

## Aggregation of Phospholipid Vesicles by Water-Soluble Polymers

D. Meyuhas,\* S. Nir,# and D. Lichtenberg\*

\*Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv 69978, Israel, and #Seagram Center for Soil and Water Sciences, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

**ABSTRACT** Water-soluble polymers such as dextran and polyethylene glycol are known to induce aggregation and size growth of phospholipid vesicles. The present study addresses the dependence of these processes on vesicle size and concentration, polymer molecular weight, temperature, and compartmentalization of the vesicles and polymers, using static and dynamic light scattering. Increasing the molecular weight of the polymers resulted in a reduction of the concentration of polymer needed for induction of aggregation of small unilamellar vesicles. The aggregation was fully reversible (by dilution), within a few seconds, up to a polymer concentration of at least 20 wt %. At relatively low phosphatidylcholine (PC) concentrations (up to ~1 mM), increasing the PC concentration resulted in faster kinetics of aggregation and reduced the threshold concentration of polymer required for rapid aggregation ( $C_A$ ). At higher PC concentrations,  $C_A$  was only slightly dependent on the concentration of PC and was approximately equal to the overlapping concentration of the polymer ( $C^*$ ). The extent of aggregation was similar at 37 and 4°C. Aggregation of large unilamellar vesicles required a lower polymer concentration, probably because aggregation occurs in a secondary minimum (without surface contact). In contrast to experiments in which the polymers were added directly to the vesicles, dialysis of the vesicles against polymer-containing solutions did not induce aggregation. Based on this result, it appears that exclusion of polymer from the hydration sphere of vesicles and the consequent depletion of polymer molecules from clusters of aggregated vesicles play the central role in the induction of reversible vesicle aggregation. The results of all the other experiments are consistent with this conclusion.

### INTRODUCTION

Polyethylene glycol (PEG) is a commonly used fusogen (Kao and Michayluk, 1974; Davidson and Gerald, 1977). It is known that, during cell fusion processes induced by PEG, patches of plasma membrane are bared of intramembrane particles and fusion occurs between protein-free lipid bilayers (Robinson et al., 1979; Knutton, 1979; Krahling, 1981; Wojcieszyn et al., 1983). The interactions of PEG with pure lipid vesicles can therefore be relevant to PEG-induced cell fusion processes.

At relatively low concentrations, PEG induces reversible aggregation of phosphatidylcholine (PC) vesicles without binding to the vesicle surface (Arnold et al., 1990). The induction of aggregation of PC vesicles occurs only when the concentration of PEG exceeds a threshold value  $C_A$  (concentration of polymer required to induce aggregation), which is a decreasing function of the molecular weight of the polymer (Yamazaki et al., 1989; Viguera et al., 1995; Tilcock and Fisher, 1982). This dependence has been previously explained as being due to two different effects of the polymer, namely, 1) dehydration of the vesicle surface due to water binding to the polymer, which increases with the

molecular weight of the polymer (Tilcock and Fisher, 1982) and 2) exclusion of PEG from the hydration sphere of the vesicles, which causes an osmotic stress due to osmolarity difference between the hydration sphere and the bulk water (Yamazaki et al., 1989).

The aggregation is also dependent on the PC concentration. For PEG of molecular weight of 6 kDa and any PC concentration above 2 mM,  $C_A$  is approximately 1.5 wt %, whereas at lower concentrations of PC,  $C_A$  increases upon decreasing the PC concentration, approaching the value of  $C_A = 4.5$  wt % at PC concentration of 0.2 mM (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; Yamazaki et al., 1989).

Dextran-induced aggregation exhibits a similar dependence of  $C_A$  on the molecular weight of the polymer (Suzumoto et al., 1980; Schachter, 1978), although this polymer is likely to have a smaller effect on the vesicle hydration sphere as it binds to the vesicles (Minetti et al., 1979; Evans and Metcalfe, 1984; Massenbourg and Lentz, 1993). When polymer solutions of similar osmotic activities were compared, e.g., 20-kDa PEG and 40-kDa dextran (MacDonald, 1985), vesicle aggregation in the dextran solutions required significantly higher polymer concentrations. As an example, for 20-kDa PEG,  $C_A = 1.5$  wt %, whereas for 40-kDa dextran  $C_A = 8$  wt %. Notably, for various dextrans,  $C_A$  was reported to depend only slightly on the molecular mass (e.g., for 40-kDa dextran, the turbidity approaches one-half of its maximal value at 6.4 wt %, whereas for 500-kDa dextran,  $C_A = 4.5$  wt %; Schachter, 1978).

Dehydration of vesicles by water-soluble polymers also affects the packing of phospholipids within the bilayers, the effect of dextran being smaller than that of PEG (Lehtonen

Received for publication 20 February 1996 and in final form 27 August 1996.

This work constitutes a part of the Ph.D. Thesis of Dina Meyuhas.

Address reprint requests to Dr. Dov Lichtenberg, Department of Physiology/Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv 69978, Israel. Tel.: 972-3-6407305; Fax: 972-3-6409113; E-mail: physisdov@ccsg.tau.ac.il.

© 1996 by the Biophysical Society

0006-3495/96/11/2602/11 \$2.00

and Kinnunen, 1994). This effect, as well as the influence of PEG on lipid transfer between bilayers and the leakage of water-soluble substances entrapped within the vesicles, may of course be related to vesicle aggregation (Wu and Lentz, 1991). However, the PEG concentrations at which these processes were observed are higher than  $C_A$ , as evaluated from the polymer-induced increase of turbidity.

Furthermore, whenever irreversible size growth of the vesicles occurs, it requires much higher PEG concentrations (Burgess et al., 1991, 1992; Massenbourg and Lentz, 1993; Lehtonen and Kinnunen, 1994). For 6-kDa PEG and PC vesicles, the latter process occurs only above 25 wt %, yielding large and oligo- or multilamellar vesicles (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982; MacDonald, 1985). The mechanism of this size growth is not completely clear. Several authors referred to it as being fusion (Hui and Boni, 1991; Boni et al., 1981, 1984); several others have presented data that indicate a lipid-mixing mechanism (Tilcock and Fisher, 1982; Hui and Boni, 1991; MacDonald, 1985). PEG-induced size growth is not due to direct binding of PEG to the PC, as shown in an experiment in which size growth was observed either by direct mixing of vesicles and PEG or mixing across a dialysis membrane (MacDonald, 1985). In fact, when the vesicles were dialyzed against an equi-osmolar solution of dextran, similar size growth occurred, despite the lack of such a process in mixtures of small unilamellar vesicle (SUV) and dextran solutions of the same composition. Notably, possible aggregation of vesicles in these compartmentalized systems was not investigated.

The current study is devoted to vesicle aggregation induced by dextran and PEG over a wide range of molecular weights and concentrations of the polymers as a function of the temperature and of the size and concentration of the vesicles. Comparison of the results of direct mixing experiments with those obtained in compartmentalized systems demonstrates the important role of polymer exclusion in the induction of reversible vesicle aggregation. The dependence of aggregation on vesicles and polymers and on temperature are consistent with this conclusion.

## MATERIALS AND METHODS

### Materials

Egg PC and dextrans of molecular weights of 10,000 (lot 35F0867), 20,000 (lot 20B0940), 40,000 (lot 52H0358), and 2,000,000 (lot 115F0236 or 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 788) 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).

### Preparation of egg PC SUV

Egg PC dissolved in  $\text{CHCl}_3$  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%  $\text{NaN}_3$ , and 10 mM Tris, pH 7.4) to form multila-

mellar vesicles (Bangham et al., 1967). This suspension was sonicated for 20 min using a probe sonicator (XL-2020 Heat System Inc., Sarmingdale, NY) as previously described (Huang, 1969). The average diameter of the resultant vesicles, as measured by quasi-elastic light scattering (QLS), was  $35 \pm 5$  nm. PC concentration was determined chemically (Dittmer and Wells, 1969).

### Preparation of egg PC large unilamellar vesicles (LUVs)

SUVs (8 mM egg PC) were mixed with 6.3 mM cholate to a final effective ratio (the ratio of cholate to PC in the vesicles) of  $R_e = 0.3$ ;  $R_c = (D_t - D_w)/L$ , where  $L$  denotes the lipid concentration,  $D_t$  denotes the total detergent concentration, and  $D_w$  is the concentration of detergent monomers (Lichtenberg, 1993). After incubation for 2 h, the cholate-containing vesicles were dialyzed four times against 500 ml of buffer A for 24 h to remove residual cholate. The average diameter of the resultant vesicles, as measured by QLS, was 80–92 nm.

### Mixing protocol

Vesicle dispersions (40 mM) were diluted in 10- to 400-fold larger volumes of polymer-containing solutions of the appropriate polymer concentration.

### Dialysis experiments

Dialysis bags (Spectra/Por membrane molecular weight cut-off (MWCO): 6000–8000 Spectrum Medical Industries, Inc., Houston, TX) containing SUV dispersions (2 mM) were immersed in solutions of fivefold larger volume containing different polymer concentrations. After 24 h of incubation, the vesicle dispersions within the dialysis bag were studied using two different protocols. In studying the aggregation induced by moderate polymer concentrations (up to 12 wt % dextran or 20 wt % PEG), 1.5 ml of the dialyzed solutions were transferred into cuvettes and the turbidity was measured at 360 nm. The turbidity was then expressed as molar turbidity (OD/M, as corrected for the dialysis-induced shrinkage). In several experiments, the turbidity inside the dialysis bag was also measured using a probe colorimeter (PC 801 colorimeter; SYBRON Brinkmann, Westbury, NY) at 470 nm, both while the bag was immersed in the polymer solution and after removing the bag from the polymer solution. In other experiments (carried out with higher polymer concentrations), the solution contained in the dialysis bag was diluted at least 30-fold in the medium used in the experiment (after dialysis and just before QLS measurement).

### Turbidity measurements

Turbidity was measured at 360 nm, using a Shimadzu UV-VIS (UV-160) spectrophotometer, against a reference of buffer A. In control experiments, the turbidities of polymer solutions (30 wt % of either dextran or PEG of various molecular weights) were measured against buffer A. The observed OD was lower than 0.002 OD units, i.e., the contribution of the polymers to the overall turbidity of the studied vesicle dispersions was negligible. Turbidities of diluted vesicle dispersions are therefore given without corrections. When measured after equilibration (up to 24 h), the dispersions were kept in sealed glassware at room temperature.

### Viscosity measurements

The viscosity was measured with 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 dilutions to avoid multiple scattering. Such dilutions have been previously shown to have only a slight effect (if any) on the vesicle size (Lichtenberg, 1993). The viscosity of all measured samples differed from that of the medium by no more than 1%.

## Centrifugation

Samples were centrifuged for 30 min ( $8000 \times g$ ) in a Sorvall RC-5C plus (DuPont, Boston, MA) centrifuge using an SM-24 rotor.

## Kinetic measurements

Polymer-induced vesicle aggregation was measured at room temperature by monitoring the time-dependent increase in the 90° scattering of light in SUV suspensions at 650 nm. Time-resolved measurements were carried out using a modular stopped-flow spectrometer (Bio-Logic, Grenoble, France). Three syringes were filled with SUVs (30 mM), polymer solution (20 wt %), and buffer A, respectively. Mixing to the final required concentrations was done by varying the volumes injected while keeping the final total volume constant (100  $\mu$ l). All of the syringes were driven simultaneously in 20 ms, and measurements began after a dead time of 1.8 ms. Scattered light passed through a cuvette model TC-100/15 (40  $\mu$ l) and a light path of 10 mm. The total flow rate was 5 ml/s.

## RESULTS

Similar to previous studies (e.g., Schachter, 1978; Sunamoto et al., 1980; Hui and Boni, 1991; MacDonald, 1985; Tilcock and Fisher, 1982; Saez et al., 1982; Boni et al., 1981, 1984), we have investigated the steady-state turbidity of mixed systems containing egg PC SUVs and water-soluble polymers of different molecular weights as functions of the polymer concentration. The stepwise increase of turbidity obtained in all of the studied systems at any polymer concentration above a threshold concentration  $C_A$  but lower than 25 wt % can be attributed to reversible vesicle aggregation, as 10-fold dilution of any of these dispersions resulted in a much larger decrease of turbidity within less than 15 s. Furthermore, the size of the vesicles, as determined by QLS after a 10-fold dilution, was identical to that observed before polymer addition (not shown).

### Dependence of $C_A$ on vesicle concentration and size, polymer molecular weight, and temperature

For any given vesicle preparation and polymer, the value of  $C_A$  depended on the concentration of PC. The example depicted in Fig. 1 for SUVs and 70-kDa dextran is very similar to previously reported data on 6-kDa PEG (Tilcock and Fisher, 1982). Specifically, at PC concentration below 1 mM,  $C_A$  decreased upon increasing the concentration of the vesicles, approaching a limiting value of approximately 3 wt % at approximately 1 mM PC.

The value of  $C_A$  as measured for 2 mM PC SUVs decreased upon increasing the molecular weight of the

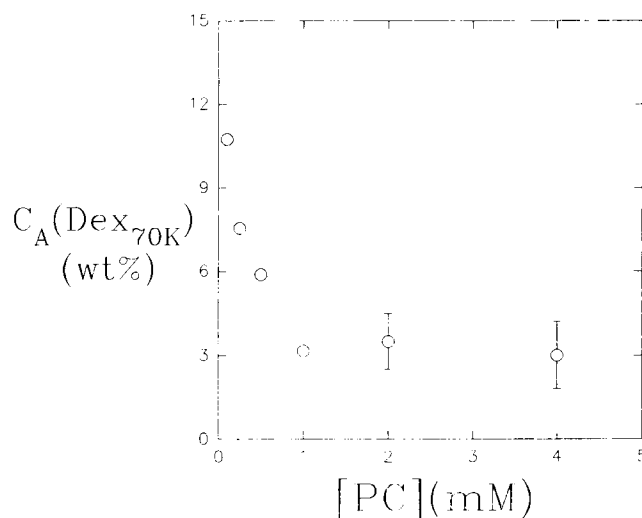


FIGURE 1 The dependence of  $C_A$  in mixtures of SUVs and 70-kDa dextran (Dex<sub>70K</sub>) on PC concentration. For each PC concentration,  $C_A$  was evaluated from the dependencies of turbidity (at 360 nm) on the polymer concentration, as measured 24 h after mixing at room temperature. Before turbidity measurements, the PC-dextran mixtures were remixed.

dextran (Fig. 2 A), in agreement with previous experiments (Schachter, 1978; Sunamoto et al., 1980). Interestingly, the value of  $C_A$  apparently coincided with the overlapping concentration ( $C^*$ ) computed from the intrinsic viscosity (given by the manufacturer) on the basis of De Gennes's equation (De Gennes, 1979) (Fig. 2 B). It thus appears that vesicle aggregation occurs when the polymer undergoes entanglement into a supramolecular structure (at  $C^*$ ). For the two PEGs studied here,  $C^*$ , as determined by the break in viscosity versus concentration curves (not shown) was equal to 0.6 wt % for 20-kDa PEG and 1.5 wt % for 6-kDa PEG, as compared with  $C_A$  values of 1 and 3 wt %, respectively. Unlike dextran, PEG, at sufficiently high con-

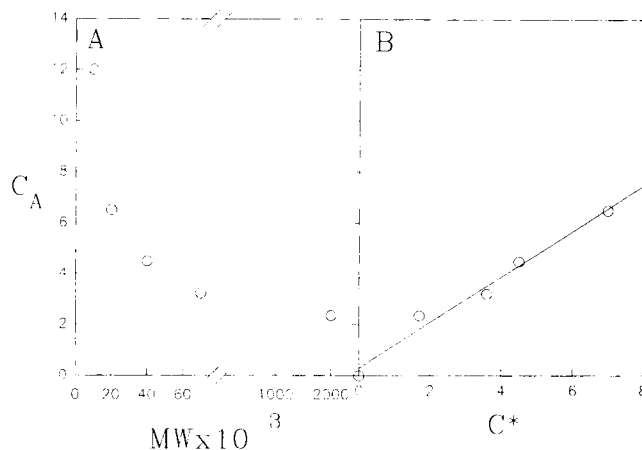


FIGURE 2 The dependence of  $C_A$  on dextran molecular weight and  $C^*$ . (A) The dependence of  $C_A$  on dextran molecular weight. (B) The dependence of  $C_A$  on  $C^*$  (the polymer overlapping concentration, as computed from the intrinsic viscosity ( $\eta_0$ ) given by the manufacturer, using the relationship  $C^* = 0.77/\eta_0$ ; De Gennes, 1979).

centrations (more than 20 wt % for both 6 and 20 kDa) caused an irreversible size growth (not shown), as previously observed by several investigators and attributed to the combined effect of complete structuring of all of the water in the system and consequent membrane destabilization (Tilcock and Fisher, 1982; Hui and Boni, 1991; Boni et al., 1981, 1984; MacDonald, 1985).

The tendency of vesicles to aggregate in polymer solutions depended also on their initial size. Thus, aggregation of LUVs (80–92 nm) in a solution of 70-kDa dextran began at a considerably lower dextran concentration than in the case of SUVs (Fig. 3), despite the much larger concentration of SUVs as compared with LUVs at the same PC concentration.

Polymer-induced aggregation of SUVs was apparently independent of temperature. The turbidities observed after 15 min of incubation of SUVs (0.5 and 2 mM PC) with varying concentrations of 70-kDa dextran (4–16 wt %) or 6-kDa PEG (2–10 wt %) at 4°C were identical to those observed after incubation at 37°C (Fig. 4). The results obtained after 1 and 21 h were indistinguishable from those observed after 15 min (not shown), indicating that a steady state was reached in all cases within less than 15 min.

**Kinetic studies**

To further characterize the polymer-induced vesicle aggregation, we have studied the time course of light scattering during the first 5 s after mixing the polymer with SUVs at

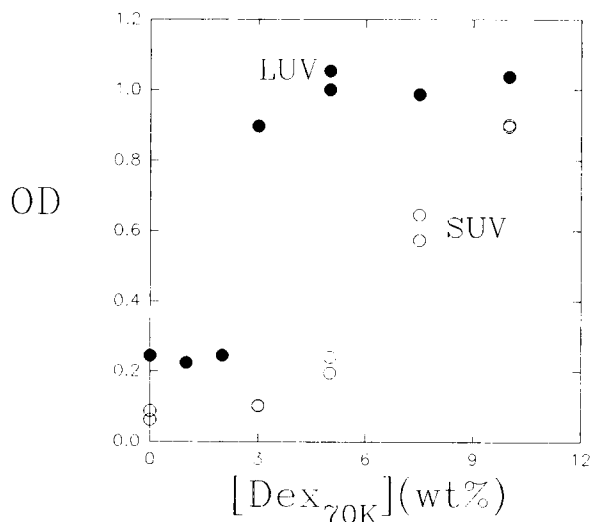


FIGURE 3 The dependence of steady-state turbidity on the concentration of 70-kDa dextran ( $Dex_{70K}$ ), as measured for SUVs and LUVs. Dispersions of 2 mM PC were prepared by mixing SUVs (32 nm diameter;  $\circ$ ) or LUVs (80–92 nm diameter;  $\bullet$ ) with varying concentrations of 70-kDa dextran. The turbidity of the samples was measured at 360 nm 24 h after mixing the vesicles with the polymer solution. The samples were remixed before being measured. After a 10-fold dilution in buffer A, the turbidity decreased to values below 0.05 OD units (not shown); i.e., the molar turbidity of aggregated vesicles decreased by more than a factor of 10, indicating that the vesicles became deaggregated.

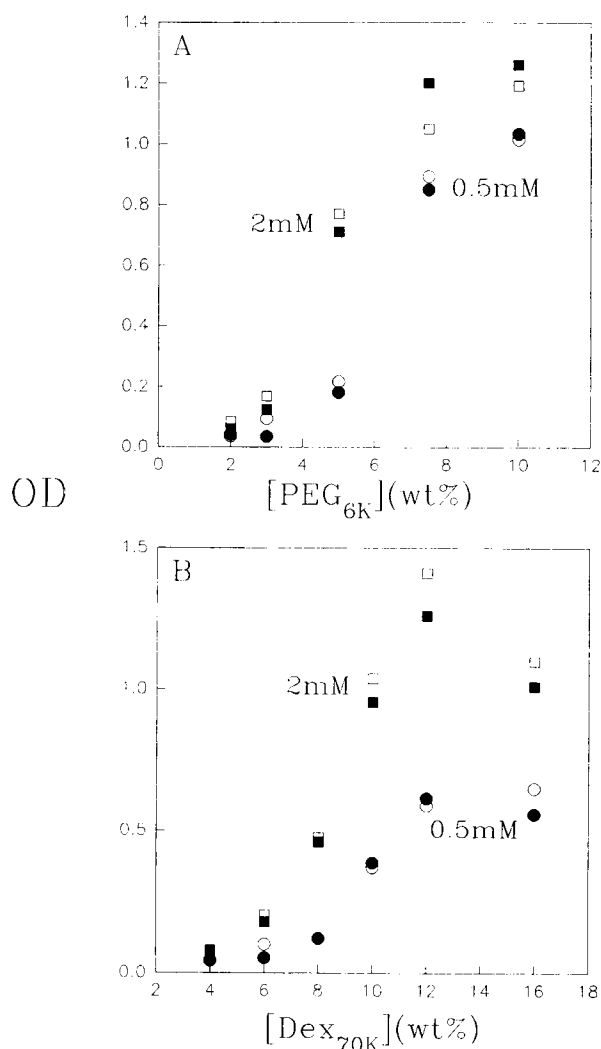


FIGURE 4 Effect of temperature on the dependence of the steady-state turbidity of PC SUVs on the concentration of 70-kDa dextran ( $Dex_{70K}$ ) and 6-kDa PEG ( $PEG_{6K}$ ). Dispersions of 2 mM PC ( $\square$  and  $\blacksquare$ ) and 0.5 mM PC ( $\circ$  and  $\bullet$ ) were prepared by mixing SUVs with varying concentrations of 6-kDa PEG (A) or 70-kDa dextran (B). The samples were kept at 4°C ( $\square$  and  $\circ$ ) or 37°C ( $\blacksquare$  and  $\bullet$ ) for 15 min and their turbidity was measured without dilution at 360 nm. Turbidity measurements carried out after 1 and 21 h were indistinguishable (not shown).

different PC concentrations. The time course observed after addition of 10 wt % 70-kDa dextran to vesicles of different concentrations demonstrates that increasing the PC concentration from 3 to 5.1 mM (Fig. 5) resulted in a monotonic increase in the steady-state intensity of scattered light. Additional increase in PC concentration resulted in an increase of the intensity of scattered light followed by a decrease. Similar behavior was previously reported by Viguera et al. (1995) for PEG 1500 and attributed to non-Rayleigh scattering. Qualitatively similar results were also observed with 5 wt % 70-kDa dextran and 5 and 10 wt % 6-kDa PEG (not shown). The inset of Fig. 5 presents the kinetics during the first 0.5 s. The kinetic profiles were analyzed in terms of the maximal rate of increase of light scattering ( $V_{max}$ ) after the

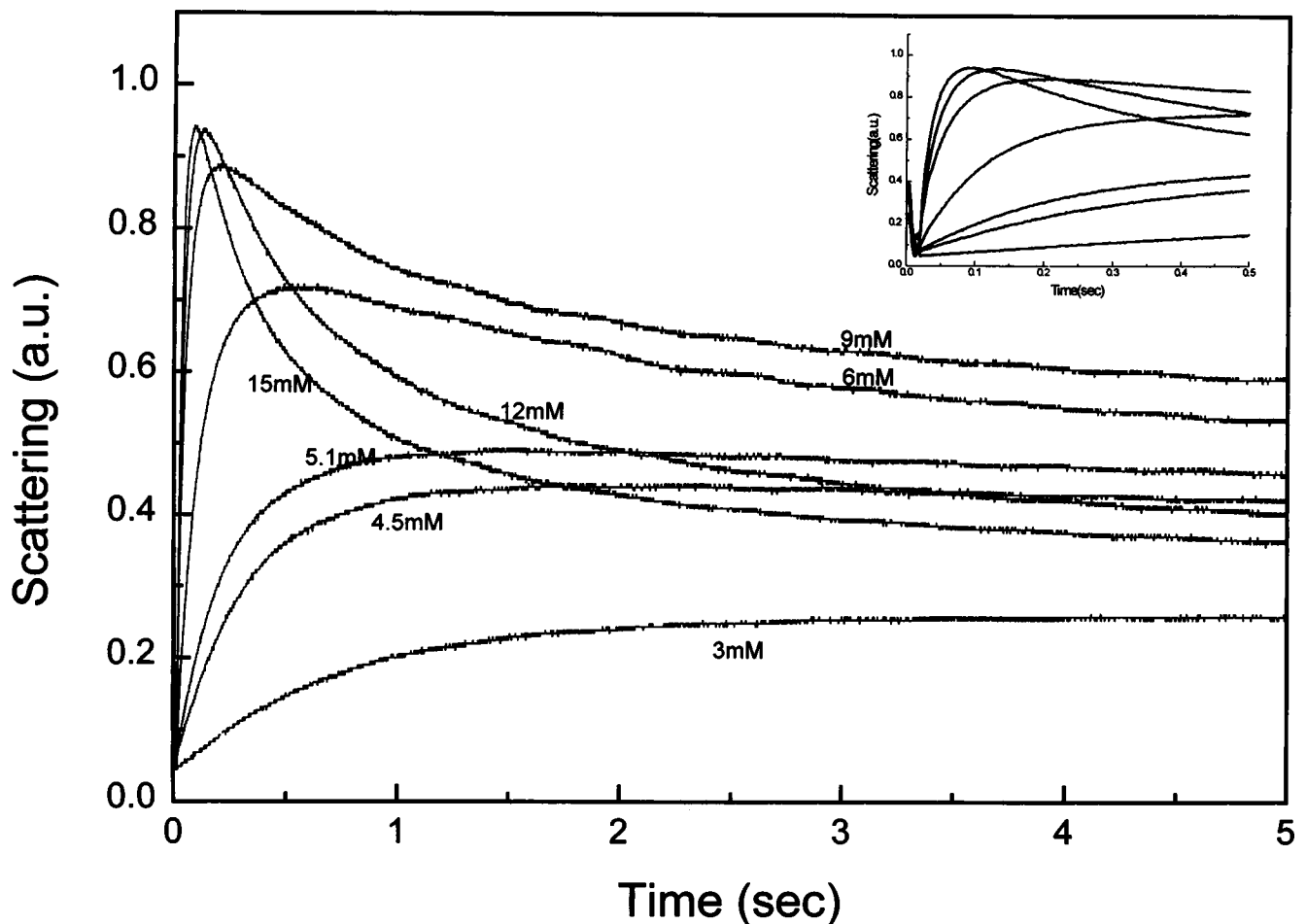


FIGURE 5 Kinetics of aggregation of SUVs in the presence of 10% 70-kDa dextran. SUVs of varying PC concentrations (3–15 mM, as indicated in the figure) were mixed with 10% 70-kDa dextran and light scattering was monitored at 600 nm for 5 s. The inset shows the kinetics profile in the first 0.5 s.

initial stage of mixing (first 20 ms in the inset of Fig. 5). Fig. 6 depicts  $\log V_{\max}$  as a function of the logarithm of PC concentration. As evident from this figure, for any given PC concentration, the aggregation induced by 5 wt % 70-kDa dextran was slower than that obtained in the presence of 5 wt % 6-kDa PEG. In fact, the rate of aggregation induced by 5 wt % 6-kDa PEG was obtained with 70-kDa dextran only when the concentration of the latter polymer was 10 wt %. Notably, when apparent equilibrium was attained, 24 h after preparation, the effects of 6-kDa PEG and 70-kDa dextran were similar and the two polymers had similar  $C_A$  values (Fig. 4). It thus appears that, when two polymers, dextran and PEG of different molecular weights, have the same potency in inducing aggregation, the dextran-induced aggregation is slower than that induced by the corresponding PEG, probably due to the binding of dextran to the vesicle surface (Minetti et al., 1979).

The dependence of the rate of aggregation on PC concentration appeared to be stepwise for all of the polymers studied. For both 5 wt % 6-kDa PEG and 10 wt % 70-kDa dextran, the rate became independent of PC concentration at

9 mM PC; for 10 wt % PEG, this value was 2.5 mM, and for 5 wt % dextran,  $V_{\max}$  reached a constant level only at PC concentrations higher than 16 mM (Fig. 6). The logarithm of the initial rate depended linearly on the logarithm of the lipid concentration up to the saturating PC concentration. For all of the studied polymers, the slope of these dependencies was  $4 \pm 0.5$ .

### Phase separation

In all of the experiments described above, the dispersions were mixed before measurement of turbidity. In fact, when mixtures containing polymer concentrations higher than  $C_A$  were incubated for 24 h, they appeared to separate into two macroscopic phases, as previously described for dimyristoylphosphatidylcholine vesicles by Lehtonen and Kinnunen (1995). Due to this phase separation, the turbidity, as measured after mixing, was always higher than that measured without mixing (not shown). However, in most cases, the phase separation was incomplete, namely, cloudy ag-

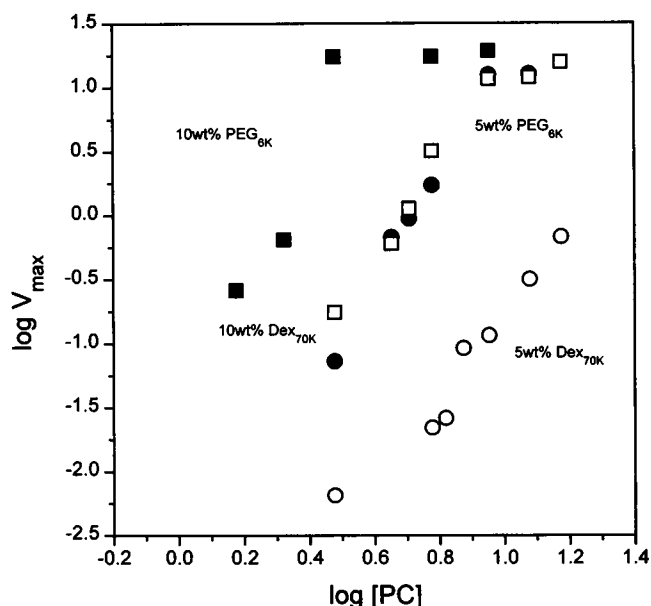


FIGURE 6 The dependence of the maximal rate of aggregation ( $V_{\max}$ ) on PC concentration. Double-logarithmic plot of the dependence of  $V_{\max}$ , as determined from kinetic profiles similar to those of Fig. 5 on PC concentration for 6-kDa PEG (■ and ●) and 70-kDa dextran (□ and ○) at concentrations of 5 wt % (○ and ●) and 10 wt % (□ and ■).

gregates appeared below a creamy floating lipid. Thirty minutes of centrifugation at  $8000 \times g$  were sufficient for complete separation, yielding relatively transparent infranatants, the turbidity of which was much lower than that obtained after remixing of the dispersions (Fig. 7 A). These infranatants contained only a fraction of the PC, as determined by chemical analysis (Fig. 7 B).

### Dialysis experiments

To evaluate the effect of depletion forces on cluster formation, we have carried out experiments similar to those of MacDonald (1985), in which the vesicles were separated from the polymer by dialysis bags. The latter studies were conducted to evaluate polymer-induced fusion processes, and the turbidity of vesicle dispersions was therefore measured only after dilution of the content of the dialysis bags, which would have resulted in deaggregation of aggregated vesicles.

As our dialysis experiments addressed aggregation of vesicles, which depends on their concentration, we have first measured the osmotically driven shrinkage of the bags, hence the increase of the vesicle concentration within the bag. As evident from the results depicted in Fig. 8, the volume within the bags decreased upon increasing the concentration of the polymer (either PEG or dextran). Despite their increased concentration, the vesicles did not aggregate. As an example, when 2 mM PC SUVs were dialyzed against a 5-fold larger volume of medium containing 12 wt % 70-kDa dextran, the steady-state molar OD of the vesicle dispersion was 32 OD units/M, which is the same molar OD

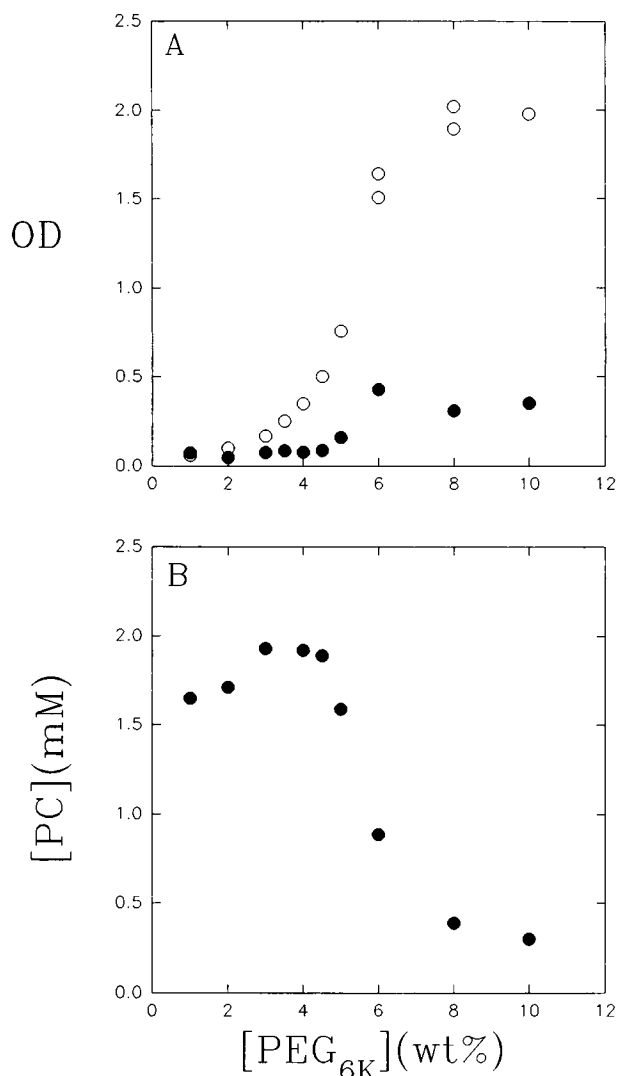


FIGURE 7 The dependence of phase separation on PEG concentration. Dispersions of 2 mM PC were made by mixing SUVs with varying concentrations of 6-kDa PEG (PEG<sub>6K</sub>). The turbidity of the samples (A) was measured at 360 nm, 24 h after mixing the SUVs with the polymer solutions. The empty symbols (○) represent the OD of the whole dispersions after remixing. The solid symbols (●) represent the OD of the infranatant obtained after 30 min of centrifugation ( $8000 \times g$ ). The PC concentration in the infranatants, as determined chemically, is presented in B as a function of PEG concentration.

as that of the vesicles before dialysis against the polymer. By comparison, the turbidity of a system of the same composition made by direct mixing of dextran (10 wt %) and PC SUVs (0.35 mM) was 26-fold higher (845 OD units/M). Noticeably, in direct mixing experiments with 2 mM PC, aggregation occurs at much lower polymer concentrations ( $C_A = 3.5$  wt %). Similar results (i.e., no increase in the molar turbidity in dialysis experiments) were observed when the vesicles were immersed in solutions of up to 20 wt % 6-kDa PEG.

In both our dialysis experiments and in the similar previous experiments (MacDonald, 1985), the turbidity of the

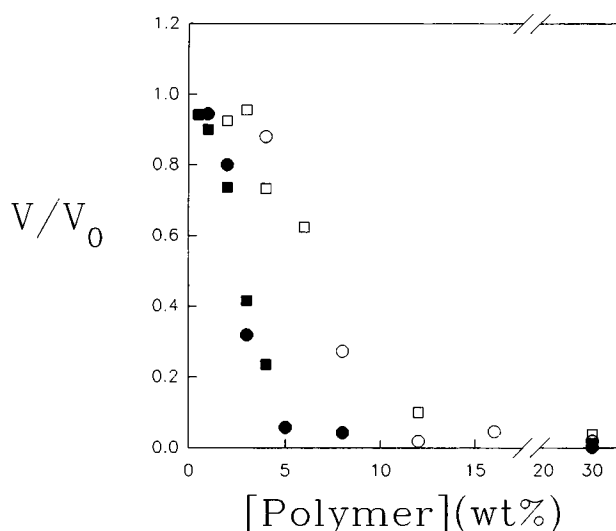


FIGURE 8 Osmotically driven shrinkage of dialysis bags. Dialysis bags were filled with 2 mM PC SUVs and immersed in fivefold larger external volumes of polymer solutions of varying concentrations. Four different polymers were used: 6-kDa PEG (●), 20-kDa PEG (■), 40-kDa dextran (○), and 70-kDa dextran (□). The volume reduction (expressed in terms of  $V/V_0$ ) was calculated from the weights of the dialysis bags before ( $g_0$ ) and after ( $g$ ) dialysis, using the correlation  $V/V_0 = (g - B)/(g_0 - B)$ , where  $B$  is the weight of the dialysis bag after removal of its content. Similar experiments carried out with dialysis bags filled with buffer A gave very similar results (not shown).

vesicle dispersions was determined after transfer of the contents of the dialysis bag into cuvettes. This could have possibly affected the results as the measured dispersions were not under the influence of the polymer solution. This possibility can, however, be ruled out as the same result (i.e., no increase of turbidity) was obtained when the turbidity within the bag was measured while the bag was still immersed in the external polymer solution, using a probe colorimeter (not shown; see Materials and Methods).

When dialyzed against much higher concentrations of either dextran or PEG, the vesicles underwent irreversible size growth, as previously reported by MacDonald (1985). Thus, when the concentrated dispersions obtained after dialysis of 2 mM PC against concentrated polymer solutions were diluted to a volume larger than 30 times the original volume and measured by QLS, the vesicles had much larger sizes than the SUVs. In fact, the size of the vesicles obtained after dialysis against a solution of 70-kDa dextran was an increasing function of the dextran concentration in the external solution (e.g., 60 nm at 20 wt %, 400 nm at 25 wt %, and more than 2  $\mu\text{m}$  at 30 wt %).

## DISCUSSION

### Vesicle-vesicle interactions

PC vesicles in their liquid crystalline phase aggregate and fuse very slowly (Lentz et al., 1987), if at all, even when

their size is sufficiently small to result in high excess free energy due to their curved surface (Grunner et al., 1988). This relative stability can be attributed, at least partially, to the layer of bound water on the surface of the vesicles, which inhibits close approach of the vesicles (Parsegian and Rand, 1991) and presents a thermodynamic barrier that prevents transfer of apolar groups to the aqueous phase (Hafeti and Hanstein, 1974).

Vesicle-vesicle interactions have been previously described (Petersen and Chan, 1978) in terms of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey and Overbeek, 1948; Kruyt, 1952), which showed that the dependence of the total energy of interaction on the distance between vesicles exhibits a deep (primary) minimum when the surface to surface distance is less than 1 nm and a shallow (secondary) minimum when the surface to surface separation is of the order of magnitude of several nanometers. As a result, the interaction between vesicles may occur either at the primary minimum, yielding close contact, termed coagulation, or at the secondary energy minimum, yielding loose association, described by the term flocculation (Petersen and Chan, 1978). Noticeably, the depth of the latter minimum is an increasing function of vesicle size (Petersen and Chan, 1978).

In most systems studied thus far, the lipid concentration was in the range of up to 10 mM. At this concentration, it can be shown that the average distance between the surfaces of neighboring vesicles of a diameter of 30 nm is approximately 60 nm. At lower PC concentrations, the average distance becomes larger (e.g., 150 nm at 2 mM) and the likelihood of formation of relatively stable vesicle clusters can be expected to decrease. In dispersions containing larger vesicles, the average distance between surfaces increases (e.g., at 10 mM, the distance between vesicles of a diameter of 90 nm is approximately 100 nm).

### Polymer-induced vesicle-vesicle interactions

Polymer-induced vesicle-vesicle interactions have been attributed to the ability of the polymer to bind and structure water, thus facilitate two essential steps in fusion: 1) bilayer contact (aggregation) and 2) destabilization of the vesicles due to formation of defects in the bilayer structure (Hui and Boni, 1991). The latter effect requires a larger degree of stripping of the membrane surface from water, which is not likely to occur when the polymer is bound to the surface (e.g., dextran).

The present study is devoted to polymer-induced aggregation. Three major factors have been considered as being significant in this process, namely, 1) dehydration of the vesicle surface, which reduces the energy barrier to close approach of the vesicles (e.g., Yamazaki et al., 1989), 2) deformation of the vesicles due to their shrinkage in a medium of higher osmolarity, which results in increased contact surface (e.g., Hui and Boni, 1991), and 3) reduction of the rate of deaggregation due to depletion forces that

result from depletion of polymer molecules from the volume elements between vesicles (Israelachvili, 1992).

The major conclusion of the present study is that depletion forces play a primary role in the polymer-induced aggregation of PC vesicles. These forces can be expected to induce aggregation when the molecular volume of the polymer is large in comparison with the average distance between vesicles. In fact, the molecular volume of the polymers investigated in this study is much smaller than the average distance between vesicles. For example, a polymer molecule of a molecular weight of  $2 \times 10^6$  Da has a self volume of approximately  $1.5 \times 10^3$  nm<sup>3</sup>. Assuming that the polymer molecule is globular, its diameter will be 14 nm, as compared with an average distance larger than 60 nm between neighboring vesicles (see above). However, when the polymer molecules become entangled, at  $C^*$  (De Gennes, 1979), their effective molecular volume grows markedly and they are depleted from volume elements between vesicles. This results in depletion forces that inhibit deaggregation of the vesicles and by that increase both the rate and extent of the overall cluster formation at  $C^*$ . This effect is likely to be larger for PEG than for dextrans, which are known to bind reversibly to the vesicles (Minetti et al., 1979).

An alternative, slightly different, approach to the effect of polymer entanglement on the flocculation of vesicles is that at  $C^*$  a network of polymer is formed from which vesicles are excluded. Polymer scaling concepts, experimentally verified by small angle neutron scattering (Abbott et al., 1992), have demonstrated that the mesh size of a solution of extensively entangled coils of PEG of molecular weights higher than 6 kDa (used in this study) at concentrations within the range of 5 to 20% is at least an order of magnitude smaller than SUVs. Hence, entrapment of vesicles within entangled webs of polymer is not very likely. This can lead to spontaneous (but not necessarily complete) separation of the system into two distinctly different phases of different densities after prolonged equilibration. The reversible flocculation of vesicles observed after shorter periods of time or lower polymer concentrations may be an intermediary state in this process of macroscopic phase separation (see below). Only at much higher polymer concentrations did irreversible size growth occur, as previously described (MacDonald, 1985; Hui and Boni, 1991; Boni et al., 1981, 1984).

All of the data of the present study are consistent with the central role of depletion forces in polymer-induced aggregation of vesicles. First of all, reversible aggregation was never observed in dialysis experiments. Both dehydration of vesicle surface and deformation of the vesicles in these experiments should be the same as in direct mixing experiments. Furthermore, in the dialysis experiments, the polymer-induced shrinkage of the bags results in increased vesicle concentration. Yet no aggregation was observed in the lack of vesicle-polymer contact at polymer concentrations that were much higher than  $C_A$ , lending strong support to our conclusion regarding the central role of depletion forces.

In dialysis experiments, size growth occurs only when the volume within the bag (and most likely within the vesicles as well) was reduced to less than a tenth of the initial volume (Fig. 8). Given the large reduction of the volume within the bag (which at 30 wt % dextran appeared to be dry), the vesicles obtained after dilution of the remaining concentrated solution were probably multilamellar (Hui and Boni, 1991; Boni et al., 1981, 1984; Saez et al., 1982; MacDonald, 1985), similar to the multilamellar vesicles obtained by hydration of dried lipids.

The value of  $C_A$  is dependent on vesicle size,  $C_A$  for LUVs being lower than for SUVs. This dependence was observed despite both the larger distance between LUVs than between SUVs of the same PC concentration (see above) and the inherent instability of the SUVs. Similar results observed for Mg<sup>2+</sup>-induced aggregation of phosphatidylserine vesicles (Nir et al., 1981) were attributed to aggregation without surface contact in a secondary minimum. Such aggregation can be expected to play a much more important role in the aggregation of LUVs as the depth of the secondary minimum is an increasing function of particle size (Petersen and Chan, 1978). The reversible polymer-induced interaction between vesicles can be described in terms of cluster formation or flocculation. Notably, for 10 mM SUVs, the surface to surface distance in the flocculated vesicles is reduced to approximately one-fourth of its original value. It is important to note that such aggregation, at a secondary minimum, can also be accompanied by larger interference effects in the intensity of the scattered light (Kerker, 1969). Upon dilution, the vesicles deaggregate severalfold more rapidly than vesicle clusters that were formed upon addition of Na<sup>+</sup> to phosphatidylserine SUVs, which aggregate at a primary minimum (Bentz and Nir, 1981; Nir et al., 1983; Peled et al., 1995). Another interpretation that can be invoked is that LUVs are more sensitive than SUVs to the effects exerted by the polymers, i.e., dehydration, deformation, and/or shrinkage. The excess pressure in the interior of SUVs is larger than in LUVs. Consequently, SUVs may be more resistant to the effect of osmotic pressure.

The dependence of  $C_A$  on PC concentration is also consistent with the central role of depletion forces. For SUVs and 70-kDa dextran, the value of  $C_A$  at PC concentrations higher than 1 mM PC is independent of the PC concentration (in agreement with the results of Tilcock and Fisher (1982) obtained for PEG) and is equal to the value of  $C^*$  (the overlapping concentration). At sufficiently high PC concentrations, exclusion of entangled polymer of high effective molecular volume (obtained at  $C^*$ ) from the vicinity of the vesicles (Evans and Needham, 1988; Van Oss et al., 1988; Arnold et al., 1990) pushes the vesicles to separate from the polymer phase, thus reducing possible deaggregation. The sharp dependence of  $C_A$  on PC concentration below this critical concentration is likely to arise from the sharp dependence of the average distance between vesicles on the concentration of the vesicles below 1 mM PC SUVs (not shown). Under these conditions, depletion can occur



only at much higher polymer concentrations, at which larger polymer webs are formed.

The lack of dependence of  $C_A$  on temperature can also be explained by similar terms. The effect of exclusion of polymer from within clusters of vesicles is apparently sufficient to reduce the rate of deaggregation to the extent that temperature changes (within the range of 4 to 37°C) have insignificant effects on the polymer concentration required for cluster formation ( $C_A$ ). Several studies on  $\text{Na}^+$ -induced aggregation of negatively charged vesicles demonstrated that the average aggregate size increased at lower temperatures (Day et al., 1980; Nir et al., 1980, 1983; Bentz and Nir, 1981; Peled et al., 1995; Wong and Thompson, 1982). This general tendency of colloidal particles to be more dispersed at higher temperatures arises from a steep increase in the rate constant of deaggregation. The absence of such an effect in polymer-induced aggregation can be interpreted to arise from a significant reduction in the rate of deaggregation of vesicles due to the exclusion of polymer molecules from the vicinity of the membranes, which inhibits the deaggregation process even at a high temperature.

### Kinetic studies

Noticeably, the rate of aggregation and the threshold polymer concentration ( $C_A$ ) depended differently on the polymer and its molecular weight. Comparison of the aggregation induced by dextran with that induced by PEG raises an intriguing, yet unresolved question: the osmotic effect of dextrans (40 and 70 kDa), as evaluated from their effect on the volume of vesicle-containing dialysis bags at equilibrium, is smaller than that of PEG (6 and 20 kDa) at the same polymer concentrations (Fig. 8). Furthermore, binding of dextrans to the vesicle surface (Minetti et al., 1979) is likely to reduce the depletion forces in comparison with PEG (Israelachvili, 1992). The much faster rate of aggregation induced by 6-kDa PEG in comparison with that induced by 70-kDa dextran (Fig. 6) is consistent with these considerations. Nonetheless, the threshold concentration ( $C_A$ ), as determined by the steady-state turbidities, is similar for the latter two polymers (Fig. 4). Hence, whereas depletion forces and osmotic effects are the major determinants of the rate of aggregation, the extent of aggregation appears to be determined only by the average size of polymer networks formed at  $C^*$ . Interpretation of this difference may be based on the assumption that the pronounced osmotic effects and depletion forces of PEG (in comparison with dextran) yield faster aggregation whereas the extent of aggregation is governed by the possibility of deaggregation, which is critically dependent only on the size of polymer webs.

Another difference between the rate and extent of aggregation is that the rate depends on vesicle concentration up to much higher PC concentrations than  $C_A$ . As an example, for

PC concentrations above 1 mM,  $C_A$  for 70-kDa dextran appears to be independent of the concentration of vesicles (Fig. 1) whereas the rate of aggregation for both 5 and 10 wt % of the same polymer is critically dependent on the concentration of PC (Fig. 6).

According to our studies, the aggregation is fourth order in vesicle concentration (Fig. 6), as compared with a second order indicated by the study of Viguera et al. (1995) for PEG 1500. In the latter work, the slope of the line that describes the dependence of  $\log V_{\max}$  on  $\log$  PC concentration was 2 and the  $V_{\max}$  values were higher than those obtained in our experiments, despite the lower PC concentrations used in the previous study. The reason for these differences is not clear to us. We tried to relate it to the different NaCl concentration used in the two studies (150 mM NaCl in our experiments as compared with 300 mM in the study of Viguera et al., 1995). However, experiments carried out in 300 mM NaCl and 5 wt % 6-kDa PEG gave the same results as those done in 150 mM NaCl (not shown), indicating that within the studied range of NaCl concentrations the rate is independent of ionic strength. Thus, the difference between the results of the two studies may either be a result of the different molecular weight of the PEG (1500 in the work of Viguera et al. as compared with higher molecular weights in our studies) or of differences in the exact composition and/or size of the PC vesicles. In fact, the tendency of the vesicles to aggregate in the studies of Viguera et al. (1995) was considerably greater than in most other systems studied thus far (Tilcock and Fisher, 1982).

For analysis of the early stages of the increase in light scattering in Fig. 5 in terms of a mass action theory (utilizing Eq. 2.24 in Nir et al., 1983) we focused on the three lowest lipid concentrations. In these early stages (up to 20–30% increase in the intensity of scattered light), interference effects are not pronounced, and in addition, ignoring deaggregation processes is also less critical than for higher lipid concentrations. This analysis gave a value of  $5 \times 10^6 \text{ m}^{-1} \text{ s}^{-1}$  ( $\pm 50\%$ ) for the forward rate constant of aggregation. This value is approximately 600-fold smaller than the value corresponding to diffusion-controlled aggregation and implies that the magnitude of the potential barrier for close approach of SUVs (probably due to depletion forces) is 6–7 kT.

In conclusion, the results of the present study indicate that depletion forces play the predominant role in polymer-induced aggregation of phospholipid vesicles. This effect, in conjunction with dehydration of the vesicle surface and shrinkage-induced deformation of the bilayers, yields relatively stable vesicle clusters, which can, however, be rapidly reversed by dilution. Only at much higher polymer concentrations do the bilayers of the aggregated (and dehydrated) vesicles become sufficiently deformed to result in vesicle fusion. The latter process can, however, be induced by alteration of the bilayer deformability, as observed upon inclusion of various membrane perturbants such as surfac-

tant, as demonstrated in the companion article appearing in this issue (Meyuhas and Lichtenberg, 1996).

We thank Drs. Misha Kozlov, Jacob Klein, Itzhak Rabin, Daniel Blank-shtein, Peter Schurtenberger, and Adrian Parsegian for several helpful discussions. The Israel Academy of Sciences is acknowledged for supporting this study.

## REFERENCES

- Abbott, N. L., D. Blankshtein, and T. A. Hatton. 1992. Protein partitioning in two-phase aqueous polymer system: a neutron scattering investigation of the polymer solution structure and protein-polymer interactions. *Macromolecules*. 25:3932–3945.
- Almog, S., B. J. Litman, W. Wimley, J. Choen, E. J. Wachtel, Y. Barenholtz, A. Ben-Shaul, and D. Lichtenberg. 1990. States of aggregation and phase transformation in the mixture of phosphatidylcholine. *Biochemistry*. 29:4582–4592.
- Arnold, K., O. Zschoering, D. Barthel, and W. Herold. 1990. Exclusion of poly(ethylene glycol) from liposome surfaces. *Biochim. Biophys. Acta*. 1022:303–310.
- Bangham, A. D., J. deGier, and G. D. Greville. 1967. Properties and water permeability of phospholipid crystals. *Chem. Phys. Lipids*. 1:225–246.
- Bentz, J., and S. Nir. 1981. Kinetic and equilibrium aspects of reversible aggregation. *J. Chem. Soc. Faraday Trans.* 77:1249–1275.
- Boni, L. T., J. S. Hah, S. W. Hui, P. Mukherjee, J. T. Ho, and C. Y. Jung. 1984. Aggregation and fusion of unilamellar vesicles by poly(ethylene glycol). *Biochim. Biophys. Acta*. 775:409–418.
- Boni, L. T., T. P. Stewart, J. L. Alderfer, and S. W. Hui. 1981. Lipid-polyethylene glycol interaction: induction of fusion between liposomes. *J. Membrane Biol.* 62:65–70.
- Burgess, S. W., D. Massenburg, J. Yates, and B. R. Lentz. 1991. Poly(ethylene glycol)-induced lipid mixing but not fusion between synthetic phosphatidylcholine large unilamellar vesicles. *Biochemistry*. 30:4193–4200.
- Burgess, S. W., T. J. McIntosh, and B. R. Lentz. 1992. Modulation of poly(ethylene glycol)-induced fusion by membrane hydration: importance of interbilayer separation. *Biochemistry*. 31:2653–2661.
- Davidson, R. L., and P. S. Gerald. 1977. Induction of mammalian somatic cell hybridization by polyethylene glycol. In *Methods in Cell Biology*, Vol. 15. D. Prescott, editor. Academic Press, London. 325.
- Day, E. P., A. Y. W. Kwok, S. K. Hark, J. T. Ho, W. J. Vail, J. Bentz, and S. Nir. 1980. Reversibility of sodium-induced aggregation of sonicated phosphatidylserine vesicles. *Proc. Natl. Acad. Sci. USA*. 77:4026–4029.
- De Gennes, P. G. 1979. *Scaling Concepts in Polymer Physics*. Cornell University Press, Ithaca, NY.
- Dittmer, J. C., and M. A. Wells. 1969. Quantitative and qualitative analysis of lipids and lipid components. *Methods Enzymol.* 14:482–530.
- Evans, E., and M. Metcalfe. 1984. Free energy potential for aggregation of mixed PC/PS vesicles in dextran solution. *Biophys. J.* 45:715–720.
- Evans, E., and D. Needham. 1988. Intrinsic colloidal attraction/repulsion between lipid bilayers and strong attraction induced by non-absorbing polymers. In *Molecular Mechanisms of Membrane Fusion*. S. Ohki, T. D. Flanagan, S. W. Hui, and E. Mayhew, editors. Plenum Publishing Co., New York. 83–100.
- Grunner, S. M., M. W. Tate, G. L. Kirk, P. T. C. So, D. C. Turner, D. T. Keane, C. P. S. Tilcock, and P. R. Cullis. 1988. X-ray diffraction study of the polymorphic behavior of N-methylated dioleoyl phosphatidylethanolamine. *Biochemistry*. 27:2853–2866.
- Hafeti, Y., and W. G. Hanstein. 1974. Destabilization of membranes with chaotropic ions. In *Methods in Enzymology*, Vol. 31. A. S. Fleischer and L. Packer, editors. Academic Press, New York. 770–790.
- Huang, S. 1969. Studies on phosphatidylcholine vesicles: formation and physical characteristics. *Biochemistry*. 8:344–352.
- Hui, S. W., and L. T. Boni. 1991. Membrane fusion induced by polyethylene glycol. In *Membrane Fusion*. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, New York. 231–254.
- Israelachvili, J. 1992. *Intramolecular and Surface Forces*, 2nd ed. Academic Press, New York.
- Kao, K. N., and M. R. Michayluk. 1974. Method for high frequency intergeneric fusion of plant protoplasts. *Planta*. 115:355–367.
- Kerker, M. 1969. *The Scattering of Light and Other Electromagnetic Radiation*. Academic Press, New York.
- Knutton, S. 1979. Studies of membrane fusion. III. Fusion of erythrocytes with polyethylene glycol. *J. Cell Sci.* 36:61–72.
- Krahling, H. 1981. Discrimination of two fusogenic properties of aqueous polyethylene glycol solutions. *Z. Naturforsch.* 36:593–596.
- Kruyt, M. K. 1952. *Colloid Science*, Vol. 1. Elsevier Publishing Co., Amsterdam.
- Lehtonen, J. Y. A., and P. K. J. Kinnunen. 1994. Changes in the lipid dynamics of liposomal membranes induced by poly(ethylene glycol): free volume alterations revealed by inter- and intramolecular excimer-forming phospholipid analogs. *Biophys. J.* 66:1981–1990.
- Lehtonen, J. Y. A., and P. K. J. Kinnunen. 1995. Poly(ethylene glycol)-induced and temperature-dependent phase separation in fluid binary phospholipid membranes. *Biophys. J.* 68:525–535.
- Lentz, B. R., T. J. Carpenter, and D. R. Alford. 1987. Spontaneous fusion of phosphatidylcholine small unilamellar vesicles in the fluid phase. *Biochemistry*. 26:5389–5397.
- Lichtenberg, D. 1993. Micelles and liposomes. In *Biomembranes, Physical Aspects*. M. Shinitzky, editor. VCH, Weinheim, Germany. 63–96.
- MacDonald, R. I. 1985. Membrane fusion due to dehydration by polyethylene glycol, dextran or sucrose. *Biochemistry*. 24:4058–4066.
- Massenburg, D., and B. R. Lentz. 1993. Poly(ethylene glycol)-induced fusion and rupture of dipalmitoylphosphatidylcholine large unilamellar extruded vesicles. *Biochemistry*. 32:9172–9180.
- Meyuhas, D., and D. Lichtenberg. 1996. Effect of water-soluble polymers on the state of aggregation, vesicle size, and phase transformations in mixtures of phosphatidylcholine and sodium cholate. *Biophys. J.* 71:2613–2622.
- Minetti, M., P. Aducci, and V. Viti. 1979. Interaction of neutral polysaccharides with phosphatidylcholine multilamellar liposomes: phase transitions studied by the binding of fluorescein-conjugated dextrans. *Biochemistry*. 18:2541–2548.
- Nir, S., J. Bentz, and N. Duzgunes. 1981. Two modes of reversible aggregation: particle size and the DLVO theory. *J. Colloid Interface Sci.* 84:266–269.
- Nir, S., J. Bentz, and A. R. Portis, Jr. 1980. Effect of cation concentrations and temperature on the rates of aggregation of alidil phospholipid vesicles: application to fusion. *Adv. Chem.* 188:75–106.
- Nir, S., J. Bentz, J. Wilschut, and N. Duzgunes. 1983. Aggregation and fusion of phospholipid vesicles. *Prog. Surf. Sci.* 13:1–124.
- Parsegian, V. A., and P. R. Rand. 1991. Process governing lipid interaction and rearrangement. In *Membrane Fusion*. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, New York. 65–89.
- Peled, C. R., G. Braun, and S. Nir. 1995. Time of equilibration in reversible aggregation of particles. *J. Colloid Interface Sci.* 169:204–213.
- Petersen, N. O., and S. I. Chan. 1978. The effects of the thermal prephase transition and salts on the coagulation and flocculation of phosphatidylcholine bilayer vesicles. *Biochim. Biophys. Acta*. 509:111–128.
- Robinson, J. M., D. S. Roos, R. L. Davidson, and M. J. Karnovsky. 1979. Membrane alterations and other morphological features associated with polyethylene glycol-induced cell fusion. *J. Cell Sci.* 40:63–75.
- Saez, R., A. Alonso, A. Villena, and F. M. Goni. 1982. Detergent-like properties of polyethyleneglycols in relation to model membranes. *FEBS Lett.* 137:323–326.
- Schachter, D. 1978. Aggregation of liposomes by dextrans of high molecular weight. *Biochem. Biophys. Res. Commun.* 84:840–844.
- Sunamoto, J., K. Iwamoto, H. Kondo, and S. Shinkai. 1980. Liposomal membranes. VI. Polysaccharide-induced aggregation of multilamellar liposomes of egg lecithin. *J. Biochem.* 88:1219–1226.
- Tilcock, C. P. S., and D. Fisher. 1982. The interaction of phospholipid membranes with poly(ethylene glycol) vesicles aggregation lipid exchange. *Biochim. Biophys. Acta*. 688:645–652.
- Van Oss, C. J., M. K. Chaudhury, and R. J. Good. 1988. In *Molecular Mechanisms of Membrane Fusion*. S. Ohki, T. D. Flanagan, S. W. Hui, and E. Mayhew, editors. Plenum Publishing Co., New York. 113–122.

- Viguera, A. R., A. Alonso, and F. Goni. 1995. Polar interface interactions: hydration pressure and membrane fusion. *Colloids Surf. B Biointerfaces*. 3:263–270.
- Verwey, E. J. W., and J. T. G. Overbeek. 1948. *Theory of Stability of Lyophobic Colloids*. Elsevier, Amsterdam.
- Wojcieszyn, J. W., R. A. Schlegel, K. Lumley-Sapanski, and K. A. Jacobson. 1983. Studies on the mechanism of polyethylene glycol-mediated cell fusion using fluorescent membrane and cytoplasmic probes. *J. Cell Biol.* 96:151–159.
- Wong, M., and T. E. Thompson. 1982. Aggregation of dipalmitoyl phosphatidylcholine vesicles. *Biochemistry*. 21:4133–4139.
- Wu, J. R., and B. R. Lantz. 1991. Mechanism of poly(ethylene glycol)-induced lipid transfer between phosphatidylcholine large unilamellar vesicles: a fluorescent probe study. *Biochemistry*. 30:6780–6787.
- Yamazaki, M., S. I. Ohnishi, and T. Ito. 1989. Osmoelastic coupling in biological structures: decrease in membrane fluidity and osmophobic association of phospholipid vesicles in response of osmotic stress. *Biochemistry*. 28:3710–3715.