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Poly(ethylene glycol)-conjugated surfactants promote or inhibit aggregation of phospholipids

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Abstract

The calcium-induced aggregation of dilauroyl phosphatidic acid (DLPA) suspensions, with or without added poly(ethylene oxide) (PEO)-conjugated surfactants containing 4 to 30 ethylene oxide subunits, were monitored by turbidity measurement and quasi-elastic light scattering (QLS). The aggregation was inhibited (protected) by the incorporated PEO surfactant for most samples, while a window for promotive effect was found for samples with low surface coverage by the PEO moiety of the incorporated surfactant. Promotion occurs only when the aggregation is slow and at a low level. The promotion is explained by the synergistic effect of PEO and divalent calcium cations when the steric repulsion is weak. The promotion/protection crossover is a display between the PEO/calcium synergistic effect and the steric repulsion.

Keywords: Poly(ethylene oxide) surfactant; Aggregation; Turbidity; Quasi-elastic light scattering; Steric stabilization; Electrostatic screening

1. Introduction

Poly(ethylene oxide) (PEO), or poly(ethylene glycol) (PEG) as the low molecular mass (M_r) compounds are called, is one of the most hydrophilic polymers. PEG is a commonly used chemical fusogen for cells and liposomes. It is also applied as a precipitating agent and cryoprotective agent for proteins and nucleic acids [1,2].

Recently, various PEG derivatives were used to stabilize liposomes for increasing efficiency in drug and/or gene delivery. Most 'stabilized' liposomes, e.g., the so-called 'stealth' liposomes [3], or 'cryptosomes' [4], contain a certain percentage of PEG-derivatized phospholipids, which reduce the uptake by mononuclear phagocytic system (MPS), prolonging thereof the circulation times. The theory of steric stabilization [5,6] has been utilized to explain these phenomena [7–9].

It has been recognized that the protection effect of PEG conjugated lipids or PEO-containing surfactant is dependent on the M_r of PEO moieties. These PEO moieties protrude from the surface of the lipid assemblies, while the hydrophobic tails of these molecules are anchored into the assemblies. In order to produce a significant inhibition of the macrophage uptake, the ethylene oxide (EO) chains used were found to be at least 50 subunits [10]; or equivalently, their M_r must

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range from 1000 to 5000 [3]. A similar observation was made in the studying of protection on protein adsorption: the degree of polymerization of PEO on the solid substrate must approach about 100 [11]. These observations indicate that the steric barrier provided by the grafted PEO must be sufficiently thick (≥ 100 Å) to overcome the effect of van der Waals and other attractive forces between the substrates to which these PEO-containing molecules are attached.

The conformation of polymers grafted to a surface have been analyzed by deGennes [12–14]. The grafted polymers assume various conformations depending on the surface density of polymers, thus providing various extent of steric barriers. The principle has been adapted to form theoretical basis for vesicle stabilization by PEG-conjugated lipids or PEO-containing surfactant [9,15]. The steric repulsion between lipid surfaces containing PEG-conjugated lipids or PEO-containing surfactants has been measured by X-ray diffraction under osmotic pressure [16] and by direct surface force measurement [17]. The results follow the theoretical prediction in general; in short range the interaction is dominated by steric repulsion, whereas at long range, by electrostatic repulsion. Little is known about an enhancement of attraction by PEG-conjugated lipids or PEO-containing surfactants that may promote vesicle aggregation.

In the present study, dilauroyl phosphatidic acid (DLPA) suspensions containing a small percentages of PEO surfactants of short (4 EO subunits) to relatively long (30 EO subunits) PEO moieties were used to monitor the kinetics and extent of calcium-induced aggregation. To our surprise, we found PEO surfactants had both protective (inhibitive) and promotive effects on the aggregation of lipid assemblies, depending on aggregation conditions. Defining these conditions will help us to a better understanding of the contribution of PEO surfactants to colloidal stabilization of phospholipid assemblies.

2. Materials and methods

DLPA was purchased from Avanti Polar Lipids (Alabaster, AL). The PEO-containing surfactants: nonyl phenol polyglycol ethers with 4, 10 and 30 EO subunits, respectively (trade names: Hostapal N-040, N-100 and N-300) were generous gifts from Hoechst Celanese Corp. (Somerville, NJ).

The stock solutions (in chloroform) containing given amount of DLPA, or mixture of DLPA and PEO surfactant with desired molar ratio, were put in a sonication tube. After evaporating the solvent on a Buchi R110 Rotavapor for about a half hour, a calculated volume of HisTes buffer (2 mM L-histidine, 2 mM Tes, and 0.02% NaN_3 (pH 7.4)) was added to the sonication tube. The lipid, or the mixture of lipid and surfactant, was dispersed by vortexing. The suspension was then sonicated in a water bath ultrasonicator (Laboratory Supplies Co., Hicksville, NY) under an atmosphere of nitrogen until a transparent suspension was obtained. Turbidity measurements were carried out on a Carl Zeiss spectrophotometer at a wavelength of 660 nm. Typically, 0.4 ml lipid suspension was put in a cuvette and 1.6 ml $CaCl_2$ solution was added and well mixed. The turbidity was measured as a function of time. The first reading of absorbance was at $t = 0.5$ min after initial mixing. Thereafter readings were taken every 0.5 or 1 min, during shaking intervals. The zero point of absorbance was calibrated with buffer solution only (well-sonicated lipid suspensions were found to have the same zero absorbance reading as that of the buffer solution) before each measurement and immediately after it, the drift was usually found to be smaller than 0.002.

Quasi-elastic light scattering (QLS), or photon correlation spectroscopy (PCS), was performed on a Model 370 Submicron Particle Sizer (Nicomp Particle Sizing Systems, Santa Barbara, CA). A Coherent Innova Argon Ion Laser (Coherent, Palo Alto, CA) with a maximum CW output of 2 W (in usual measurements, the output was not larger than 0.2 W). The photon count rates were at all times adjusted to about 300 kHz. The apparatus was calibrated by measuring the size of monodispersed polystyrene latex spheres of known sizes. The sample preparation and manipulation were identical to those in the turbidity measurement. In a typical experiment, 0.5 ml suspension was mixed with 2.0 ml buffer or $CaCl_2$ solution of a desired concentration in a cuvette. It usually takes 2 min to acquire sufficient data for a good correlation curve after the well-mixed sample was put into the sample holder, then the volume-weighted mean particle size and polydispersity were

determined for the initial suspensions as well as after $CaCl_2$ solutions were added. All the turbidity and QLS experiments were performed at room temperature (23°C).

The nature and size of lipid dispersions were examined by both negative staining and freeze fracture electron microscope as previously described [18]. The majority of samples examined were small unilamellar vesicles. Due to the detergent-like effect of added PEG-surfactants [17], collapsed vesicles and disc micelles were observable in samples containing 1 or higher mol% of PEG-surfactant. Micellation thresholds were higher for surfactants containing shorter PEG chains. The micellation thresholds were much lower than those reported by Kenworthy et al. [19] using PEG-conjugated phospholipids. Sonication may also contribute to the trapping of samples in the micellar form. Whatever was the morphology of the suspended particles, the outer surface was expected to be a monolayer of phospholipid and detergent mixture. This would not influence our aggregation assay since the initial particle sizes for all samples were the same, as measured by QLS, and surfactant to lipid molar ratios S/L on the particle surface were not changed for the samples containing PEG-surfactant. The particles are referred to as lipid 'assemblies' herein.

3. Results

A simple way to monitor the kinetics of aggregation of lipid assemblies is by turbidity measurement. A typical set of experimental results is shown in Fig. 1A–D. In Fig. 1A, a dramatic change in the sample turbidity due to Ca^{2+}-induced aggregation for the 1.0 mM DLPA assemblies is evident: upon adding of $CaCl_2$ solution to a final concentration of 1.0 mM or less, no turbidity change could be detected; when Ca^{2+} was added to a concentration of 2.0 mM, aggregation became so fast that only one data point (absorbance reading at $t = 0.5$ min) could be obtained, before clumping and precipitation occurred; at an even higher concentration of Ca^{2+}, e.g., 3.0 mM, clumping and precipitation immediately occurred, before any reading could be taken. Fig. 1B–D show the

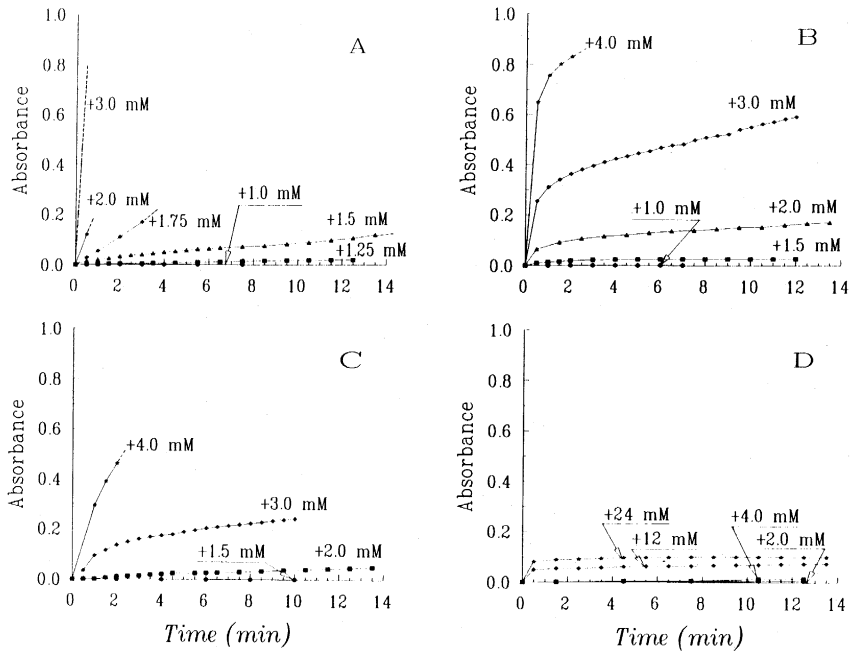

Fig. 1. Time course of Ca^{2+}-induced aggregation, monitored by turbidity measurement (absorbance at 660 nm), for sonicated suspensions containing (A) 1.0 mM DLPA only, as a control sample; (B) 1.0 mM DLPA and 2 mol% Hostapal N-040; (C) 1.0 mM DLPA and 2 mol% Hostapal N-100; and (D) 1.0 mM DLPA and 2 mol% Hostapal N-300. The Ca^{2+} concentration after initial mixing is indicated for each curve. The dashed line denotes that clumping occurs and no stable reading could be obtained.

aggregation behaviors for the corresponding PEO-surfactant containing DLPA suspensions (2 mol% of Hostapal N-040, N-100, or N-300 in Fig. 1B, C, or D, respectively). With increasing PEO chain length (the number of EO subunits increased from 4 to 30), the suspensions could tolerate higher calcium concentration before clumping and precipitation set in, i.e., 4.0 mM in Fig. 1B and C and even more than 24 mM in Fig. 1D. Another way of comparison is to look at the curves corresponding to the same added calcium concentration, e.g., 2.0 mM. With increasing PEO chain length, both the initial aggregation rate (defined as the initial slope of the curve, dD/dt, at $t \rightarrow 0$, where D denotes the optical density at 660 nm and t is the measuring time), and the 'final' D_{10} (at $t = 10$ min) became lower. An obvious protection (inhibition) effect against aggregation by PEO chains on the lipid surface was evident from this set of data.

QLS was applied to measure the size change of the particles during the aggregation process and the extent of aggregation. The QLS results of a set of experiments under identical conditions to that in Fig. 1A to D are shown in Fig. 2A–D. The results presented a similar protection effect with increasing PEO chain length. This set of direct size measurement curves supported the turbidity measurement as a good monitoring of the aggregation process. Furthermore, this set of QLS data showed that the initial sizes in the control sample (pure DLPA suspension) and PEO-surfactant containing samples were the same within the experimental uncertainties, i.e., all were about 30 nm in diameter.

Since many factors (lipid concentration, surfactant to lipid molar ratio, divalent calcium cation to phosphatidic acid ratio, etc.) may influence the aggregation process, we have examined several major parameters in the following sets of experiments.

First, aggregation was monitored at a reduced surfactant to lipid molar ratio (S/L) of 1 mol%, keeping the lipid concentration unchanged (1.0 mM). The curves are shown in Fig. 3A–D). As expected, lower S/L ratio reduced the protection ability for the PEO-surfactant containing particles. When we compared the corresponding curves in Fig. 3 and Fig. 1, it was obvious that for any particular Ca^{2+} concentration, e.g., 2.0 mM, the initial rates as well as the 'final' D_{10} of the curves in Fig. 3 were always higher than that in Fig. 1. Increasing inhibition against aggregation was also found with increasing PEO chain length at this low S/L ratio (Fig. 3A–D). However, a

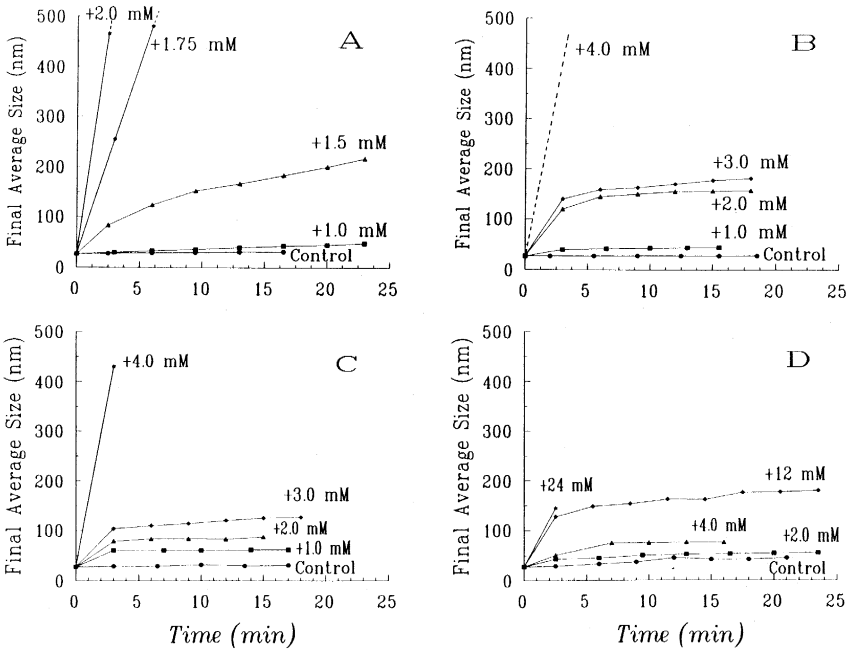

Fig. 2. Same as in Fig. 1, but monitored by QLS, final average size measurement.

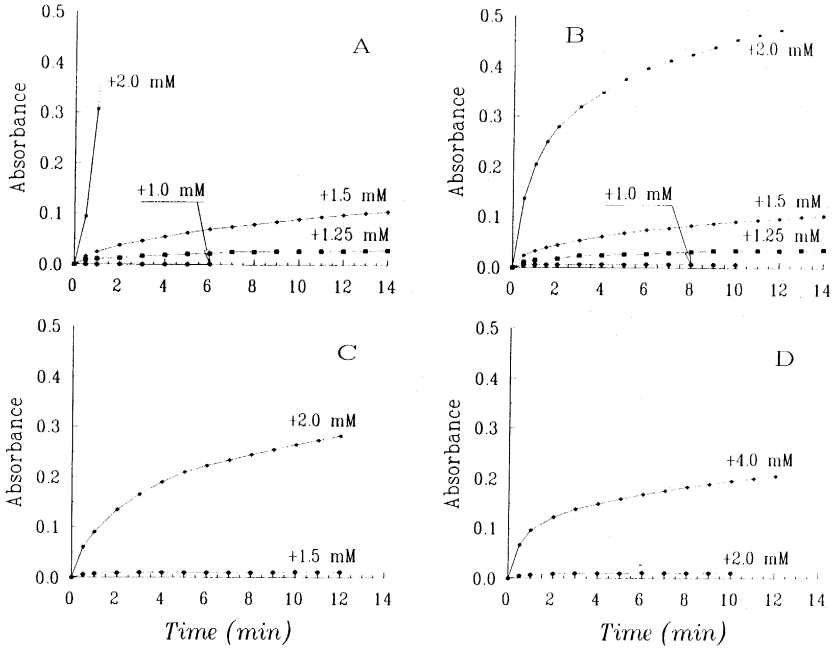

Fig. 3. Same as in Fig. 1, but for B, C, and D, sample containing 1 mol% of corresponding PEO-surfactant.

weak *promotion* effect of PEO surfactant (Hospatal N-040) was found by comparing the corresponding 1.5 mM and 1.25 mM Ca^{2+} curves in Fig. 3A and B. This promotion effect was not yet very obvious for these two pairs of curves.

The promotion effect was more apparent in more

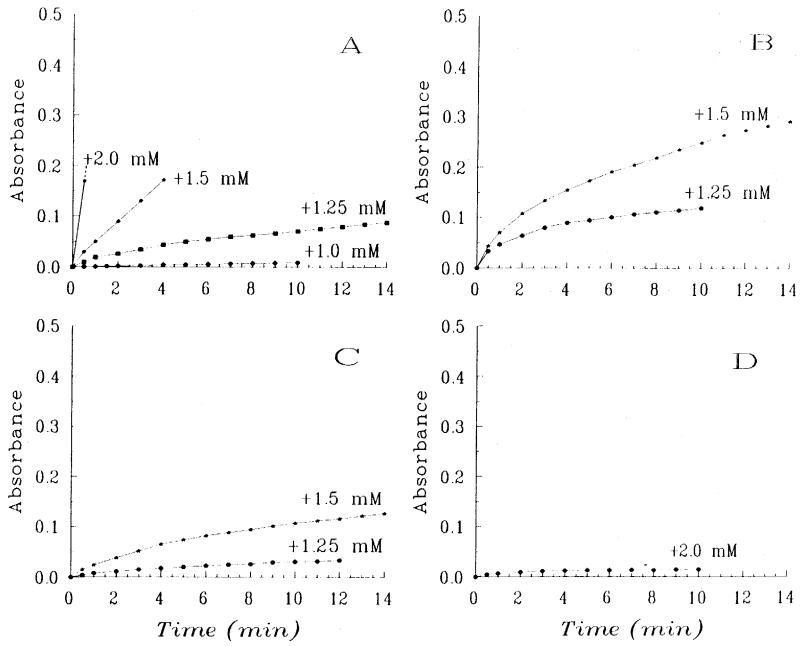

Fig. 4. Same as in Fig. 3, but all four samples are diluted twofold. DLPA concentration is 0.5 mM for B to D.

diluted samples when the aggregation rate was lower. By simply diluting (without changing the S/L molar ratio) the lipid concentration to 0.5 mM for all four samples (one control and three surfactant containing samples), more favorable conditions for aggregation detection could be reached for 1.5 mM and 1.25 mM of added Ca^{2+}. Under these conditions, the promotion effect was more apparent (comparing, for example, the corresponding 1.25 mM Ca^{2+} curve in Fig. 4A and B). For samples containing longer PEO chains (Fig. 4C and D), the protection effect was still predominant.

Another set of data for samples with higher lipid concentration but lower S/L ratio (4.0 mM lipid and 0.5 mol% PEO-surfactants) is shown in Fig. 5A–D. Under these conditions, a very strong promotion effect was found for the two samples containing relatively short PEO chains ($n = 4$ and 10) surfactants (comparing Fig. 5A with B and C), but the protective effect was barely detectable for that containing longest PEO chain ($n = 30$) surfactant (Fig. 5D). For instance, the aggregation was much more rapid and to a much greater extent in samples containing Hostapal N-040 and N-100 than in control samples, with up to 3.0 mM of added Ca^{2+}. For samples containing Hospatal N-300, only a weak promotion was noted at low calcium concentration (2.0 mM). This was the only case where we found Hostapal N-300 had promotion effect, while in all other cases it had protection effect.

It seems that promotion effect occurs only under conditions that aggregation is just begin to occur, i.e., at low (usually < 1) Ca^{2+}/PA^{-} ratio, low S/L molar ratio and mostly with short chain PEO. To summarize and analyze the data, some of which are shown in Fig. 1, Fig. 3, Fig. 4 and Fig. 5, we attempt to correlate the promotion/protection phenomena with the initial rates of aggregation to provide the plot shown in Fig. 6: the promotion/protection parameter R is defined as the common logarithm of the ratio of the initial aggregation rate of PEO-surfactant containing sample to that of the corresponding control sample under identical conditions

$$R = \log\{[\mathrm{d}D/\mathrm{d}t]/[\mathrm{d}D/\mathrm{d}t]_{\mathrm{o}}\}$$

This parameter is plotted against the initial rates of control samples, $[\mathrm{d}D/\mathrm{d}t]_{\mathrm{o}}$. Each data point in the plot represents a ratio of a pair of initial aggregation

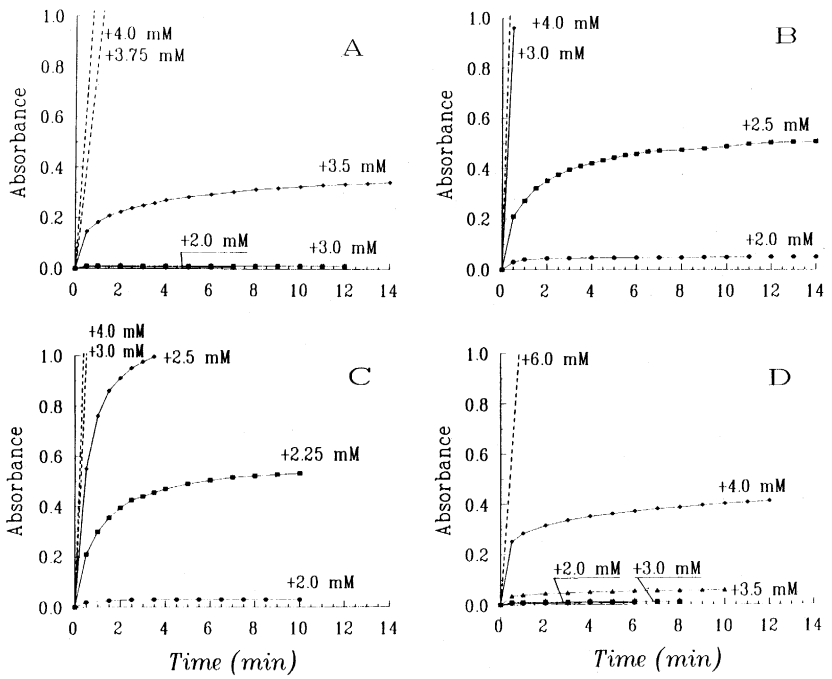

Fig. 5. Same as in Fig. 1, but DLPA concentration is 4.0 mM for all four samples and for B, C, and D, sample containing 0.5 mol% of corresponding PEO-surfactant.

rates; positive points (ratios > 1) represent promotion and negative ones (ratios < 1) protection. Those data pairs whose initial rates for control samples are too rapid to measure are excluded. These missing points always represent strong protection effect, and would be at the far lower right in Fig. 6.

Since the promotion/protection crossover seems to depend on S/L as well as on PEO chain length, both of which are related to the percentage of surface area covered by PEO, the points in Fig. 6 are grouped according to their 'surface coverage' which is determined by PEO chain length (n) and the surfactant to lipid molar ratio (S/L). For a first approximation, each lipid molecule has a surface area of 0.75 nm^2 [9]. The Flory radii of the PEO chains may be estimated as 0.80 nm, 1.7 nm, and 2.5 nm for $n = 4$, 10, and 30, respectively. These radii correspond the areas of 2.0, 9.0, and 20 nm^2, respectively, on the assembly surface to be covered. The ratios of these areas to that of one lipid molecule, 0.75 nm^2, are 2.6, 12, and 26 for $n = 4$, 10, and 30 respectively. Expressing our data for different S/L molar ratios and PEO chain lengths in terms of 'surface coverage', we found that our data fall into two groups: high coverage ($> 4\%$) and low coverage ($< 4\%$). The data points in Fig. 6 form two distinct bands by this grouping criterion. Promotion is found only at very low initial rates for samples of all coverages. At higher initial rates, the promotion effect for low coverage samples diminishes, while protection effect sets in for high coverage samples. The samples with even higher coverage ($> > 4\%$) always have stronger protection effect.

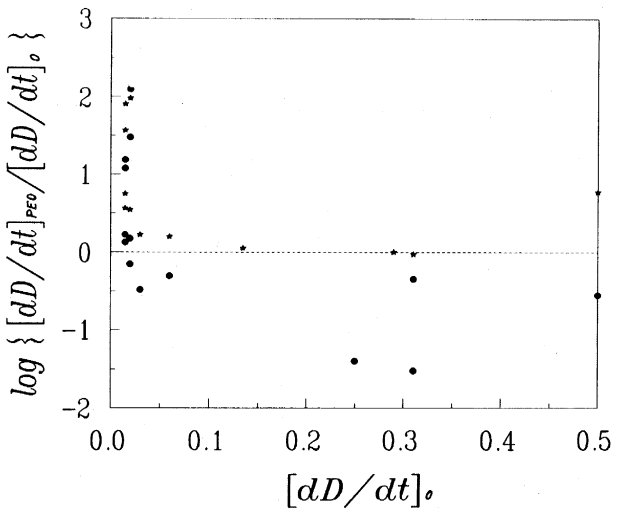

Fig. 6. Grouping promotion/protection by the initial rate of aggregation (see text for details). Positive points represent promotion and negative ones, protection. ★ Denotes a data point from a low surface coverage ($< 4\%$) sample, ● from a high surface coverage ($> 4\%$) sample.

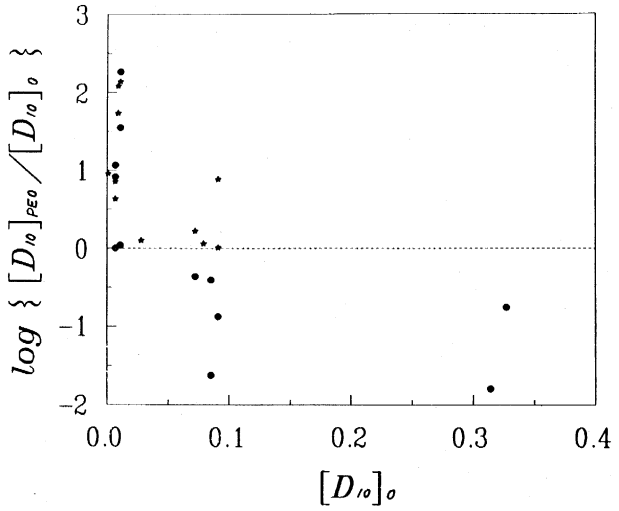

Fig. 7. Grouping promotion/protection by the 'final' D_{10} of aggregation. Legends are the same as in Fig. 6.

A similar way to summarize and analyze the initial rates using another criterion, the 'final' D_{10} (at $t = 10$ min, not in an equilibrium state), is plotted for all possible data pairs and shown in Fig. 7. The same promotion/protection trend again appeared as two bands, resembling those in Fig. 6.

4. Discussion

Despite the fact that steric stabilization has been used to stabilize colloidal solutions for more than six millennia [6], and the intensive study of its mechanisms for more than a half century, starting from the DLVO (Derjaguin-Landau-Verwey-Overbeek) theory to the Alexader–de Gennes model [12–14], the origin of this interaction is still controversial [20]. Recent rapid development of stabilized liposomes in drug and gene delivery requires more thorough and clear theory to explain the phenomena.

Several reports have demonstrated the protective (inhibitive) effect of surface residing PEO derivatives or other macromolecules such as ganglioside GM_1 on

liposome aggregation and fusion [7,21,22]. Force measurements also showed that steric force is responsible for retarding close approach of surfaces containing surface residing PEO derivatives [16,17]. But to our knowledge, the present work is the first to show that PEG conjugating molecules have an aggregation promotion effect.

The obvious contribution of PEG-containing surfactant is the steric hindrance it imposes on the approaching lipid surfaces in the aggregation process. The percentage of lipid surface area covered by the PEG moiety of PEG-containing surfactant can be estimated by the equivalent PEG volume [9]. The molecular masses of the PEG moieties of Hospatal N-040, N-100 and N-300 are 193, 457 and 1337 respectively, so their respective mole percentages for complete coverage of lipid surfaces are approximately 20%, 8% and 4% [9]. The estimate for Hospatal N-040 is not accurate because the non-applicability of short chain polymers to a spherical or semi-spherical volume. If steric hindrance of aggregation/fusion is interpreted to be the prevention of lipid-to-lipid contact by the hemispherical volume of the PEG moiety of PEG-containing surfactant, then by simple geometry, we may calculate the minimum PEG-free area needed for lipid–lipid contact between two lipid spheres of a given size (see Appendix A). We also calculated the corresponding minimum mole percentages of PEG-containing surfactant to prevent lipid–lipid contact between two lipid spherical surfaces of 15 nm in radius, using again the values for the hemispherical footprint of PEG and a surface area of lipid molecule of 0.75 nm^2 [9]. These percentages are 2.44%, 1.19% and 0.71% for Hospatal N-040, N-100 and N-300 respectively. These minimal mole percentages for aggregation prevention, rather than those required for full surface coverage, are more relevant in setting the upper limit of the aggregation promotion window. Indeed we observed that the crossover from promotion to inhibition of aggregation/fusion occur at mole percentages of 1% to 0.5% of PEG-containing surfactants, and in a decreasing order of N-040 > N-100 > N-300 as predicted.

The above calculation assumes that PEG-containing surfactant molecules distribute evenly over the lipid surface. These minimum percentages may be different if the PEG-containing surfactant molecules are distributed randomly rather than evenly. However, since these PEG-containing surfactant molecules move laterally over the lipid surface in time, the values derived from a static model are only an approximation. In the kinetic process of aggregation, the steric hindrance of PEG-containing surfactant is expressed mainly in modulating the probability of having available area for lipid–lipid contact during random particle approach. The probability for vesicle encounter is higher when the lipid concentration is higher. The probability of having a random clearing area for lipid–lipid contact is also higher when the mole percent of surfactants is lower and the reaction rate is slower. This is indeed the case in our measurement of aggregation kinetics. The changeover from inhibition to promotion of aggregation kinetics is observed at high lipid concentration and low PEG coverage (Figs. 4–7) and at low rate of reaction (Fig. 6).

What is the nature of promotion of aggregation by PEG-containing surfactant? To understand the cause for the dual effects of PEG-containing surfactant on lipid aggregation, we must consider the changes of physical environment induced by the grafting of PEG-containing surfactant on lipid assemblies. A theoretical analysis of polymer mediated interaction between charged lipid assemblies and electrolyte solutions is given by Raudino and Bianciardi [15]. According to their analysis, water soluble polymers such as PEG in bulk solution have many effects. Some of those effects, such as the depletion of polymer segments near the lipid surface and the consequent alteration of osmotic forces near the lipid surface, are more relevant to polymers in solution. The detailed contributions of these factors to lipid interfacial interaction have been measured by us [23]. For our case here, the PEG moiety is held close to the lipid surface, while the bulk solution is free of polymers. The most relevant factors, apart from steric hindrance, are (1) the alteration of dielectric characteristics of the lipid environment, and (2) the enhancement of lipid–ion interaction.

The dielectric properties of PEG solution has been measured by Arnold et al. [24]. The dielectric constant varies non-linearly with the concentration of PEG in solution, decreasing from the value of 80 for pure water (concentration of PEG is zero) to about 50 at 50% of PEG, depending on the molecular mass of the PEG. Considering the case of a lipid surface

containing 1 mol% of Hospatal N-040, N-100 or N-300, respectively 5%, 12.5% or 25% of the surface area is covered by the PEG moiety. The surface dielectric constant is slightly lowered ($< 10\%$) by the incomplete PEG shell, according to the values obtained by Arnold et al. [24], thence the electric field from the charged DLPA surface extends slightly further away than that of the control DLPA surface without PEG-containing surfactant. However, the slight change in electric field profile does not seem to be fully responsible for the promotion effect.

A theoretical analysis of PEG effect on lipid assemblies in electrolyte solutions [15] indicates that the presence of low concentrations of PEG favors ion-charged lipid interaction, because of polymer fluctuation. The theory was proposed to be the basis for the synergistic effect of calcium ion and PEG in inducing liposome aggregation and fusion [15]. The effect could be applicable to the case of incomplete coverage of the lipid surface by grafted PEG. At a concentration of 0.5 mol% of Hospatal N-040, N-100 or N-300, the percentage of PEG covered area is only 2.5%, 6.25% and 12.5% of the total lipid surface area. The uncovered DLPA area is sufficiently large for lipid–lipid contact. Enhanced calcium binding to these areas will promote the aggregation/fusion process. We did not determine the lower limit of PEG-containing surfactant concentration of the promotion window, but we expect that the promotion effect would diminish at a PEG-containing surfactant concentration somewhat less than 0.5 mole, when the ion binding enhancement also diminishes with PEG-containing surfactant concentration.

Could this promotion be caused by a calcium modification of the PEO moiety? First, with calcium concentration in the submolar range, we expect only an extremely weak salt effect on PEO chains. This was verified by a set of comparison experiments: replacing negatively charged DLPA by neutral egg lecithin and keep all other conditions identical, we found no calcium-induced turbidity change in samples containing PEO-surfactants (data not shown). Therefore, the promotion effect cannot be explained by the direct salt effect on PEO chains only. Secondly, in the presence of negatively charged DLPA, surface-bound calcium may modify the aggregation behavior of the PEO-containing particles by changing the conformation of the PEO moiety and/or surface hydrophilicity. Although this is possible due to the strong electrostatics, dehydration and rearrangement effects of calcium on the surface of DLPA particles, we need further direct experiments to support this hypothesis.

Although the promotion condition window in our case is narrow with respect to charge neutralization, PEO-coverage and reaction rate, it should be taken into consideration when applying the steric repulsion principle to liposome stabilization. Our results demonstrate that incorporating PEO-containing lipid in liposomes does not invariably lead to stabilization. This is especially important for the in vivo experiments with the calcium ion concentration is in the millimolar region, where the combined effect of nonionic PEO and divalent calcium cations may play some role.

Acknowledgements

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Appendix A

Let the radius of the hemispherical PEG moiety of the surfactant to be r, and the radius of the lipid

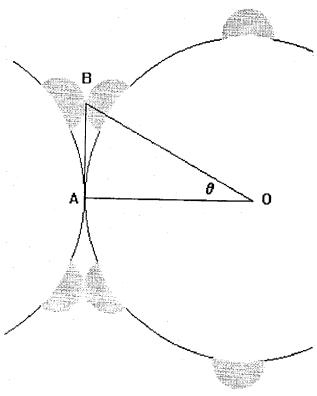

Fig. 8. The geometry for minimal lipid surface clearing which allows lipid–lipid contact between adjacent vesicles containing hemispherical PEG moieties (grey shaded) on their surfaces. The size of PEG moieties are greatly exaggerated in the drawing for clarity.

vesicle (or other spherical assemblies) to be R. In our case, $R \gg r$. The minimal bilayer surface clearing which allows lipid–lipid contact between adjacent vesicles is depicted in Fig. 8. Here the PEG moieties of adjacent vesicles on the periphery of the clearing area just touch. The solid angle θ subtaining the cone defined by rotating OB about OA, as a fraction of 4π, gives the approximate clearing area as a fraction of the entire vesicle surface. When $R \gg r$, this fraction may be approximated as $2\cos^{-1}\{(R + r)/R\}/2\pi$.

Using this clearing area fraction and the areas occupied by single lipid molecules and PEG moieties, the mole percent of PEG-containing surfactant at the critical concentration for each molecular mass moiety may be evaluated.

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