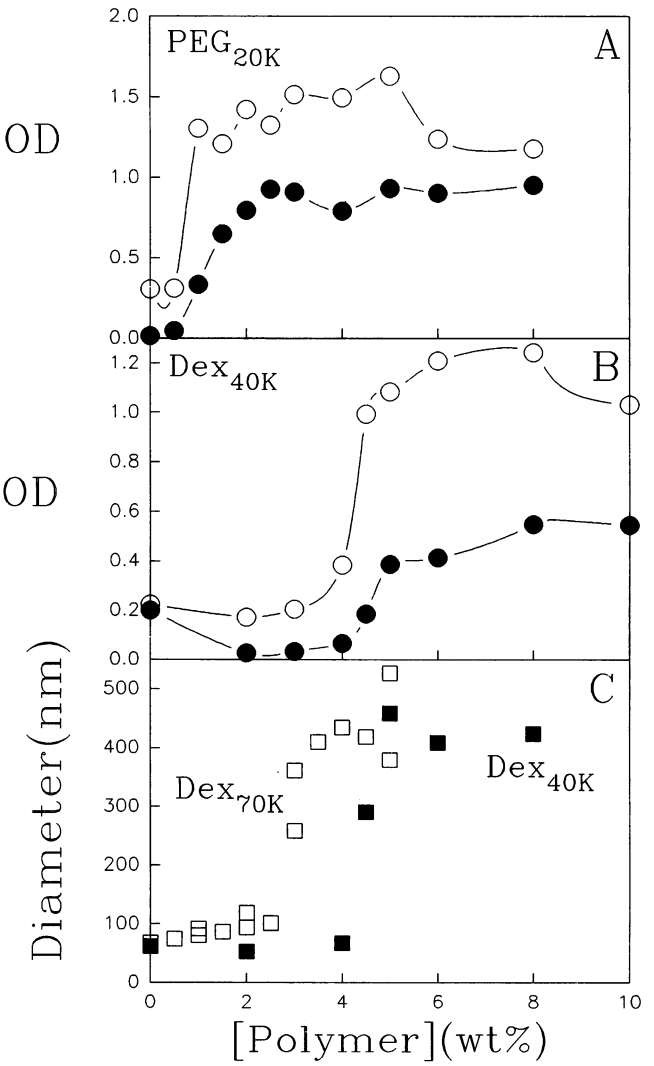

FIGURE 3 The dependence of the steady-state turbidity and hydrodynamic diameter of cholate-containing vesicles (R_e = 0.3) on polymer concentration. Dispersions of 2 mM PC were made by a 2-fold dilution of a mixed micellar system of 4 mM PC and 9 mM cholate in buffer A containing varying concentrations of (*A*) 20-kDa PEG (PEG_{20K}) or (*B*) 40-kDa dextran (Dex_{40K}). The turbidity of the samples as is (○) or after 10-fold dilution (●) was measured after 24 h. The relatively small effect of dilution on the OD can be attributed to the multiple scattering in the undiluted samples (see also Fig. 1). (*C*) Depicts the hydrodynamic diameter of the vesicles prepared in the presence of 70-kDa dextran (Dex_{70K}; □) or 40-kDa dextran (■) as measured by QLS after 30-fold dilution in buffer A.

Dependence of aggregation and size growth on vesicle composition

The aggregation and size growth of vesicles in the presence of 20-kDa PEG was studied as a function of the vesicle composition (expressed in terms of the effective ratio R_e). At any given R_e within the range of R_e = 0.18–0.3, the dependence of turbidity of diluted samples on PEG concen-

tration was parallel to that of the undiluted samples (Fig. 4), indicating that within this range of R_e values aggregated vesicles underwent relatively rapid size growth.

At lower R_e values, reversible aggregation required lower polymer concentrations than irreversible size growth. As an example, at R_e = 0.1, aggregation occurred at 4 wt % PEG whereas size growth occurred only at 6 wt %. By comparison, in the absence of cholate, aggregation required merely 1 wt %, but size growth required 25 wt %. Hence, relatively low cholate content decreased the tendency of the vesicles to undergo aggregation, probably due to electrostatic repulsion between the negatively charged vesicles (Yamazaki et al., 1989), but increased the tendency of aggregated vesicles to fuse into large ones. Only at much higher R_e values (R_e = 0.3) did the turbidity increase occur at PEG concentrations similar to those required in the absence of cholate, but unlike in the absence of cholate, the aggregated vesicles fused. In other words, under these conditions, the irreversible polymer-induced increase of turbidity can be attributed to vesicle fusion. The same trend was observed in the presence of dextran (not shown).

Kinetic studies of aggregation and size growth

To further explore the mechanism of dextran-induced aggregation and size growth, we have examined the kinetics of the turbidity increase observed at varying concentrations of PC and 70-kDa dextran at two different R_e values. A solution containing SUVs was diluted to a final PC concentration varying from 0.1 to 8 mM in media containing varying cholate and dextran concentrations (3–10 wt %). Two series of experiments were carried out. In one of these series, the cholate concentrations were chosen to yield vesicles of R_e = 0.3, and in the other series R_e was kept constant at R_e = 0.22. In both experiments, the polymer-induced aggregation and/or size growth was followed by monitoring the turbidity of the samples as a function of time. As dilution of the dispersions results in deaggregation of vesicle clusters but does not affect the size of individual vesicles, the time course of size growth was also followed by measuring the turbidity of diluted aliquots of the same solutions as a function of time. Fig. 5 presents the time dependencies of turbidity as measured continuously (lines) as well as after dilution (points). These results show that, first, increasing the dextran concentration from 5 wt % (Fig. 5 *A*) to 10 wt % (Fig. 5 *B*) enhanced the maximal rate of turbidity increase (V_{max}) and eliminated the apparent latency before the increase of turbidity both in the continuously measured samples and in the diluted aliquots. On the other hand, the extent of aggregation and size growth (as evaluated from the

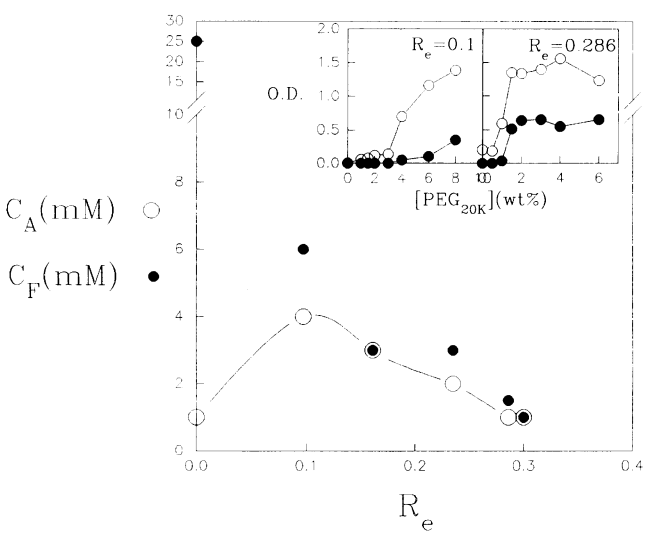

FIGURE 4 Dependence of threshold concentration of 20-kDa PEG for aggregation and for size growth on the affective ratio R_e. Dispersions of 1.85 mM PC and varying cholate concentrations (1.45–4.45 mM) of final R_e values of 0.1–0.3 were made by dilution of dispersion of 40 mM PC in buffer A containing the appropriate concentrations of cholate and varying concentrations of 20-kDa PEG. The turbidity of the samples as is (○) or after 10-fold dilution (●) was measured after 24 h (*inset*). The value of the threshold PEG concentration required for aggregation (○) or for size growth (●) is presented as the function of the R_e value.

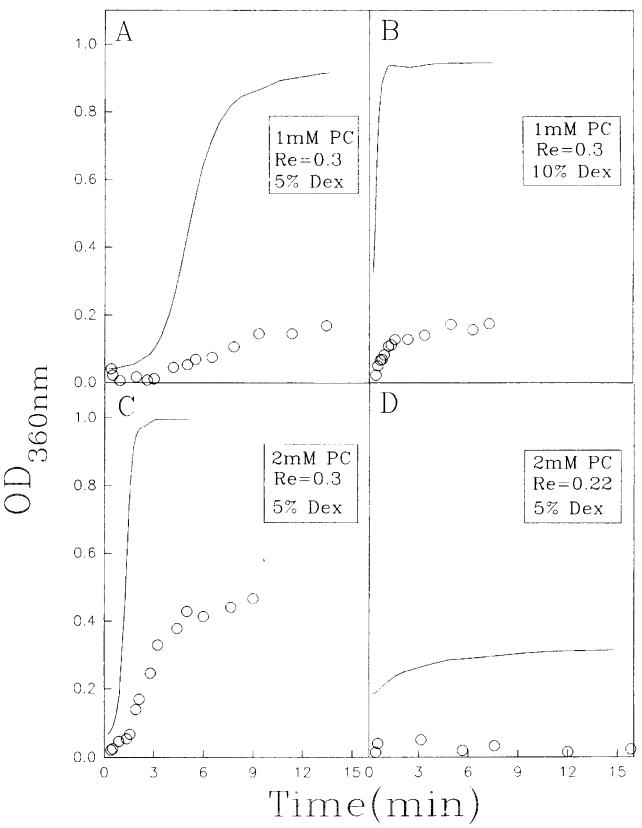

FIGURE 5 Kinetics profiles of turbidity increase of cholate-containing vesicles in the presence of 70-kDa dextran. Dispersions of 1 or 2 mM PC were made by 20- to 40-fold dilution of 40 mM SUVs in buffer A containing the appropriate cholate concentration to final R_e values of 0.3 or 0.22 and 5 wt % or 10 wt % 70-kDa dextran. The turbidity was measured continuously (——) or after 10-fold dilution (○). Note that in *C* 10-fold dilution resulted in a relatively small decrease of OD, as in Figs. 1 and 3. The final PC concentration, R_e value, and the dextran concentration are indicated in the figure.

steady-state turbidity of diluted and undiluted systems, respectively) were not affected by the increase of dextran concentration. Second, increasing the concentration of PC from 1 mM (Fig. 5 *A*) to 2 mM (Fig. 5 *C*) had qualitatively similar effects on both the latency and the maximal rate to those obtained upon increasing the dextran concentration. Third, the effect of R_e on the rate of vesicle aggregation and size growth at constant PC and dextran concentrations is complex. Exposure of cholate-containing vesicles of R_e = 0.22 (at PC concentration of 2 mM) to a 5 wt % dextran solution resulted in a rapid but relatively small increase in the turbidity of the dispersion (Fig. 5 *D*). When diluted, at any time point during the first 15 min after dextran addition, the turbidity was similar to that of the initial vesicle system, indicating that the polymer did not induce irreversible size growth. Similar turbidity was also observed when measured after dilution after 24 h of incubation in the presence of 5 wt % dextran (not shown). The vesicles present in the latter dispersion had a mean diameter of approximately 100 nm, i.e., only somewhat larger than those formed in the absence of dextran. By contrast, under the same experimental conditions, vesicles of R_e = 0.3 aggregated and grew in size in less than 15 min (Fig. 5 *C*) into vesicles of a mean size of approximately 500 nm, as measured by QLS after a 30-fold dilution. Thus, increasing R_e (from 0.22 to 0.3) increased the extent of the overall process of aggregation and size growth but reduced the initial rate of aggregation (note the lag in Fig. 5 *C*), probably due to increased electrostatic repulsion. In other words, 5 wt % 70-kDa dextran was sufficient to induce rapid but limited and reversible aggregation of vesicles containing 18% cholate (R_e = 0.22) whereas higher polymer concentrations were required to induce growth of those vesicles. When mixed with increasing dextran concentrations, the size of the vesicles of this composition (R_e = 0.22), as measured by QLS after a 30-fold dilution, increased monotonically with 70-kDa dextran concentration from 180 nm at 7 wt % to 360 nm at 8 wt % and 600 nm at 10 wt % dextran (not shown).

The latency τ, which in many cases preceded the increase in turbidity, depended on R_e as well as on both the PC and dextran concentrations. Fig. 6 *A* presents this latency (τ), as measured for both aggregation and size growth, as functions of the concentration of dextran at a constant PC concentration of 2 mM at two different R_e values of 0.3 and 0.22. As evident from this figure, increasing the dextran concentration enhanced the rate of both aggregation and size growth at R_e = 0.3 and of the size growth of vesicles of R_e = 0.22. The aggregation of (the less charged) vesicles of R_e = 0.22 was not preceded by an observable latency at any dextran concentration of 5 wt % or higher. For vesicles of R_e = 0.3, increasing the concentrations of dextran from 3 to 7 wt % shortened the latency before size growth from more than 200 s to merely 50 s.

Fig. 6 *B* presents the dependence of the latency τ on the concentration of PC for both aggregation and size growth of vesicles of R_e = 0.3 at a constant dextran concentration of 5 wt %. These dependencies show that increasing the PC

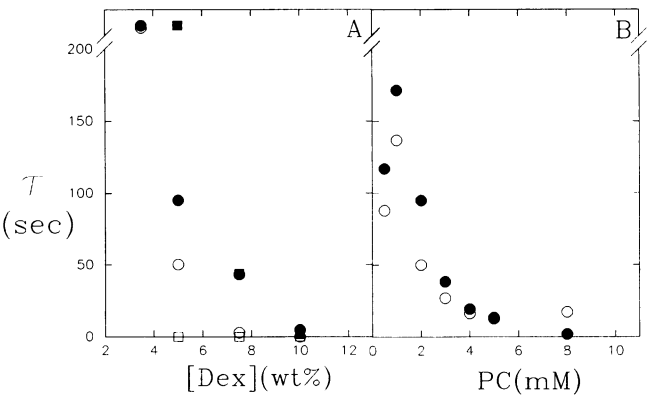

FIGURE 6 Dependence of the latency on dextran and PC concentrations. Kinetics profiles, as those presented in Fig. 5, were analyzed in terms of the latency τ. The latency is presented as a function of the dextran concentration (wt %; *A*) or as a function of the PC concentration (*B*). The final R_e value was 0.22 (□) or 0.3 (○). The open symbols represent aggregation profiles (without dilution); the solid symbols represent size-growth profiles (after 10-fold dilution).

concentration promotes aggregation and size growth, decreasing the latency before both of these processes. Noticeably, the latency before aggregation was, in all cases, shorter than the latency before size growth, indicating that aggregation occurred before size growth. It thus appears that vesicles first aggregate to form clusters and only when the cholate content of the bilayers is sufficiently large do the vesicles fuse into larger vesicles. Increasing the concentrations of either PC or dextran promote both of these steps.

Dialysis experiments

In an attempt to evaluate the role of depletion forces in polymer-induced aggregation and size growth, we have carried out dialysis experiments in which dialysis bags containing PC-SUV (2 mM) were immersed in a fivefold excess of a buffered medium containing cholate (4.5 mM). After 24 h of equilibration at room temperature, the cholate-containing vesicles of an effective ratio R_e = 0.3 had a mean hydrodynamic diameter of 75 nm (as measured by QLS). Polymer was then added to the external medium and the system was incubated for additional 24 h. Results of two representative experiments carried out with 12 and 16.8 wt % 6-kDa PEG are presented in Table 2. In both of these experiments, the volume within the bag was reduced during dialysis due to osmotically driven water flow out of the bag. As the dialysis bag is permeable to cholate, the shrinkage of the bag is accompanied by re-equilibration of the cholate so that the concentration of cholate monomers in water (D_w) in both compartments remain equal. For any given R_e, the partitioning of cholate between the vesicle bilayers and aqueous medium is given by a constant $K = D_b/[(L + D_b)D_w]$ (Lichtenberg, 1985). This means that $K = R_e/[(1 + R_e)D_w]$. Hence, as D_w does not change, R_e does not change either. In fact, when the medium contained 16.8 wt % PEG, the volume decreased to merely 15% of the original volume.

TABLE 2 Vesicle size growth in dialysis experiments

Initial composition					Final composition (after dialysis)			
Bag		External medium			Bag			
Volume	PC (mM)	Volume	Cholate (mM)	PEG (wt %)	Volume	PC (mM)	R_e	Diameter (nm)*
4	2.0	20	4.5	–	3.7	2.16	0.3	75
4	2.0	20	4.5	12	2.2	3.64	0.3	73
4	2.0	20	4.5	16.8	0.6	13.33	0.3	70

*Measured by QLS after a 10-fold dilution.

Yet, despite the consequent sevenfold increase of the PC concentration in the bag, the equilibrated system contained vesicles of mean diameter of 70 nm (as measured by QLS). This result indicates that direct interaction between the vesicles and the polymer are important in inducing size growth of cholate-containing vesicles.

DISCUSSION

Three major conclusions can be drawn from this work. First, both dextran and PEG have only minor effects (if any) on the phase behavior of PC/cholate mixtures (e.g., Fig. 1). Second, the negatively charged bile salt sodium cholate reduces the tendency of phospholipid vesicles to undergo polymer-induced aggregation. This is evident from both the increased threshold in polymer concentration (C_A) required for aggregation of vesicles with up to approximately 10 mol % cholate (e.g., Fig. 4) and from the much slower rate of turbidity increase observed for cholate-containing vesicles (Figs. 5 and 6) in comparison with the rate observed in the absence of cholate (Fig. 5 in Meyuhas et al., 1996). Third, by contrast, the tendency of cholate-containing vesicles to undergo polymer-induced size growth is much higher than that of pure PC vesicles. In fact, in the absence of cholate in the vesicles, dextran of any molecular weight induces only reversible aggregation, whereas in the presence of approximately 25 mol % cholate in the bilayer (R_e = 0.3), size growth occurs at any 70-kDa dextran concentration above 3.5 wt % (Fig. 3). Furthermore, 20-kDa PEG was reported to induce size growth of PC vesicles only when its concentration was higher than 25 wt % (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982; Meyuhas et al., 1996), whereas cholate-containing vesicles of R_e = 0.3 undergo size growth when the concentration of PEG of the same molecular weight is as low as 1 wt % (at R_e = **0.3**).

In interpreting these results, it should first be recalled that in the absence of cholate the threshold concentration above which vesicle aggregation occurred rapidly in polymer/vesicle mixtures coincided with the overlapping concentration of the polymer (C^*). This result was taken as evidence for the importance of depletion forces, which are likely to increase at C^* and function to separate vesicle clusters from the overlapping polymer in the solution (Meyuhas et al., 1996). The same forces play a similar role in the aggregation (and size growth) of cholate-containing vesicles. Hence, when the vesicles were separated from the polymer solution by a dialysis membrane, they neither aggregated nor grew even when the vesicle dispersions became very concentrated due to the difference in osmolarity between the interior of the dialysis bag and the external polymer solution and despite the consequent deformation of vesicles, which can be expected when the vesicles lose most of their entrapped aqueous volume.

In direct mixing experiments and moderate cholate concentrations (e.g., at R_e = 0.1), electrostatic repulsion between the negatively charged cholate-containing vesicles reduces the tendency of the vesicles to flocculate. As a consequence, the threshold polymer concentration required for aggregation of the vesicles was higher than C^*. Hence, overlapping of polymer molecules, at C^*, is not sufficient to induce clustering of these vesicles, and aggregation occurs only when much stronger forces are applied (e.g., due to much higher polymer concentrations). Furthermore, when the polymer concentrations were elevated to values above the new (higher) threshold, aggregation was very slow in comparison with that observed for vesicles without cholate and is often preceded by an apparent latency (Fig. 5, *A* and *C*). To explain such a time course, it is important to note that, unlike in the pure PC vesicles, in cholate-containing vesicles, a barrier to close approach of vesicles is likely to result from a balance between attractive forces (mostly depletion forces) and repulsive interactions (mostly electrostatic repulsion). At any given PC concentration, increasing the cholate content (hence the charge density) elevates this barrier whereas increasing the polymer concentration reduces it, thus shortening the apparent lag, until, at sufficiently high polymer concentrations, aggregation becomes very rapid and the kinetic profiles lose their sigmoidal characteristics. The sigmoidal nature of the kinetics observed under most conditions can be taken as an indication that vesicle aggregation induces further aggregation. The probable cause of this induction of further aggregation can be related to depletion forces, which are the major driving force for vesicle aggregation. The detailed mechanism responsible for this phenomenon is not clear. One possibility is that aggregation of vesicles not only reduces the polymer concentration in the immediate gap between the membranes of the aggregated vesicles but also extends to volume elements in the vicinity of surfaces that are still available for

interaction with additional vesicles. Hence, aggregation of vesicles can result in increased efficacy of depletion forces in promoting further aggregation.

In contrast to the effect of cholate on the tendency of the vesicles to aggregate, the tendency of aggregated vesicles to fuse increases markedly in the presence of cholate. As an example, large PC vesicles, of sizes similar to those of PC/cholate systems of $R_e = 0.3$, do not undergo irreversible size growth at polymer concentrations lower than 20 wt % 20-kDa PEG (MacDonald, 1985). Contradistinctively, irreversible size growth occurs when vesicles of $R_e = 0.3$ are mixed with 20-kDa PEG at much lower concentrations (1 wt %; Fig. 4).

The effect of cholate on the fusion of aggregated vesicles depends on the cholate concentration within the vesicle bilayers. Thus, at $R_e = 0.1$, aggregation requires 4 wt % of 20-kDa PEG (as compared with 1 wt % in pure PC vesicles) whereas irreversible size growth requires higher polymer concentration ($C_F = 8$ wt %, as compared with $C_F = 25$ wt % in the absence of cholate). For vesicles with higher cholate content, aggregation always results in irreversible size growth. For vesicles of $R_e = 0.18$, $C_A = C_F = 3$ wt % (as compared with $C_A = 1$ wt % and $C_F = 25$ wt % observed in the absence of cholate). Further increase of R_e results in a greater tendency of the vesicles to undergo size growth. As a consequence, the vesicles undergo irreversible size growth at 1 wt % 20-kDa PEG. This, despite the reduced tendency of the vesicles to aggregate, is expected in view of the larger electrostatic repulsion between them. The mechanism involved in size growth of aggregated vesicles depends upon the actual forces responsible for the size growth. When a relatively moderate force is applied (at relatively low polymer concentrations), the resultant vesicles are essentially unilamellar and the mechanism may be similar to that involved in the fusion of gel-phase (deformed) SUVs (Lichtenberg and Thompson, 1991). At higher osmotic forces, the vesicles shrink rapidly and the whole aggregate of vesicles fuses into a large multilamellar vesicle. Such liposomes were produced only when pure PC vesicles were subjected to the very high osmotic stress upon mixing with high concentrations of PEG (>25 wt %; Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982; MacDonald, 1985).

In conclusion, the combined effects of polymer-induced dehydration of the surface of vesicles, depletion forces, and membrane deformation is sufficient to result in vesicle size growth of PC vesicles only at very high polymer concentrations, if at all. Cholate-containing vesicles, on the other hand, undergo irreversible size growth at much lower polymer concentrations, depending on the cholate concentration. Such interactions occur only when the polymer is directly mixed with the vesicles, probably because depletion forces play a central role in inducing close approach of the vesicles. The occurrence of fusion of aggregated cholate-containing vesicles at relatively low polymer concentrations is consistent with the idea that close approach is sufficient to induce size growth of vesicle bilayers only when sufficiently large fluctuations occur in the membranes. Such fluctuations may be rate limiting in the size growth of vesicles with moderate cholate content (e.g., $R_e = 0.1$), but at higher R_e values they may result in instantaneous fusion of aggregated vesicles. The cholate content required for rapid size growth in the presence of polymers is only slightly smaller than that required for the induction of instantaneous activation of phospholipase-A_2-catalyzed hydrolysis of the phospholipids. The cholate content of the vesicles at which this hydrolysis becomes instantaneous has been interpreted as a percolation point at which the structural fluctuations in the membrane become very large (Gheriani-Gruszka et al., 1988). The increase in the vesicle's tendency to fuse probably results from similar, cholate-induced, structural and dynamic modifications.

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