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Phase Behavior and Aggregate Structure in Mixtures of Dioleoylphosphatidylethanolamine and Poly(Ethylene Glycol)-Lipids

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ABSTRACT Cryo-transmission electron microscopy has been used to investigate the phase behavior and aggregate structure in dilute aqueous mixtures of dioleoylphosphatidylethanolamine (DOPE) and poly(ethylene glycol)-phospholipids (PEG-lipids). It is shown that PEG-lipids (micelle-forming lipids) induce a lamellar phase in mixtures with DOPE (inverted hexagonal forming lipid). The amount of PEG-lipid that is needed to induce a pure dispersed lamellar phase, at physiological conditions, depends on the size of the PEG headgroup. In the transition region between the inverted hexagonal phase and the lamellar phase, particles with dense inner textures are formed. It is proposed that these aggregates constitute dispersed cubic phase particles. Above bilayer saturating concentration of PEG-lipid, small disks and spherical micelles are formed. The stability of DOPE/PEG-lipid liposomes, prepared at high pH, against a rapid drop of the pH was also investigated. It is shown that the density of PEG-lipid in the membrane, sufficient to prevent liposome aggregation and subsequent phase transition, depends on the size of the PEG headgroup. Below a certain density of PEG-lipid, aggregation and phase transition occurs, but the processes involved proceed relatively slow, over the time scale of weeks. This allows detailed studies of the aggregate structure during membrane fusion.

INTRODUCTION

Dioleoylphosphatidylethanolamine (DOPE) forms an inverted hexagonal (H_{II}) phase above 10–15°C at near neutral or acidic pH (Cullis and De Kruijff, 1978). The H_{II} phase coexists with a water phase in the dilute region of the phase diagram. Poly(ethylene glycol) derivatized phospholipids (PEG-lipids), on the other hand, form spherical micelles in dilute aqueous solution (Kenworthy et al., 1995; Edwards et al., 1997). The phase behavior of mixtures of PEG-lipids and lamellar (L_α) forming lipids has been investigated by means of NMR (Kenworthy et al., 1995), x-ray diffraction (Kenworthy et al., 1995; Hristova et al., 1995), and in dilute aqueous solution by electron spin resonance (ESR) (Belsito et al., 2000) and cryo-transmission electron microscopy (c-TEM) (Edwards et al., 1997). The general results are that at some critical concentration of PEG-lipid in the membrane, which depends on the lipid composition and the size of the PEG headgroup, micelles are formed. In an earlier study we showed that before micelles are formed (which may be cylindrical or spherical, depending on lipid composition), a formation of bilayer disks takes place (Edwards et al., 1997).

It is clear that PEG-lipids should exhibit a complementary shape to that of lipids, preferring an inverted lipid phase, such as DOPE. By adding PEG-lipids to DOPE, we therefore expect a phase transition to a lamellar phase. An estimation of the amount of PEG-lipid needed to induce a pure (dispersed) lamellar phase is accordingly of interest to determine. In addition, intermediate structures formed in the phase transition region should display similarities to structures formed during temperature- or pH-induced L_α-to-H_{II} phase transition in, for example, pure phosphatidylethanolamine (PE) systems. Furthermore, the amount of PEG-lipid that can be incorporated in the bilayer before any structural transitions or micelle formation occur should give information about the effect of lipid composition on the phase behavior in phospholipid/PEG-lipid systems.

PEG-lipids have been utilized by Holland et al. (1996a) to promote the formation of a lamellar phase in mixtures of DOPE/cholesterol. At PEG-lipid concentrations of ~10 mol %, a large isotropic signal dominated the ^{31}P-NMR spectrum. This signal was attributed by Holland et al. to arise from small vesicles and lipidic particles. In the present study, we show that in mixtures of DOPE and low concentrations of PEG(750)-lipid or PEG(2000)-lipid, particles with a fine inner texture coexist with liposomes. Koynova et al. (1999) found in mixtures of dielaidoylphosphatidylethanolamine (DEPE) and DMPE-PEG(550) a cubic phase between the L_α and H_{II} phases, above 5 mol % DMPE-PEG(550) and at ~75°C. In relation to this result, we discuss the possibility of cubic phase formation in the present systems.

Cubic phases are usually difficult to disperse. However, in recent studies, Gustafsson et al. (1996, 1997) have shown that glycerol monooleate (GMO)-based cubic phases may be dispersed if a steric stabilizer is added. The triblock copolymer Poloxamer 407 ($PEO_{98}PPO_{67}PEO_{98}$) was used as dispersing agent. This polymer has two water-soluble PEO (PEG) chains and a more hydrophobic middle block that may be adsorbed at, or incorporated in, the surface of the particle. Although the molecular nature of such triblock copolymers may seem very different from PEG-lipids, the two types of dispersing agents may have a similar effect on the colloidal stability of the dispersed particles. In the

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present study we show that PEG-lipids do indeed act as stabilizers for the presumably cubic particles.

PE systems are the most commonly investigated lipid systems concerning the L_α-to-H_{II} transition (in dilute aqueous solution). In principal, there are two ways to generate the phase transition in these systems. The phase transition may be induced by increasing the temperature above the lamellar-to-inverted hexagonal phase transition temperature, T_H (Cullis and De Kruijff, 1978; Ellens et al., 1986, 1989; Allen et al., 1990; Siegel et al., 1994). Alternatively, the lamellar phase may be dispersed as liposomes at high pH (pH $>$ 9) where the PEs are negatively charged and therefore prefer to adopt the L_α phase. The phase transition is then induced by a rapid decrease of the pH to near neutral or acidic pH (Ellens et al., 1986; Siegel and Epand, 1997), where the PEs are zwitterionic and prefer inverted lipid phases, given that the temperature is above T_H.

The development of c-TEM has greatly improved the understanding of the mechanisms involved as detailed information about intermediate structures may be obtained with a minimum of sample perturbation (Siegel et al., 1994; Siegel and Epand, 1997). However, in most cases, samples from PE systems intended for c-TEM investigations must be viewed within a very short period of time after preparation. This is especially obvious in the case of the pH-induced phase transitions. In these cases, aggregation occurs very rapidly, within seconds, which ultimately precludes the possibility of c-TEM observation (Siegel and Epand, 1997). In this study we report on DOPE/PEG-lipid systems where aggregation and phase transitions occur over the time scale of days or even weeks. This enables detailed studies of the structure and phase behavior as well as the colloidal stability.

MATERIALS AND METHODS

Materials

1,2-Dioleoylphosphatidylethanolamine (DOPE) and PEG-lipids, with PEG of molar mass 2000 or 750 covalently attached via a carbamate linkage to 1,2-distearoylphosphatidylethanolamine (DSPE-PEG(2000) or DSPE-PEG(750)), were purchased from Avanti Polar Lipids (Alabaster, AL). The lipids were used without further purification. All other salts and reagents were of analytical grade and were used as received.

Sample preparation

Three types of dispersion procedures were employed as described below.

DOPE/PEG-lipid mixtures were prepared by co-dissolving the lipids in chloroform. The chloroform was removed under vacuum for at least 12 h. A glycine buffer (20 mM glycine, 150 mM NaCl, pH 9.6, 0.1 mM EDTA) was then added to the lipid films to give a total lipid concentration of 0.5 wt % (5 mg of lipid/ml). The samples were then subjected to at least 10 freeze-thaw cycles (including freezing in liquid nitrogen and thawing at room temperature), and the homogeneity of the dispersions was checked by visual inspection and by observation between crossed polarizers. Large unilamellar liposomes were produced by multiple extrusion of the lipid mixtures through polycarbonate filters (pore size 100 nm) mounted in a LiposoFast mini-extruder from Avestin (Ottawa, Canada). The extruded liposomal samples were then diluted with glycine buffer to 3.6 mg of lipid/ml. To investigate the pH dependence of the phase behavior, the pH was rapidly dropped to pH 5 by mixing the lipid suspension with an equal volume of an acetic acid/acetate buffer (50 mM NaAc, 100 mM NaCl, 0.1 mM EDTA, pH 4.5), as described by Siegel and Epand (1997). Samples for c-TEM were flushed with nitrogen and incubated in the dark at 25°C until TEM observation.

Dried lipid films were obtained as described above. Hepes buffer (20 mM Hepes, 150 mM NaCl, pH 7.4, 0.01% NaN_3) was then added to the dried lipid films to give a total lipid concentration of 1 wt %. The samples were thereafter subjected to at least 10 freeze-thaw cycles (including freezing in liquid nitrogen and heating to 40°C) with intermittent vigorous vortex mixing. The homogeneity of the dispersions was checked by visual inspection and by observation between crossed polarizers. It should be emphasized that samples with low concentrations of PEG-lipid required a larger number of freeze-thaw cycles to obtain homogeneous dispersions. Pure DOPE forms an inverted hexagonal phase above 10–15°C and was, as expected, poorly dispersible at pH 7.4. The samples were put in small glass vials, flushed with nitrogen, and incubated in the dark at 25°C for the indicated periods of time.

Dried lipid films of the lipid mixtures were obtained as described above. Hepes buffer was added to give a total lipid concentration of 1 wt %. The samples were then subjected to ultrasonic irradiation using a Soniprep 150 from MSE Scientific Instruments (Crawley, UK). The sonication was performed in periods of ~15 min at an amplitude of 8–10 μm. Visual inspection of the samples was employed between the periods of sonication to check sample homogeneity. The temperature during sonication was controlled by means of a water bath held at 20–25°C. Typically, for samples with low concentrations of PEG-lipid, four periods of sonication were necessary to disperse the lipids. After sonication, the samples were centrifuged at low speed to remove any metal debris from the probe, flushed with nitrogen, and incubated in the dark at 25°C for the indicated periods of time.

Turbidity measurements

A Hewlett Packard 8453 UV-visible spectrophotometer connected to a LAUDA RC6 CP thermostat set to 25°C was used at a wavelength of 350 nm. Lipid suspensions at pH 9.6 prepared as above at 3.6 mg of lipid/ml were mixed directly into quartz cuvettes with equal volumes of acetic acid/acetate buffer at pH 4.5. The resulting pH was measured on separately prepared samples and was shown to be pH 5. The turbidity of the dispersions was followed as a function of time up to 15 h.

Cryo-transmission electron microscopy

Electron microscopy investigations were performed with a Zeiss 902 A instrument, operating at 80 kV. The sample preparation procedure, described in detail in a recent review (Almgren et al., 2000), consists in short of the following. A thin film of the sample solution was prepared by a blotting procedure, performed in a custom-built environmental chamber with controlled temperature (25°C) and humidity. A drop of the solution was placed onto a copper EM grid coated with a perforated polymer film. Excess solution was removed by means of a filter paper, leaving a thin film of the solution to span the holes of the polymer film. Vitrification of the thin film was achieved by rapidly plunging the grid into liquid ethane held just above its freezing point. The vitrified specimen was thereafter transferred to the microscope. The temperature was kept below 108 K during both the transfer and viewing procedures to prevent sample perturbation and formation of ice crystals.

Some specific features of the c-TEM preparation and viewing procedures need to be emphasized. First, the copper EM grids used in the present study were coated with holey polymer films after which a carbon layer was evaporated onto both sides of the grids. The carbon layers confer mechan-

ical stability to the vitrified water matrix. However, the carbon layers are radiation sensitive, and radiation damage of the carbon/polymer films is evident even at very small electron exposures. The result is a bubbly appearance of the carbon/polymer films in the c-TEM micrographs (see Results). In this respect it is important to emphasize that the bubbly appearance of the polymer support films does not indicate radiation damage of the vitrified sample film spanning the holes in the support film. The electron exposures were determined, without inserted sample but at the same intensity as for the samples, via a microscope-built-in sensor and were between 5 and 15 $e^-/Å^2$, depending on the magnification. Second, the extent of under-focus varies somewhat between the presented images. This is mainly due to small variations in film thickness and/or a slightly buckled surface. Large under-focusing results in decreased resolution. However, this effect does not affect the general appearance of the observed structures.

RESULTS

Phase transitions induced by pH changes

As shown in previous studies, pure DOPE can be dispersed as liposomes at high pH (Ellens et al., 1986). At high pH the primary amine in the PE headgroup is deprotonated and the phospholipid acquires a negative charge. By rapidly lowering the pH (pH $\leq$ 5), a phase transition to H_{II} occurs, which for DOPE at 25°C takes place within seconds. To investigate the effect of adding PEG-lipids to the DOPE membrane on the pH-induced phase transition, we followed the process by turbidity and c-TEM measurements.

As shown in Fig. 1, the turbidity of liposomes containing 2.5 mol % PEG(2000)-lipid or 6 mol % PEG(750)-lipid changed very little during the time scale of the experiment. In contrast, the turbidity of liposomes containing 3 mol % PEG(750)-lipid increased several-fold. As expected, pure DOPE liposomes aggregated within seconds and a phase separation took place (not shown). The results indicate that 2.5 mol % PEG(2000)-lipid or 6 mol % PEG(750)-lipid is sufficient to prevent rapid aggregation and subsequent phase transitions in these systems. In fact, 1 month after preparation, the above samples showed only a small turbidity increase. These results are in agreement with results presented by Holland et al. (1996b) who showed that addition of 2 mol % DMPE-PEG(2000) to DOPE/POPS (1/1) liposomes was sufficient to inhibit Ca^{2+}-induced fusion. It is also clear that for 3 mol % PEG(750)-lipid, aggregation occurs but proceeds much slower than for pure DOPE liposomes.

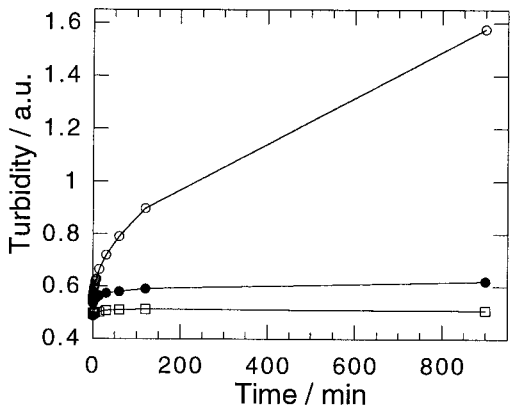


FIGURE 1 Turbidity of extruded DOPE/PEG-lipid liposomes (1.8 mg lipid/ml) as a function of time after a pH jump from pH 9.6 to pH 5. ○, 3 mol % PEG(750)-lipid; □, 6 mol % PEG(750)-lipid; ●, 2.5 mol % PEG(2000)-lipid. Liposomes were prepared as described in Materials and Methods.

C-TEM investigations of the above samples revealed that for the samples containing 2.5 mol % PEG(2000)-lipid or 6 mol % PEG(750)-lipid at pH 5, very little change occurred compared with the same samples at pH 9.6 (not shown). However, prolonged incubation resulted in a slightly larger fraction of aggregated liposomes, although it is not possible by c-TEM to determine the absolute magnitude of aggregation. In contrast, as shown in Fig. 2, the sample containing 3 mol % PEG(750)-lipid displayed a significantly different behavior. After 20 h of incubation, a substantial number of liposome clusters were observed (Fig. 2 *b*). Further incubation resulted in the formation of particles with a seemingly dense inner structure (Fig. 2 *c*). After 1 month of storage, the dispersion was highly turbid and relatively large particles displaying a dense inner structure were observed (Fig. 2 *d*). Interestingly, partly fused liposomes appeared at the surfaces of the particles. We also note that although the majority of the material in the samples at pH 9.6 was found in nicely shaped unilamellar liposomes (Fig. 2 *a*), a minor fraction of the particles displayed invaginations or was observed as small clusters of liposomes.

It may of course be argued that hydrolysis of DOPE, which is expected to be faster at pH 5 compared with near neutral pH, can cause the observed structural transitions (Fig. 2, *a–d*). We therefore extracted the lipids using a Bligh-Dyer extraction (Bligh and Dyer, 1959) and subsequently investigated the composition using thin-layer chromatography ($CHCl_3$:MeOH:H_2O, 65:25:4 (v/v/v)). We found that after 2 months of storage at pH 5, the samples contained surprisingly little lyso-PE. Although we could not determine the absolute amount of lyso-PE, it was likely well below 10% as judged by the intensities of the ninhydrin-positive spots from DOPE and lyso-PE. Furthermore, because the samples containing 2.5 mol % PEG(2000)-lipid or 6 mol % PEG(750)-lipid showed very little change compared with the reference liposomes at pH 9.6, we conclude that the structural transitions occurring during the first weeks of incubation are probably not affected by lipid hydrolysis.

Phase behavior of DOPE/PEG(2000)-lipid

In the following section, results from dispersions in Hepes buffer (pH 7.4) prepared by freeze-thaw cycling will be presented.

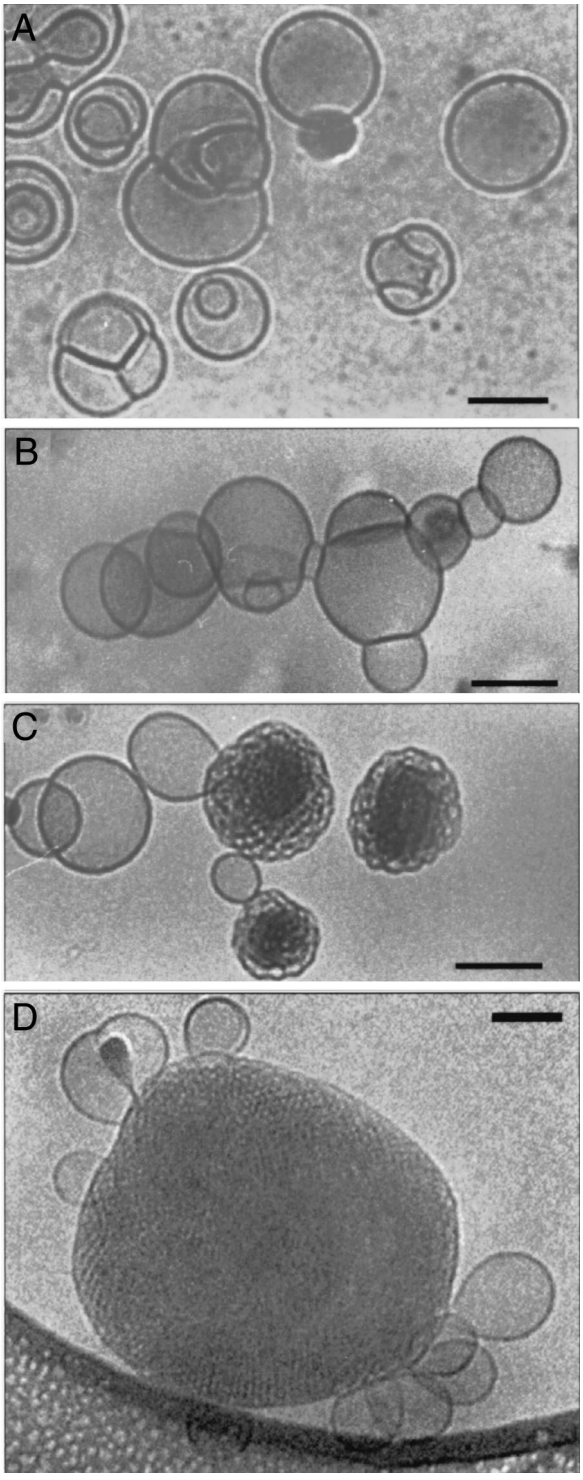


FIGURE 2 Cryo-TEM micrographs of extruded DOPE/PEG(750)-lipid liposomes (1.8 mg lipid/ml) containing 3 mol % PEG(750)-lipid at pH 9.6 incubated for 24 h (*a*), pH 5 incubated for 24 h (*b*), pH 5 incubated for 4 days (*c*), and pH 5 incubated for 1 month (*d*). See text for more information. The dark dots seen in *a* represent ice crystals deposited on the sample surface after vitrification. Liposomes were prepared at pH 9.6 and the pH was reduced to pH 5 by mixing the liposomal dispersions with an equal volume of acetic acid/acetate buffer, as described in Materials and Methods. Bar, 100 nm.

Fig. 3 shows micrographs obtained from DOPE/PEG(2000)-lipid samples. At low PEG-lipid concentration (2.5 mol %), the coexistence of large liposomes and particles displaying complex morphologies can be seen (Fig. 3 *a*). Liposomes showing membrane passages or interlamellar attachments (ILAs) can also be observed. The density of such membrane passages varied to a large degree between the observed particles. The time dependence of the phase and structural behavior is clearly displayed in Fig. 3 *b* where the micrograph is obtained from the same sample but after prolonged storage (2 weeks). Now, particles with a dense inner structure of hexagonal symmetry can be observed in coexistence with relatively large liposomes. The periodicity of the inner structure may be estimated to be approximately 130 Å (±15%). Note also that the outermost surface region displays lamellar features and that the even contrast of the particles suggests that they are relatively thin or flat objects. In principle, it is possible that the hexagonal symmetry of the inner structure is a result of superposition of stacks of membranes oriented at different angles. However, the observed aggregates are discrete particles, and it seems unlikely that such a large number of particles should display the same type of inner structure, with the same periodicity, if it were due to stacks of membranes oriented at different angles. It should be emphasized that the colloidal stability of the dispersion with 2.5 mol % PEG(2000)-lipid was limited, and after approximately 1 month of storage, sedimented material could be observed by visual inspection. Increasing the concentration of PEG(2000)-lipid to 8 mol % resulted in a dispersion consisting of essentially only large liposomes (Fig. 3 *c*). Note that (spherical) particles larger than approximately 500 nm in diameter cannot be observed using c-TEM. Furthermore, because the thickness of the films spanning the holes of the c-TEM grid varies, being thinner in the center of the hole and thicker at the edges, there is a risk of misleading size determinations. Therefore, if accurate size distributions are to be determined, inspection of a large number of micrographs as well as repeated preparations is necessary. It is also possible that other types of structures are present in the samples but not observable by c-TEM. However, judging from a large collection of micrographs, it appears that a pure (dispersed) lamellar phase is reached at ~8 mol % PEG(2000)-lipid. The appearance of samples with 8–17.5 mol % PEG-lipid was very similar; large and predominantly unilamellar liposomes were observed in this region. Above 17.5 mol % PEG-lipid, coexistence of liposomes of varying size, small disk-like fragments and spherical micelles was observed as shown in Fig. 3 *d*. To find the phase boundary between the lamellar and micellar phase, the concentration of PEG-lipid was progressively increased. At 60 mol % PEG-lipid, the majority of the observed structures were spherical micelles although a minor fraction of rather small unilamellar liposomes still existed (not shown). We conclude that the micellar one-phase region is

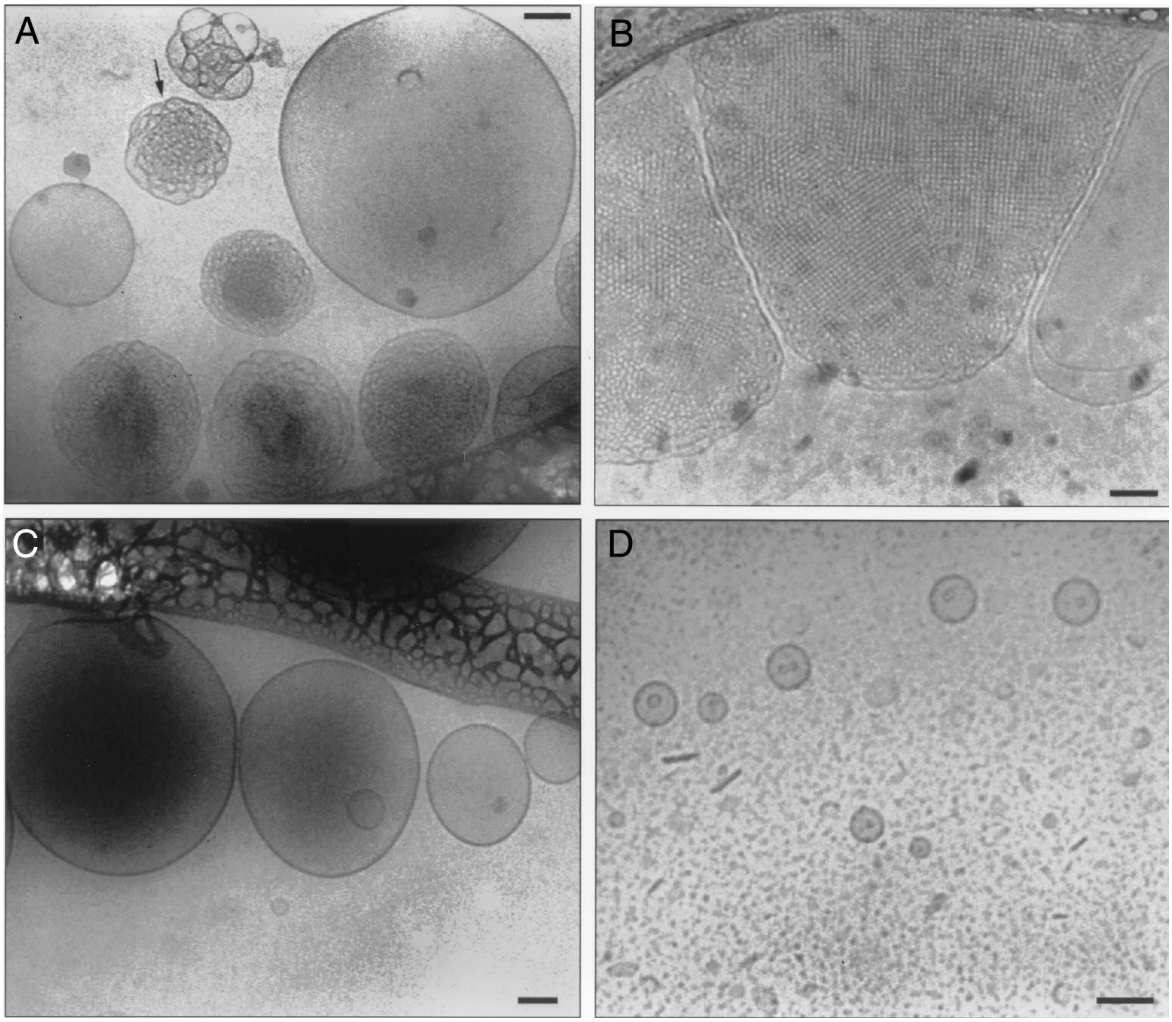


FIGURE 3 Cryo-TEM micrographs of freeze-thaw cycled dispersions (1 wt % lipid, pH 7.4) of DOPE and PEG(2000)-lipid in concentrations of 2.5 mol % incubated 2 days (*a*), 2.5 mol % incubated 2 weeks (*b*), 8 mol % incubated 2 days (*c*), and 50 mol % incubated 2 days (*d*). The arrow in *a* denotes a membrane passage or interlamellar attachment (ILA) as seen from the side. Particles displaying an inner texture of hexagonal symmetry with a periodicity of approximately 130 Å can be observed in *b*. Note also disks and spherical micelles in *d*. The disks are easiest observed edge-on (dark lines) and the spherical micelles are observed as small dark dots. See text for more information. The samples were prepared as described in Materials and Methods. Bar, 100 nm.

reached at a concentration of PEG(2000)-lipid slightly larger than 60 mol %.

Effect of dispersion procedure on aggregate structure

One difficulty regarding the phase behavior of lipid systems in dilute aqueous solution is to determine whether the observed structures depend on the preparation procedure and, in relation to this, whether the structures that are formed represent dispersed structures of equilibrium phases. To address the issue of different dispersion techniques, we prepared samples containing low amounts of PEG-lipid using sonication. Sonication typically yields a higher-energy input than freeze-thaw cycling, and for dispersed phosphatidylcholine (PC) lamellar phases this means that small unilamellar liposomes with radii of ~15 nm are formed. Fig. 4 shows micrographs obtained from sonicated DOPE/PEG(2000)-lipid dispersions in Hepes buffer. At 2.5 mol % PEG-lipid (Fig. 4 *a*), liposomes with membrane passages (ILAs), small liposomes, and particles with more densely spaced membrane passages can be observed. This result is similar to the micrographs shown in Fig. 3, *a* and *b*, obtained from freeze-thaw cycled dispersions. There are, however, some differences in that the particles seen in Fig. 4 on average are smaller and do not display the same ordered inner structure as the larger particles in Fig. 3 *b*. The fact that the particles are smaller is not surprising considering the higher-energy input during sonication. The development of particles displaying ordered inner structures may be size

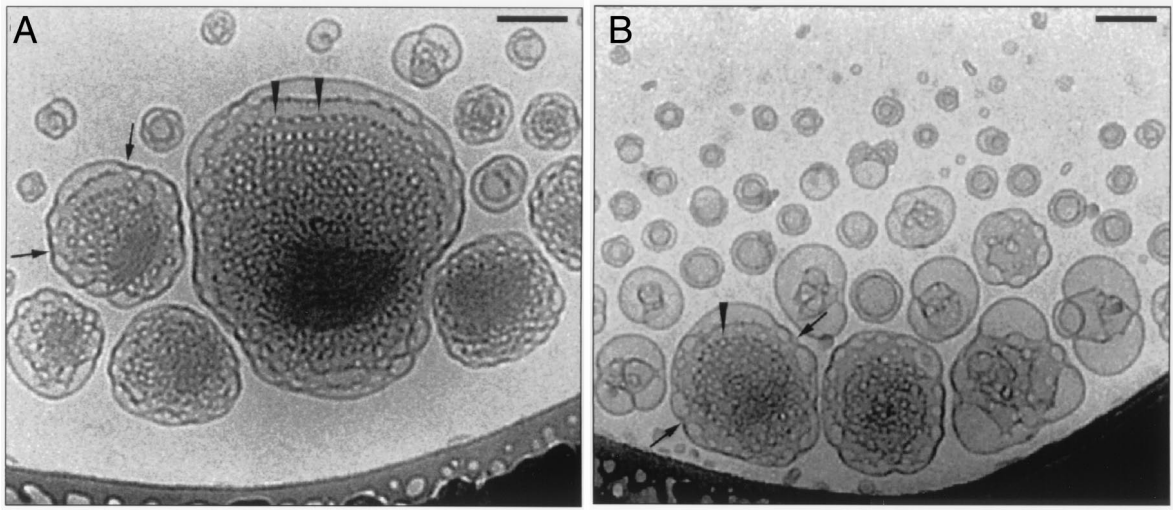


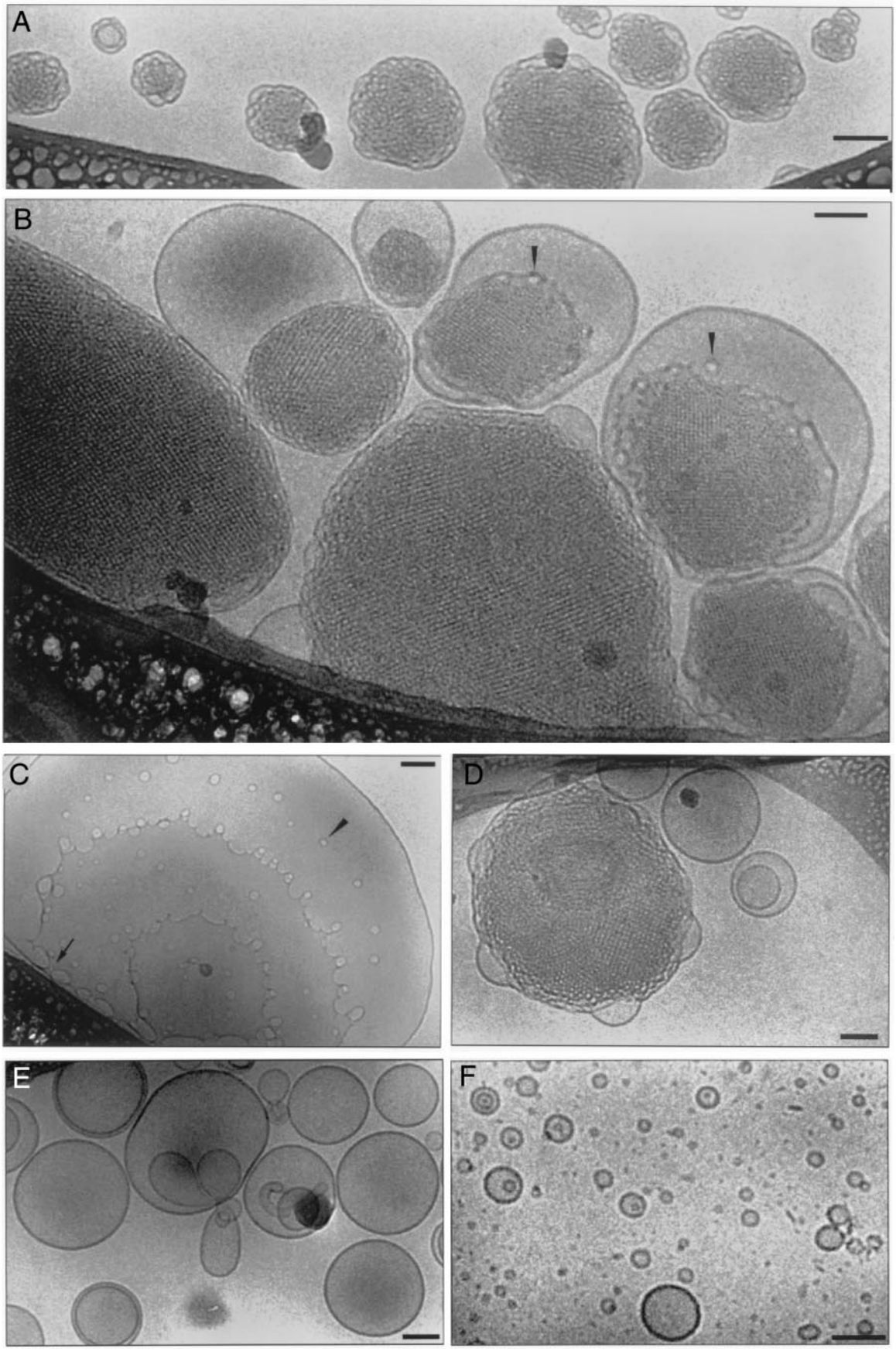
FIGURE 4 Cryo-TEM micrographs of sonicated dispersions (1 wt % lipid, pH 7.4) of DOPE and PEG(2000)-lipid in concentrations of 2.5 mol % incubated 2 days (*a*) and 5 mol % incubated 4 days (*b*). Arrows denote ILAs as seen from the side. Arrowheads denote ILAs viewed down their axes. See text for more information. The samples were prepared as described in Materials and Methods. Bar, 100 nm.

and time dependent, which can explain the absence of ordered inner structures of the relatively small particles formed during sonication. Note that although the above results show that the structural behavior does not strongly depend (except particle size) on the actual dispersion procedure (given that one starts from the same conditions in terms of pH, salt content, etc.), further studies are needed to establish the exact nature of the equilibrium phases in the transition region between H_{II} and L_{α}.

Phase behavior of DOPE/PEG(750)-lipid

The PEG(750) headgroup is considerably shorter than PEG(2000), and from simple shape considerations we therefore expect that more PEG(750)-lipid must be added to reach a pure dispersed lamellar phase and that the lamellar phase (liposomes) should be able to accommodate more PEG(750)-lipid compared with PEG(2000)-lipid before micelle and/or disk formation occurs. Below we show the results of the c-TEM investigations of the DOPE/PEG(750)-lipid system.

Fig. 5 *a* displays a micrograph obtained from a sonicated sample with 3 mol % PEG(750)-lipid. The presence of relatively small particles with membrane passages and seemingly dense inner structures can be observed. In addition, small liposomes were present in the sample. Prolonged incubation resulted in an increased turbidity, and as shown in Fig. 5 *b*, larger particles with a periodic dense inner structure can be observed. The periodicity of the inner structure is estimated to be 100 Å (±15%). Most of the particles displayed lamellar features at the surface. As is also evident, many of the aggregates consisted of areas with the dense inner structure, ILAs, and areas resembling that of normal liposomes. We also found large lamellas with a more or less random location of ILAs as shown in Fig. 5 *c*. The colloidal stability of this dispersion was limited, and after 1 month of storage, sedimented material was observed. Note also that this sample was prepared by sonication (in Hepes buffer) because we found that freeze-thaw cycling was insufficient to obtain a homogenous dispersion at pH 7.4. At 6 mol % PEG(750)-lipid, structures similar to those shown in Fig. 5 *b* were observed, coexisting with large liposomes as shown in Fig. 5 *d*. This sample was prepared by freeze-thaw cycling, but we also used sonication to disperse the sample. The sonicated sample displayed similar structures, although the average size of the particles was smaller (not shown). In contrast to the sonicated dispersion with 3 mol % PEG(750)-lipid, we could not detect any particle growth on the time scale of 1 month. Thus, it seems that 6 mol % PEG(750)-lipid is sufficient to prevent aggregation in accordance with the results described above for the pH-jump investigation. Still, at 10 mol % PEG(750)-lipid, particles with membrane passages of varying density were observed, although the majority of the material was found in liposomes of varying size (approximately 100–500 nm in diameter; not shown). At 15 mol %, however, the only observed structures were relatively large and predominantly unilamellar liposomes, as shown in Fig. 5 *e*. We therefore conclude that a pure (dispersed) lamellar phase is reached at ~10–15 mol % PEG(750)-lipid. The first evidence of micelle formation was found at 35 mol % where liposomes coexisting with small disks and spherical micelles were observed (Fig. 5 *f*). At 80 mol % PEG(750)-lipid, most of the liposomes were solubilized into spherical micelles (not shown). Complete solubilization is therefore expected to occur at concentrations slightly above 80 mol % PEG(750)-lipid.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DISCUSSION

The intermediate structures that are proposed to form upon membrane fusion have been documented also in studies of the L_α-to-H_{II} phase transition in model systems (Siegel et al., 1994; Siegel and Epand, 1997). This fact makes the study of the L_α-to-H_{II} phase transition interesting from both a fundamental and biological point of view. DOPE-based sterically stabilized liposomes have lately received attention (Kirpotin et al., 1996; Slepushkin et al., 1997) due to the potential pH sensitivity of these liposomes, which may be advantageous for intracellular release of encapsulated pharmacological agents. The combination of an increased in vivo stability, due to the PEG-induced steric stabilization, and the potential pH sensitivity makes DOPE/PEG-lipid liposomes attractive aggregates for drug delivery purposes (Slepushkin et al., 1997).

Structures formed at low PEG-lipid concentration

The pH-jump experiments in the present study indicate that the addition of only 2.5 mol % PEG(2000)-lipid or 6 mol % PEG(750)-lipid stabilizes the lamellar phase of the DOPE-based dispersions also at acidic pH. This is in accordance with previous studies on similar systems (Holland et al., 1996b). However, there is a critical PEG-lipid concentration, which depends on the length of the PEG headgroup, below which liposome aggregation, fusion, and subsequent phase transition can occur. This was clearly displayed by the sample containing 3 mol % PEG(750)-lipid where the turbidity measurements indicated a relatively slow but significant aggregate growth. The c-TEM investigation of the above sample showed that there is an initial liposome aggregation with a subsequent structural transition into relatively large particles displaying a dense inner structure (Fig. 2, *a–d*). The nature of the particles that formed at low pH will be discussed in more detail below. The system provides an interesting experimental model because the structural transitions occur over the time scale of weeks. In contrast, the pure DOPE system transforms under the same conditions into the reversed hexagonal phase within seconds.

To investigate the phase and structural behavior at near neutral pH, we prepared samples in Hepes buffer (pH 7.4), using two different dispersion techniques, freeze-thaw cycling and sonication. In both the PEG(2000)- and PEG(750)-lipid systems, particles with dense periodic inner textures of hexagonal symmetry were observed in coexistence with liposomes (Figs. 3 *b* and 5, *b* and *d*). Similar structures were observed regardless of dispersion technique, although the average size of the particles was smaller when using sonication. This can be attributed to the higher-energy input during sonication compared with freeze-thaw cycling. Interestingly, these structures show distinct similarities to those observed by Gustafsson et al. (1997) in dispersions of GMO/Poloxamer 407, at relatively low polymer content. In this case, the particles were proposed to originate from the cubic C_D structure. In a recent study, particles showing inner structures with hexagonal symmetries were observed upon dispersion of an L_3 phase in mixtures of sodium cholate and GMO in brine (Gustafsson et al., 1999). Also in this case, the observed periodicity was proposed to originate from a cubic phase structure. Furthermore, in a c-TEM study of liposome fusion, Basáñez et al. (1997) found structures similar to those observed in the present study in mixtures of PC, PE, cholesterol, and diacylglycerol. Based on x-ray diffraction data, the authors concluded that the observed aggregates represented dispersed particles of cubic structure. It is also noteworthy that PEG(550)-lipids have been found to induce a cubic phase in mixtures with DEPE at relatively high temperatures (Koynova et al., 1999). We therefore propose that the observed aggregates, at low PEG-lipid concentrations, most likely represent dispersed cubic-phase particles coexisting with liposomes. It must be emphasized that it is not possible by c-TEM to unambiguously determine the exact nature of the structures formed. In this respect, the results obtained warrants further studies of the phase behavior in a wider range of lipid concentrations using, for example, x-ray diffraction.

The sample containing only 3 mol % PEG(750)-lipid is especially interesting. Sonication of this sample initially produced relatively small liposomes and particles displaying ILAs of varying density (Fig. 5 *a*). After 3 weeks of storage, larger particles were observed that displayed the dense inner structure discussed above (Fig. 5 *b*). Furthermore, ILAs were frequently observed in the vicinity of the areas of hexagonal symmetry. Therefore, it seems that, for this particular composition, fusion of particles occurs, producing ILAs and subsequent transformation into the proposed cubic-phase structures. This scheme is in accordance with the so-called stalk mechanism of membrane fusion, extensively discussed in previous studies (Siegel and Epand, 1997; Siegel, 1999; Chernomordik, 1996; Siegel and Banschbach, 1990). It is also noteworthy that the appear-

FIGURE 5 Cryo-TEM micrographs of sonicated or freeze-thaw cycled dispersions (1 wt % lipid, pH 7.4) of DOPE and PEG(750)-lipid in concentrations of 3 mol % incubated 2 days (*a*), 3 mol % incubated 3 weeks (*b*), 3 mol % incubated 3 weeks (*c*), 6 mol % incubated 2 days (*d*), 15 mol % incubated 2 days (*e*), and 35 mol % incubated 2 days (*f*). The arrowheads in *b* and *c* denote ILAs viewed down their axes. The arrow in *c* denotes a membrane passage (ILA) as seen from the side. The larger particles in *b* display dense inner textures of hexagonal symmetry and lamellar features at the outermost surface regions. Note also that some of the particles in *b* display membrane passages in the vicinity of the dense structures. In *f*, rather small unilamellar liposomes coexisting with small disks (observed as dark lines in an edge-on projection) and spherical micelles (small dark dots) can be observed. See text for more information. The samples were prepared as described in Materials and Methods. Bar, 100 nm.

ance of the particles formed by sonication at pH 7.4 is very similar to those formed in the pH-jump experiment of the sample containing 3 mol % PEG(750)-lipid, discussed above.

Sonicated samples containing 2.5 mol % PEG(2000)-lipid (Fig. 4 *a*) or 6 mol % PEG(750)-lipid showed no particle growth on the time scale of the investigation. Again this is in accordance with the pH-jump experiments. Note that in the pH-jump experiments of these dispersions, the particles essentially remained as nonaggregated liposomes whereas the sonicated (or freeze-thawed) samples displayed particles exhibiting an inner texture. This clearly emphasizes the importance of liposome aggregation as an initial step in the phase transformation and possibly also reflects the profound hysteresis often associated with the L_α-to-Q_{II} phase transition (Lindblom and Rilfors, 1989).

Structures formed at intermediate to high PEG-lipid concentration

As has been shown in previous studies on similar DOPE-based systems, PEG-lipids promote the formation of a lamellar phase (Holland et al., 1996a). In the case of PEG(2000)-lipid, approximately 8 mol % is needed to produce a pure dispersed lamellar phase (Fig. 3 *c*). As expected from simple shape considerations, more PEG(750)-lipid was required to obtain preparations consisting of only liposomes. The lamellar/water two-phase region was in this case reached at ~10–15 mol % PEG(750)-lipid (Fig. 5 *e*). Unilamellar liposomes of varying size were predominantly found in the region 8–17.5 mol % PEG(2000)-lipid and 15–35 mol % PEG(750)-lipid. Above the bilayer-saturating concentrations of PEG-lipids, we observed coexistence of liposomes, small disks, and spherical micelles. In comparison with previous results obtained in lamellar-forming lipid/PEG-lipid systems, a number of differences can be noted. First, the amount of PEG-lipid that can be incorporated in the liposomal membrane before micelle formation is much higher in the present systems compared with the corresponding PC or PC/cholesterol systems (Edwards et al., 1997; Belsito et al., 2000). In these systems, a critical concentration of ~10 mol % PEG(2000)-lipid was determined at which micelle and/or disk formation occurred. In the case of the PEG(750)-lipid, the corresponding critical concentration was found to be ~20–25 mol % (our own unpublished results). Second, in the DOPE systems, spherical micelles are formed (Figs. 3 *d* and 5 *f*) whereas in the egg lecithin (EPC)/PEG-lipid systems, cylindrical micelles were the first micellar aggregates observed (Edwards et al., 1997). In this respect, the DOPE systems show similarities to the PC/cholesterol/PEG-lipid systems where first disks and then spherical micelles were formed at higher PEG-lipid concentration (Edwards et al., 1997).

The shift of the critical concentration for micelle formation to higher PEG-lipid content in the DOPE systems can be qualitatively understood using simple shape or curvature considerations. The DOPE molecule has the shape of an inverted truncated cone (at near neutral or acidic pH) and therefore prefers aggregates of negative curvature (bending toward the aqueous phase). Micelles and also small disks are aggregates of positive curvature where in the latter case the rim of the disks is highly bent toward the apolar part of the aggregate. Accordingly, it is reasonable to assume a higher cost in curvature energy for micelle formation in the DOPE/PEG-lipid system than for the PC/PEG-lipid system, given that the PC-lipid prefers aggregates of zero curvature (lamellar phase). Of course, this also depends on the magnitude of the monolayer elastic bending modulus (Helfrich, 1973), which in the above argument is assumed to be of similar magnitude for both systems.

The shape of the micelles that are formed in coexistence with the liposomes, at a critical concentration of PEG-lipid, is not so straightforward to understand. The problem of predicting the shape of phospholipid/surfactant mixed micelles has been analyzed theoretically by Kozlov et al. (1997). In the study it is shown that the type of micelles formed upon membrane solubilization is determined by the difference of spontaneous curvature of surfactant and lipid, the magnitude of the elastic bending modulus as well as the Gaussian curvature modulus, and the temperature. The derived expression showed that negative values of the Gaussian curvature modulus in combination with a large difference of surfactant and lipid spontaneous curvature favor the formation of disk-like or spherical micelles over cylindrical micelles. In this respect it is interesting to note that theoretical treatments of bilayers decorated with polymers (Porte and Ligoure, 1995; Szleifer et al., 1998) show that at high polymer densities, a spontaneous formation of liposomes may occur. Szleifer et al. (1998) attributed this to a decrease of the Gaussian curvature modulus with increasing PEG-lipid concentration, i.e., larger negative values of the modulus. As an example, spontaneous liposome formation was calculated to occur above ~12 mol % PEG(2000)-lipid in the EPC membrane (Szleifer et al., 1998). Although this treatment did not take into account that disk and cylindrical micelle formation occurs at ~10 mol % PEG(2000)-lipid in the EPC system, it may indicate that a large and negative value of the Gaussian curvature modulus is to be expected in the DOPE membrane, in which 17.5 mol % PEG(2000)-lipid could be incorporated. According to Kozlov et al. (1997), this would favor spherical micelles over cylindrical micelles. It should be emphasized that the issue of spontaneously formed liposomes at true equilibrium has not been dealt with in the present study. In lack of more experimental data we have here assumed that the liposomes represent a dispersed lamellar phase.

Implications for the use of DOPE-based liposomes as drug delivery vehicles

DOPE, or more generally PE liposomes, are interesting candidates for use in liposomal drug delivery (Slepushkin et

al., 1997; Holland et al., 1996b; Kirpotin et al., 1996). However, it is possible to prepare pure DOPE liposomes only at relatively high pH. On the other hand, the addition of PEG-lipids to DOPE facilitates liposome formation also at physiological conditions as shown in the present study and previously by others (Holland et al., 1996a; Kirpotin et al., 1996). Our results show that to obtain a dispersed phase consisting of only liposomes (predominantly unilamellar), 8 mol % PEG(2000)-lipid and >10 mol % of PEG(750)-lipid must be added to the DOPE system at physiological conditions. This is in qualitative agreement with the results obtained in the DOPE/cholesterol/PEG(2000)-lipid system investigated by means of NMR and freeze-fracture electron microscopy (Holland et al., 1996a). However, the reader should bear in mind that the obtained structures may be somewhat dependent on the preparation procedure. Kirpotin et al. (1996) reported that only 3 mol % PEG(2000)-lipid was sufficient to prepare small unilamellar DOPE liposomes at pH 7.2. In this case the liposomes were prepared by hydration and subsequent extrusion through polycarbonate filters and were characterized by dynamic light scattering and entrapped volume. However, neither of these methods gives any information on the morphology of the obtained particles.

The ability of DOPE/PEG-lipid liposomes to undergo the lamellar-to-inverted lipid phase transition at reduced pH depends critically on the density of PEG-lipids in the membrane and the size of the PEG headgroup. We show in the present study that ~2.5 mol % or 6 mol % of PEG(2000)-lipid or PEG(750)-lipid, respectively, is enough for inhibiting the phase transition at low pH (Fig. 1). Several PEG-lipid conjugates have been prepared where the linkage between the PEG polymer and the lipid anchor is cleavable by a number of different mechanisms (Gerasimov et al., 1999; Kirpotin et al., 1996). The disappearance of the PEG polymer from the liposome surface results in liposomes consisting of mainly DOPE. Given that the pH is low enough, these liposomes will undergo a phase transition to inverted lipid phase and presumably release encapsulated material during this process. Clearly, if the aim is to induce such a phase transition or to render the liposomes more prone to membrane fusion, our present and previous results indicate that the rate of the cleaving reaction must be sufficiently fast so as to reduce the density of PEG-lipids within a reasonable time scale.

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