

## Effect of Polyethyleneglycol-Phospholipids on Aggregate Structure in Preparations of Small Unilamellar Liposomes

K. Edwards, M. Johnsson, G. Karlsson, and M. Silvander

Department of Physical Chemistry, Uppsala University, S-75121 Uppsala, Sweden

**ABSTRACT** Phospholipids with covalently attached poly(ethylene glycol) (PEG lipids) are commonly used for the preparation of long circulating liposomes. Although it is well known that lipid/PEG-lipid mixed micelles may form above a certain critical concentration of PEG-lipid, little is known about the effects of PEG-lipids on liposome structure and leakage at submicellar concentrations. In this study we have used cryogenic transmission electron microscopy to investigate the effect of PEG(2000)-PE on aggregate structure in preparations of liposomes with different membrane compositions. The results reveal a number of important aggregate structures not documented before. The micrographs show that enclosure of PEG-PE induces the formation of open bilayer discs at concentrations well below those where mixed micelles begin to form. The maximum concentration of PEG-lipid that may be incorporated without alteration of the liposome structure depends on the phospholipid chain length, whereas phospholipid saturation or the presence of cholesterol has little or no effect. The presence of cholesterol does, however, affect the shape of the mixed micelles formed at high concentrations of PEG-lipid. Threadlike micelles form in the absence of cholesterol but adapt a globular shape when cholesterol is present.

### INTRODUCTION

Because of the biocompatible lipid matrix and the possibility of incorporating both water-soluble and hydrophobic components, liposomes were suggested early as ideal vehicles for drug delivery. The use of liposomes as vehicles for drug delivery, however, was soon found to be very limited because of the short survival time of liposomes in blood. Within minutes after injection, the liposomes are destabilized by interactions with phospholipases and lipoproteins. In addition, and even more discouraging, liposomes are quickly recognized and removed by elements of the host defense system, the reticuloendothelial system (RES) (Woodle and Lasic, 1992; Patel, 1992).

Despite serious efforts, these problems proved difficult to overcome and led to a period of scepticism about the medical utility of liposomes. Hope was regained in 1987, however, as it was shown that the blood survival time for liposomes could be substantially increased by incorporation of the glycolipid ganglioside  $G_{M1}$  (Allen and Chonn, 1987). Unfortunately, because it is derived from bovine brain,  $G_{M1}$  is not ideal for clinical use. The search for a synthetic lipid that could improve circulation times eventually led to the development of polymer grafted bilayers (Lasic and Martin, 1995, and references therein). Several polymer lipids give rise to prolonged circulation times, but the most well studied are those consisting of polyethyleneglycol (PEG) grafted onto phosphatidylethanol amine (PE).

The reasons for the extended circulation times of polymer grafted liposomes are still somewhat unclear. The formation

of a steric barrier around the liposome, however, appears to be of major importance (Woodle and Lasic, 1992; Needham et al., 1992). By incorporation of a well-balanced amount of polymer lipids, the interactions between blood proteins, such as lipoproteins and phospholipases, and the liposome membrane may be effectively hindered. Furthermore, experimental results suggest that the polymer barrier prevents the attachment of certain opsonin proteins (Patel, 1992) and hampers the liposome uptake by macrophages (Liu and Liu, 1996).

Sterically stabilized or so-called Stealth liposomes have lately come into widespread use, and intense research has led to the development of several liposomal drugs that are currently in late-stage clinical trials or are already on the market (Lasic and Martin, 1995; Chonn and Cullis, 1995; Woodle, 1995).

The majority of the sterically stabilized liposomes used for drug delivery are small, uncharged, and composed of long saturated lipids in combination with high concentrations of cholesterol. Although this combination has proved effective in prolonging the circulation time of conventional, unprotected liposomes, the effect of parameters such as size and charge are doubtful in combination with polymer grafted bilayers. Furthermore, experimental results indicate that the prolonged circulation obtained with PEG-PE is independent of both cholesterol and phospholipid saturation (Woodle et al., 1992). Strong gel-phase bilayers are often recommended, however, for the incorporation of maximum amounts of polymer lipid. Both the range and magnitude of the steric barrier are strong functions of PEG-lipid concentration and PEG size, and comparably high PEG-lipid concentrations are required for efficient steric barrier formation (Kenworthy et al., 1995a). At high concentrations of polymer lipid, the interaction between different polymer chains gives rise to a considerable lateral pressure in the liposome membrane (Hristova and Needham, 1995; Hristova et al.,

*Received for publication 9 December 1996 and in final form 27 March 1997.*

Address reprint requests to Dr. Katarina Edwards, Department of Physical Chemistry, Uppsala University, Box 532, S-75121 Uppsala, Sweden. Tel.: 46-18-183668; Fax: 46-18-508542; E-mail: edwards@fki.uu.se.

© 1997 by the Biophysical Society

0006-3495/97/07/258/09 \$2.00

1995). To prevent the membrane from rupturing, this lateral pressure must be balanced by bilayer cohesion, which in turn can be increased by the inclusion of cholesterol (Needham and Nunn, 1990). The condensing effect obtained by the inclusion of cholesterol, particularly in combination with saturated lipids, also helps reduce membrane permeability and leakage of entrapped solutes.

The liposome membrane, however, is often found to disintegrate at polymer lipid concentrations well below those where the lateral pressure exceeds the bilayer cohesion. In the absence of phospholipids, the PEG-PE molecules form micelles in aqueous solution, and it is well known that high concentrations of PEG lipids may induce a transition from bilayer to micellar phase (Woodle and Lasic, 1992; Kenworthy et al., 1995b). Thus, when the total amount of PEG-PE in the liposome preparation reaches a limiting concentration, it becomes more energetically favorable to form lipid/polymer lipid mixed micelles than bilayers. When this point, which inevitably leads to dissolution of at least part of the liposome population, occurs depends on a number of factors. The lipid composition of the membrane and the length of the PEG-PE polymer chain are, of course, major factors that govern the phase behavior and structures formed (Hristova et al., 1995; Bedu-Addo and Huang, 1995). The nature and concentration of the encapsulated drug may also be of importance, and the behavior may, in addition, be expected to vary significantly, depending on both temperature and salt concentration.

The matter is further complicated by the fact that the transition to mixed micelles may occur via intermediate structures, such as open or perforated bilayer aggregates that severely decrease, or even abolish, the encapsulation efficiency of the liposomal preparation. This phenomenon, although known to occur frequently upon the addition of conventional micelle-forming surfactants to liposome preparations (Edwards et al., 1989, 1993; Vinson et al., 1989; Walter et al., 1991; Silvander et al., 1996), has unfortunately not received much attention in systems containing PEG-lipids. In summary, thorough determinations of phase behavior and aggregate morphology are essential for elucidating the maximum concentration of PEG-lipid that can be incorporated without altering the structure or integrity of the liposomes.

The effects of PEG-PE on structure and phase behavior in systems containing liposomes with varying bilayer compositions have been investigated in a number of studies (Kenworthy et al., 1995b; Hristova et al., 1995; Bedu-Addo and Huang, 1995). These studies give good estimates of the amount of PEG-lipid needed to induce mixed micelle formation in systems of varying composition. However, the methods used, such as x-ray, NMR, and differential scanning calorimetry, require comparably high sample concentrations and, furthermore, cannot readily detect subtle changes in aggregate morphology that may take place within the bilayer or micellar phases. This makes them ill-suited for detailed investigations of aggregate structure in

the extremely dilute liposome solutions used for drug delivery.

Electron microscopy constitutes an alternative method for the determination of aggregate morphology in dilute lipid solutions. Lipid aggregates, such as liposomes and micelles, are, however, very labile and extremely sensitive to small changes in concentration and composition. Thus the drying and staining procedures used in conventional sample preparation often introduce severe artefacts.

Cryo-transmission electron microscopy (c-TEM) is a relatively new technique that offers unique possibilities for direct visualization of labile microstructures in dilute aqueous solutions (Dubochet et al., 1988; Almgren et al., 1996). By this technique, direct and detailed information may be obtained with a minimum disturbance of the original sample structure.

In the present study we have used c-TEM to carry out a systematic study of the effect of PEG-PE on aggregate structure in samples containing small unilamellar liposomes with varying bilayer composition. During the investigations we have paid particular attention to the aggregates formed at submicellar concentrations of PEG-lipid. The results show that major rearrangements, which are interesting not only from a fundamental point of view but also have important consequences for the development of liposomal drug delivery systems, take place in this concentration regime.

## MATERIALS AND METHODS

### Materials

Egg yolk lecithin (EPC) of grade 1 was purchased from Lipid Products (Nutfield, England). The other phospholipids, 1,2 distearoylphosphatidylcholine (DSPC) and 1,2-dipalmitoylphosphatidylcholine (DPPC) were bought from Avanti Polar Lipids (Alabaster, AL). PEG-lipids, with PEG of molar mass 2000 Da covalently attached to 1,2- distearoylphosphatidylethanolamine (PEG(2000)-DSPE) or 1,2- dipalmitoylphosphatidylethanolamine (PEG(2000)-DPPE), were also purchased from Avanti Polar Lipids.

### Preparation of liposomes

Lipid mixtures were prepared by codissolving the lipids and polymer-lipids in chloroform, removing the chloroform by evaporation under vacuum, and thereafter redissolving the dry lipids in buffer containing 0.15 M NaCl and 20 mM HEPES (pH 7.4). The lipid mixtures were subjected to at least eight freeze-thaw cycles (including freezing in liquid nitrogen and heating to above 70°C). Small unilamellar liposomes were produced by multiple extrusion of the lipid mixtures through polycarbonate filters (pore size 100 nm) mounted in a LiposoFast miniextruder from Avestin (Ottawa, Canada).

### Cryo-transmission electron microscopy

The technique, which has been described in detail elsewhere (Dubochet et al., 1988; Bellare et al., 1988), consists in short of the following. Thin (10–500 nm) sample films were prepared under controlled temperature (25°C) and humidity conditions within a custom-built environmental chamber. The films were thereafter vitrified by quick freezing in liquid ethane and transferred to a Zeiss EM 902 transmission electron microscope for examination. To prevent sample perturbation and the formation of ice

crystals, the specimens were kept cool (below 108 K) during both the transfer and viewing procedures. All observations were made in zero-loss bright-field mode and at an accelerating voltage of 80 kV.

For the evaluation of the c-TEM micrographs, it is important to realize that the two-dimensional projection of a closed liposome will appear as a circular object with enhanced contrast around the rim. This is due to the fact that the projected thickness of the bilayer shell is at maximum at the edges. The projection of a flat bilayer disc will, on the other hand, appear even in contrast right up to the edge.

## RESULTS

### Effect of cholesterol on the morphology of phospholipid liposomes

The ability of cholesterol to increase the cohesive strength and reduce the membrane permeability of phospholipid bilayers is well known (Needham and Nunn, 1990; Grit and Crommelin, 1993). For this reason, most lipid mixtures used to produce liposomes for drug delivery contain cholesterol, typically in concentrations of ~30–50 mol%. Although the phase behavior and mechanical properties of lipid/cholesterol systems have been well studied, the effect of cholesterol on the shape and structure of small unilamellar liposomes has not been thoroughly investigated. We therefore examined liposomes prepared in the presence of varying amounts of cholesterol by means of cryo-TEM. As shown in Fig. 1, samples containing EPC and 50 mol% cholesterol or more display a strong tendency to form elongated or even tube-shaped liposomes (Fig. 1 *b*). The deviation from a spherical shape implies the possibility of segregation of the lipid and cholesterol components. This may in turn affect the distribution of PEG-lipids in the membrane, with possible consequences for the overall range and magnitude of the PEG-mediated steric repulsion. To avoid this problem we chose to restrict the cholesterol content in all liposome preparations to 40 mol%.

Interestingly, at very high cholesterol concentrations, where formation of microcrystals of cholesterol monohydrate (Collins and Philips, 1982) was evident, the liposomes were found to revert back to a spherical shape (Fig. 1 *c*).

### Aggregate structure in liposomal preparations containing DSPC, cholesterol, and DSPE-PEG

The most commonly used lipid mixtures for the preparation of sterically stabilized liposomes are those containing the 18-carbon, fully saturated, phospholipid distearoylphosphatidylcholine (DSPC). It is well documented that liposomes composed of this lipid and cholesterol display both long circulation times and low leakage rates, possibly the two most crucial parameters for the development of successful liposomal delivery systems (Gregoriadis, 1995). We have therefore concentrated our investigations on liposomes with bilayers composed of cholesterol and DSPC.

Fig. 2 shows the sequence of structural transitions observed as the concentration of PEG-lipid is increased in preparations containing DSPC and 40 mol% cholesterol.

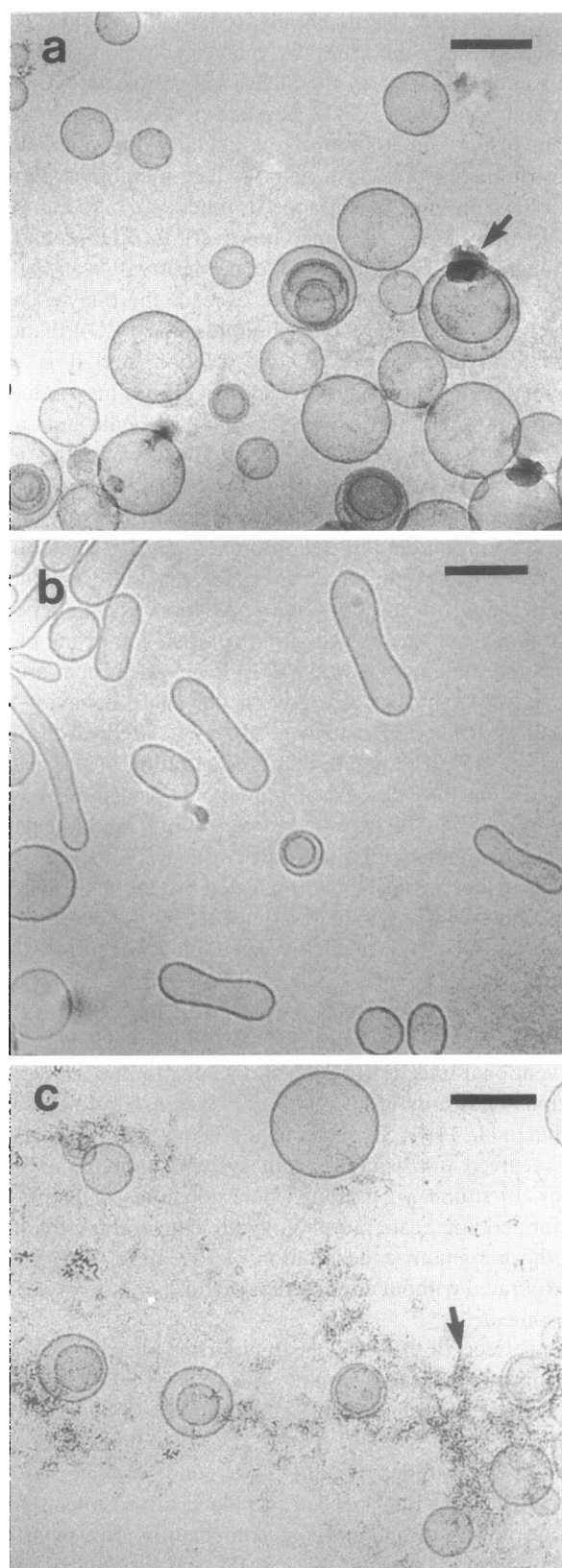


FIGURE 1 Extruded samples containing EPC and cholesterol in molar ratios of (a) 60:40, (b) 40:60, and (c) 15:85. The arrow in *a* denotes an ice crystal deposited on the sample surface after vitrification. Note elongated liposomes in *b* and crystalline particles, denoted with an arrow, in *c*. Bar = 100 nm.

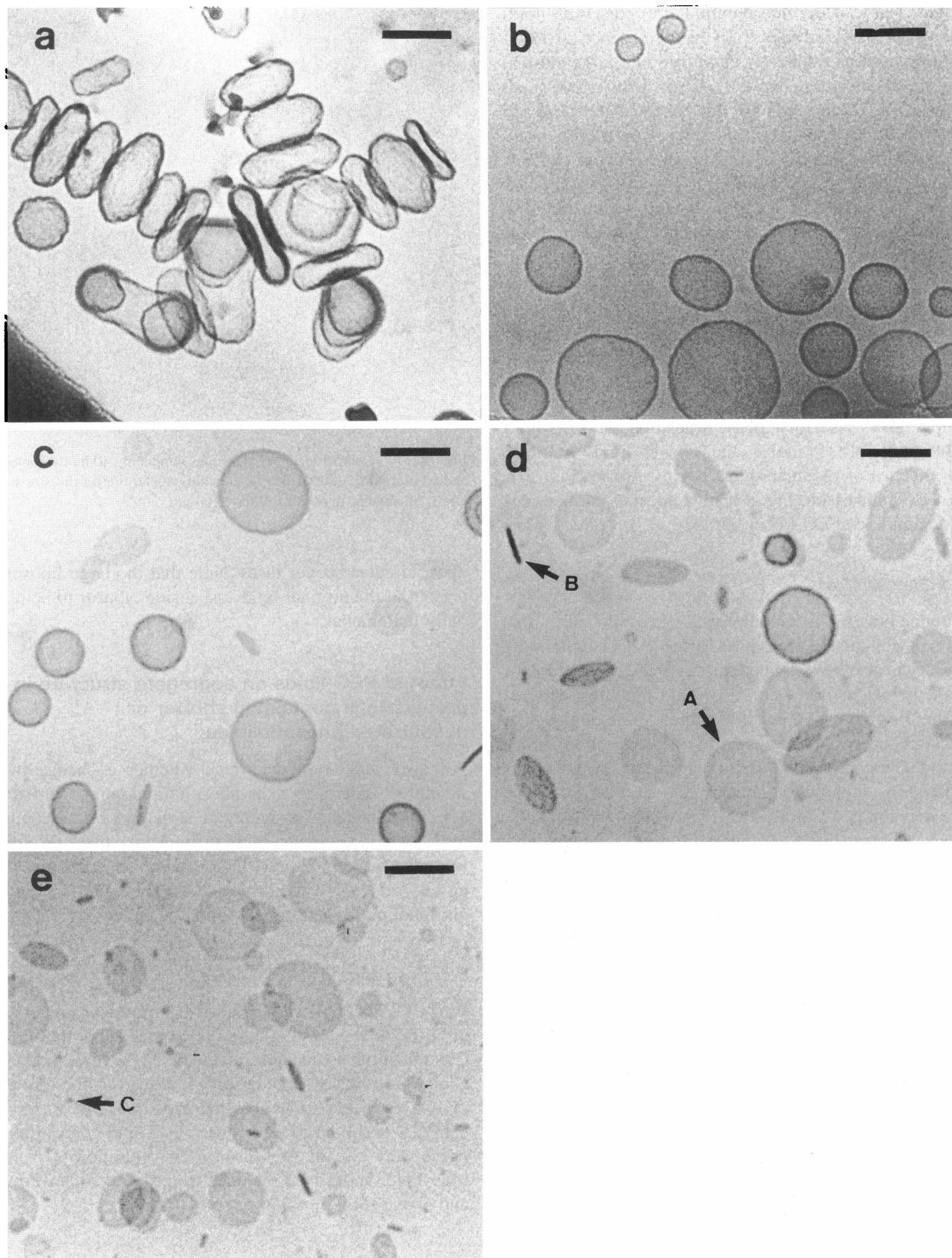


FIGURE 2 Extruded DSPC samples containing 40 mol% cholesterol and PEG(2000)-DSPE in concentrations of (a) 0 mol%, (b) 5 mol%, (c) 12 mol%, (d) 15 mol%, and (e) 20 mol%. Arrows in *d* denote bilayer discs as observed face-on (A) and edge-on (B). Arrows in *e* denote a globular micelle. See text for more information. Bar = 100 nm.

(The ratio between the phospholipid (PC plus PEG-lipid) and cholesterol was always 3:2.) In the absence of PEG-lipid the liposomes frequently aggregate into large clusters of deformed liposomes (Fig. 2 *a*). Upon inclusion of small amounts of PEG-lipid, the liposomes become spherical and appear to be well separated in the micrographs (Figs. 2 *b*). The same general appearance is observed for all samples containing up to 10 mol% PEG-lipid. Above this concentration, however, the micrographs reveal the formation of a new aggregate structure. In coexistence with the closed liposomes, open bilayered discs are now frequently observed (Figs. 2 *c*, *d*, *e*). The majority of the discs appear circular, with smooth edges, and can be seen face on, edge on, and at all projections in between. As the total concentration of PEG-lipid in the samples increases, the number of discs becomes progressively larger, at the expense of the closed liposomes. Eventually the increasing amount of PEG-lipid gives rise to a phase transition; at PEG-lipid concentrations of 15 mol% and above, lipid/PEG-lipid mixed micelles of globular shape begin to form (Fig. 2, *e* and *f*). For comparison, Fig. 3 shows micelles formed in a 10 mM sample of PEG-PE in buffer.

#### Nonextruded samples

To examine how the filtration procedure, employed to produce small unilamellar liposomes, affects the sample morphology, we also investigated the aggregate structure in some of the original, nonextruded, lipid mixtures. The results from this study showed that, although the overall size of the aggregates decreases because of the filtration procedure, the aggregate structure remain unchanged. As an example, Fig. 4 shows a nonextruded DSPC/cholesterol sample containing 12 mol% PEG-PE. As observed for the extruded samples (Fig. 2 *d*), intact liposomes coexist with

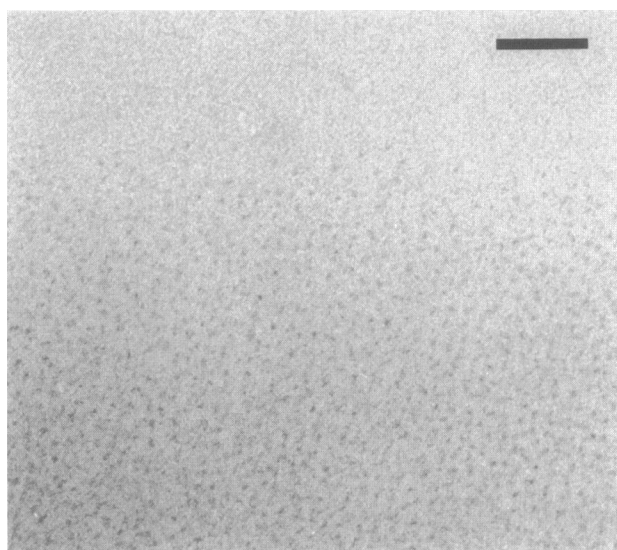


FIGURE 3 Globular micelles as observed in a sample containing 10 mM PEG(2000)-DSPE. Bar = 100 nm.

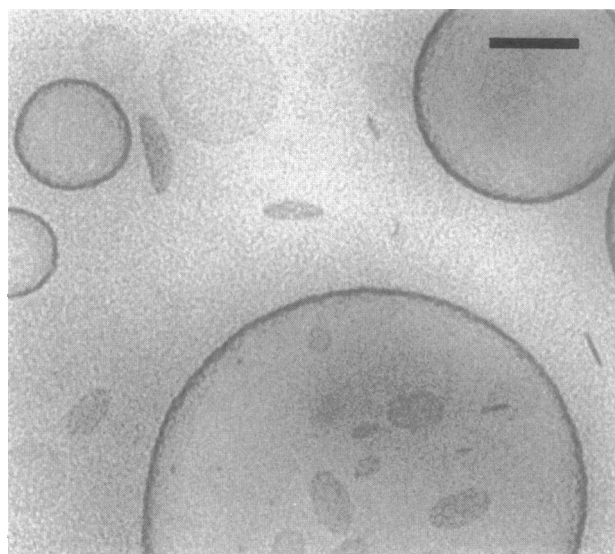


FIGURE 4 Nonextruded DSPC sample, containing 40 mol% cholesterol and 12 mol% PEG(2000)-DSPE, showing circular bilayer discs as well as intact unilamellar liposomes. Bar = 100 nm.

open, circular bilayer discs. Note that the large liposomes, present in the original lipid suspension, appear to be essentially unilamellar.

#### Effect of PEG-lipids on aggregate structure in preparations containing shorter or unsaturated phospholipids

We also wanted to determine whether a change in the saturation or length of the phospholipid component would affect the sequence of aggregate structures or the composition at which the structural transitions take place. We therefore also performed cryo-TEM studies on liposomes composed of EPC and the 16-carbon, fully saturated, phospholipid dipamitoylphosphatidylcholine (DPPC).

##### Samples containing DPPC

When DPPE-PEG was included in liposomal preparations of DPPC and cholesterol, the same sequence of morphological transitions as that observed with DSPE-PEG and DSPC/cholesterol bilayers was observed. However, the formation of bilayer discs, as well as the appearance of mixed micelles, was shifted toward lower PEG-lipid concentrations. Discs were already present at a concentration of 8 mol% PEG-lipid, and mixed micelles were observed at 12 mol% and above (Fig. 5, *a* and *b*, respectively).

##### Samples containing EPC

In contrast, no significant change in the amount of PEG-lipid needed for the inducement of discs or mixed micelles was observed when DSPC was exchanged for EPC. Discs were again formed at ~10 mol%, and mixed micelles ap-



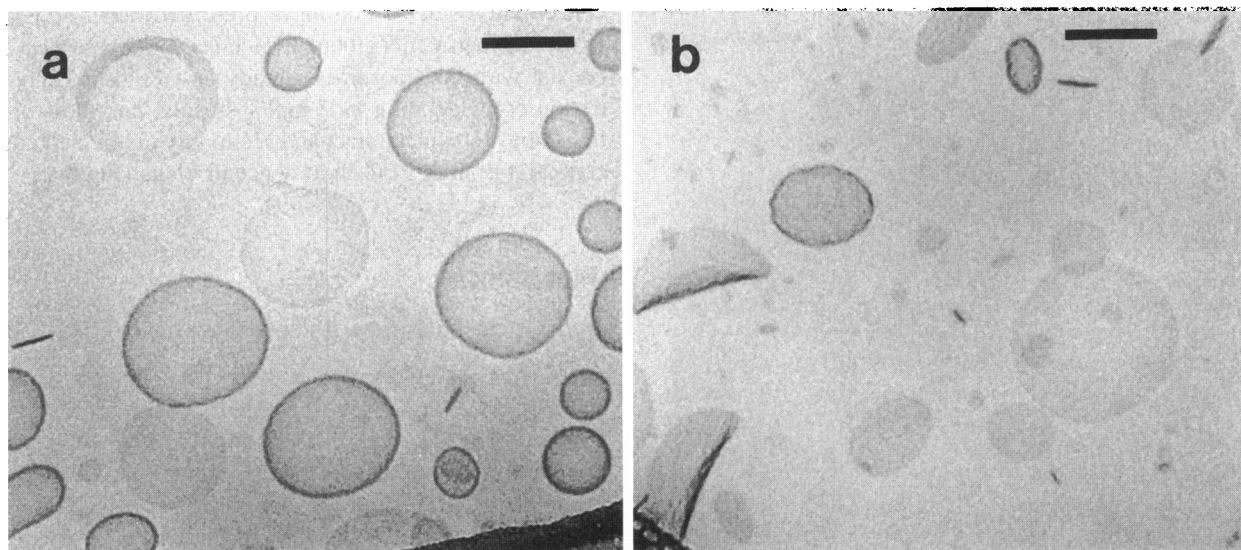


FIGURE 5 Extruded DPPC sample containing 40 mol% cholesterol and (a) 8 mol% PEG(2000)-DPPE or (b) 12 mol% PEG(2000)-DPPE. Bar = 100 nm.

peared when the PEG-lipid concentration reached  $\sim 15$  mol%. Fig. 6 shows two samples containing 17 and 40 mol% DSPE-PEG, respectively, and illustrates how the number of globular micelles gradually increases as the PEG-lipid concentration becomes greater.

#### Sequence of morphological changes in the absence of cholesterol

To investigate whether cholesterol had any effect on the aggregate structures formed in the PEG-lipid/lipid-system, we repeated the cryo-TEM study of EPC liposomes in the absence of cholesterol. As seen in Fig. 7 *a*, open bilayered structures are again formed when the PEG concentrations

exceed 10 mol%. In this system the open structures, however, are much more polydisperse in shape and often display uneven edges. Another difference is visualized in Fig. 7 *a*; in the absence of cholesterol, mixed micelles begin to form somewhat earlier and, more importantly, adapt a threadlike shape. The threadlike micelles become more obvious as the PEG concentration increases (Fig. 7 *b*), and at high PEG-lipid concentrations they can be seen to coexist with globular micelles (Fig. 7 *c*).

#### External addition of PEG-lipid

In an attempt to investigate the solubilizing power of the PEG-lipid, PEG(2000)-DSPE was added externally, from a

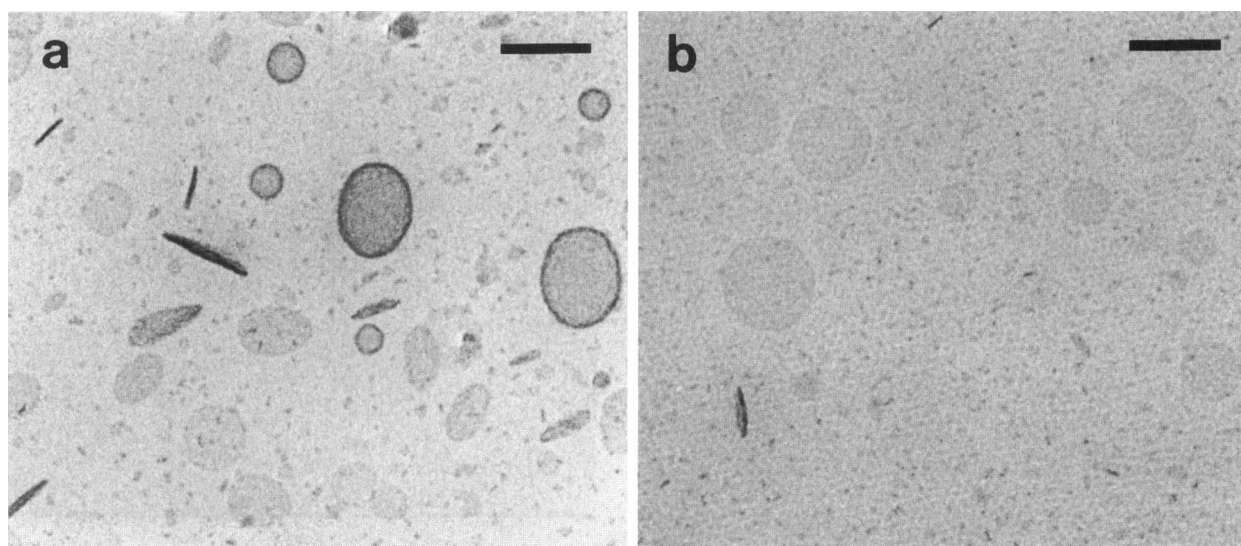


FIGURE 6 Extruded EPC sample containing 40 mol% cholesterol and (a) 17.4 mol% PEG(2000)-DSPE or (b) 40 mol% PEG(2000)-DSPE. Bar = 100 nm.

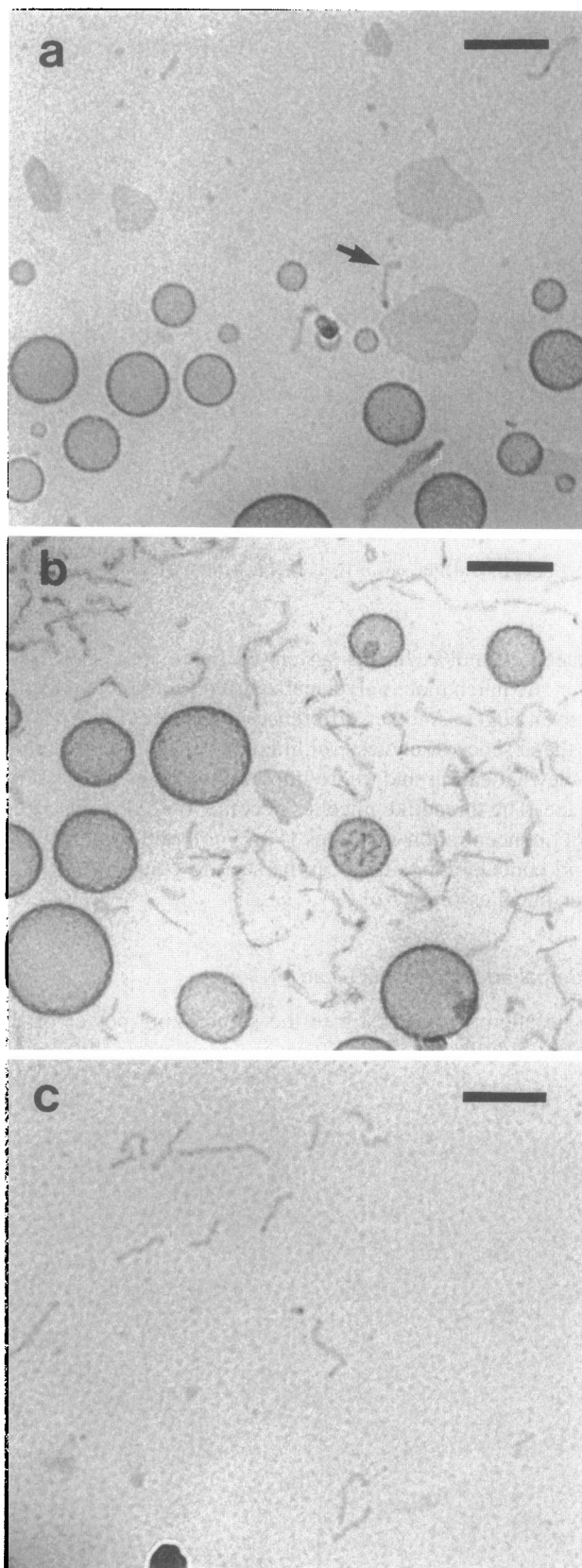


FIGURE 7 Extruded EPC sample containing (a) 11 mol% PEG(2000)-DSPE, (b) 23 mol% PEG(2000)-DSPE, and (c) 40 mol% PEG(2000)-DSPE. Note irregular shape of bilayer fragments. The arrow in *a* denotes a threadlike micelle. Bar = 100 nm.

concentrated stock solution, to preformed EPC liposomes. In these experiments globular micelles could be observed to coexist with the liposomes already at a PEG-lipid concentration corresponding to 5 mol% (results not shown). The liposomes remained intact and seemingly unaffected, also at comparably high PEG-lipid concentrations (Fig. 8).

## DISCUSSION

### Aggregate structure as revealed by c-TEM

The results obtained in this study reveal new information on phase behavior and aggregate structure in the lipid/polymer-lipid systems most commonly used to produce sterically stabilized liposomes. Several aggregate structures, never documented before in these systems, have been discovered by c-TEM in both the bilayer and micellar phases.

Perhaps the most important finding is that the transition from the lamellar to the micellar phase proceeds via an intermediate state comprising bilayer discs. Once a liposome is transformed into such an open structure its encapsulation ability, and thus its value as a vehicle for delivery of drugs (at least water soluble ones), is lost. We therefore propose that it is the formation of bilayer discs, and not mixed micelles, that sets the upper limit to the amount of PEG-lipid that can be incorporated into the sterically stabilized liposomes.

The formation of open bilayer aggregates, as an intermediate structure during the liposome-to-micelle transition, is not unique to the lipid/polymer lipid systems investigated in the present study. Open structures and disc-like bilayer fragments have been documented during solubilization of liposomes by a number of conventional surfactants (Edwards et

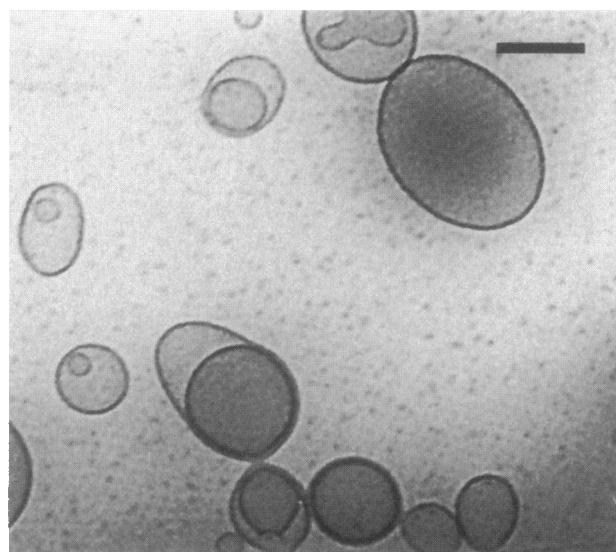


FIGURE 8 Extruded EPC sample after external addition of 23 mol% PEG(2000)-DSPE. Intact liposomes and globular micelles coexist. Note the absence of discs. Bar = 100 nm.

al., 1989, 1993; Vinson et al., 1989; Walter et al., 1991; Silvander et al., 1996).

In dispersions of lamellae-forming lipids, small bilayer fragments, or discs, are normally very short-lived. This is primarily due to the thermodynamically unfavorable exposure of hydrophobic material at the edges of the flat structures. To reduce the contact between water and the exposed hydrocarbon chains, the discs either close up on themselves and form liposomes, or fuse and grow into infinite bilayer sheets. The discs may, however, be stabilized in the presence of molecules, such as micelle-forming surfactants, which have the ability to shield the open edges and thus reduce the edge energy (Lasic, 1982; Fromherz, 1983; Edwards and Almgren, 1992; Edwards et al., 1993). Apparently a similar phenomenon takes place in the lipid/polymer-lipid mixtures; the PEG-lipids, with their bulky polymer chains, may easily be accommodated into the highly curved monolayer of lipids needed to protect the disc edge. Furthermore, the presence of the polymer chains sterically obstructs both closure and fusion of the discs.

The nearly perfectly circular shape of the discs obtained in the presence of cholesterol (see, for instance, Figs. 2 *d*, 5 *b*, and 6 *a*), as compared to the much more irregular shape adapted in the absence of cholesterol (Fig. 7 *a*), is noteworthy. Because, for a given area, a circular shape minimizes the circumference of the disc, the above observation indicates that the shielding of the disc edge is more energetically expensive in the presence of cholesterol. A plausible explanation for this may be found if we consider the effect of cholesterol on the cohesion and stiffness of the membrane. Curving the membrane, to protect the hydrocarbon chains around the rim of the disc, requires stretching of the lipid headgroup area. Cholesterol is well known to increase the resistance of lipid membranes to area dilatation; inclusion of 40 mol% cholesterol has been determined to increase the elastic area expansion modulus threefold, from 190 to 600 dyn/cm (Needham and Nunn, 1990). The creation of a highly curved lipid layer at the disc edge is thus more unfavorable, and the tendency to minimize the circumference of the discs greater, in the presence of cholesterol.

The reluctance of cholesterol-containing aggregates to form highly curved surfaces also manifests itself in the shape of the mixed micelles formed at higher PEG-lipid concentrations. For DSPC it has been shown, by the use of a number of techniques such as NMR, differential scanning calorimetry, and absorbance measurements, that inclusion of PEG(2000)-DSPE in concentrations above 15–20 mol% gives rise to the formation of micelles (Kenworthy et al., 1995b). Later investigations, by means of x-ray diffraction, indicate that micelles form at roughly the same PEG-lipid concentration in systems composed of DSPC/cholesterol and EPC (Hristova et al., 1995). This agrees well with the results obtained in our study, as does the observation that a decrease in phospholipid chain length decreases the amount of PEG-lipid needed to induce micelle formation. (In sharp contrast, Bedu-Addo and Huang (1995) report that the inclusion of 30 mol% cholesterol or more into DPPC liposomes completely inhibits the formation of lipid/PEG-lipid mixed micelles.)

The c-TEM results, however, do provide additional important information about the shape and character of the lipid/polymer lipid mixed micelles. In the absence of cholesterol, the bilayer discs are progressively solubilized into threadlike micelles (Fig. 7, *a* and *b*). At high PEG-PE concentrations, the discs disappear completely, and the threadlike micelles convert into globular micelles (Fig. 7 *c*). In contrast, no threadlike micelles are observed in the presence of cholesterol. With increasing concentration of PEG-PE, globular micelles may be seen in coexistence with the bilayer discs (Fig. 6 *a*). The discs appear very stable against solubilization into mixed micelles; a considerable number of discs can still be observed at very high concentrations of PEG-PE (Fig. 6 *b*). Thus segregation of the components into lipid/cholesterol discs saturated with PEG-lipid and globular micelles composed primarily of PEG-PE is likely to take place.

The low solubilization power of PEG-PE is further demonstrated by the micrographs obtained after the external addition of PEG-PE to preformed EPC liposomes. Globular micelles were observed at PEG-PE concentrations as low as 5 mol%, and samples containing 23 mol% PEG-PE still show intact liposomes (Fig. 8). The absence of threadlike micelles implies that no, or very little, phospholipid is solubilized into the PEG-PE micelles.

### Implications for the development of liposomal drug delivery systems

So far, the design of successful liposomal delivery systems has been based largely upon empirical experiences—an approach that is both time-consuming and expensive. As shown in the present study, c-TEM constitutes a very powerful method for straightforward determination of aggregate structure in the liposomal preparations. The results reported in the study show, for instance, that incorporation of more than 10 mol% PEG-lipid into the DSPC and EPC lipid mixtures most commonly used for liposome preparation is meaningless. At higher PEG-lipid concentrations the preparation will contain a large fraction of open bilayer discs, structures that are of no use as vehicles for the delivery of water-soluble drugs. Although the presence of cholesterol does not seem to affect the maximum amount of PEG-lipid that can be incorporated, it is likely that other modifications of the liposome membrane (for example, to achieve specific targeting) may seriously affect the bilayer properties. It is therefore important that all alterations in membrane composition are followed by investigations of the liposome structure.

In addition to structural investigations, reliable means of measuring leakage, or release, of the entrapped substance are of great importance. The liposomes must not only possess long circulation times, but should also be able to retain their cargo long enough to reach their target. In the case of sterically stabilized liposomes, this may cause a dilemma;



high concentrations of PEG-lipid are desired for maximizing the survival time in blood, but may induce not only a change in the aggregate structure but also an undesirable increase in membrane permeability. It has been shown that micelle-forming surfactants may significantly increase the leakage of liposome-entrapped substances at concentrations well below those inducing detectable alterations of the liposome structure (Edwards and Almgren, 1992). Hence there are good reasons to believe that the incorporation of PEG-lipid may seriously affect the encapsulation efficiency, also at concentrations below those where bilayer discs begin to form. We have initiated an investigation of the effect of PEG-phospholipids on liposome permeability.

Financial support from the Swedish Research Council for Engineering Sciences is gratefully acknowledged.

## REFERENCES

- Allen, T. M., and A. Chonn. 1987. Large unilamellar liposomes with low uptake by the reticuloendothelial system. *FEBS Lett.* 223:42–46.
- Almgren, M., K. Edwards, and J. Gustafsson. 1996. Cryotransmission electron microscopy of thin vitrified samples. *Curr. Opin. Colloid Interface Sci.* 1:270–278.
- Bedu-Addo, F. K., and L. Huang. 1995. Interaction of PEG-phospholipid conjugates with phospholipid: implications in liposomal drug delivery. *Adv. Drug Delivery Rev.* 16:235–247.
- Bellare, J. R., H. T. Davis, L. E. Scriven, and Y. Talmon. 1988. Controlled environment vitrification system (CEVS): an improved sample preparation technique. *J. Electron Microsc. Tech.* 10:87–111.
- Chonn, A., and P. R. Cullis. 1995. Recent advances in liposomal drug-delivery systems. *Curr. Opin. Biotechnol.* 6:698–708.
- Collins, J. J., and M. C. Phillips. 1982. The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine. *J. Lipid Res.* 23:291–298.
- Dubochet, J., M. Adrian, J. J. Chang, J. C. Homo, J. Lepault, A. W. McDowell, and P. Schultz. 1988. Cryo-electron microscopy of vitrified specimens. *Q. Rev. Biophys.* 21:129–228.
- Edwards, K., and M. Almgren. 1992. Surfactant-induced leakage and structural change of lecithin vesicles: effect of surfactant headgroup size. *Langmuir.* 8:824–832.
- Edwards, K., M. Almgren, J. Bellare, and W. Brown. 1989. Effects of Triton X-100 on sonicated lecithin vesicles. *Langmuir.* 5:473–478.
- Edwards, K., J. Gustafsson, M. Almgren, and G. Karlsson. 1993. Solubilization of lecithin vesicles by a cationic surfactant: intermediate structures in the vesicle-micelle transition observed by cryo-transmission electron microscopy. *J. Colloid Interface Sci.* 161:299–309.
- Fromherz, P. 1983. Lipid-vesicle structure: size control by edge-active agents. *Chem. Phys. Lett.* 94:259–266.
- Gregoriadis, G. 1985. Fate of liposomes in vivo and its control: a historical perspective. In *Stealth Liposomes*. D. Lasic and F. Martin, editors. CRC Press, Boca Raton, FL. 7–12.
- Grit, M., and D. J. A. Crommelin. 1993. Chemical stability of liposomes: implications for their physical stability. *Chem. Phys. Lipids.* 64:3–18.
- Hristova, K., A. Kenworthy, and T. J. McIntosh. 1995. Effect of bilayer composition on the phase behaviour of liposomal suspensions containing poly(ethylene glycol)-lipids. *Macromolecules.* 28:7693–7699.
- Hristova, K., and D. Needham. 1995. Phase behaviour of a lipid/polymer-lipid mixture in aqueous medium. *Macromolecules.* 28:991–1002.
- Kenworthy, A. K., K. Hristova, D. Needham, and T. J. McIntosh. 1995a. Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol). *Biophys. J.* 68:1921–1936.
- Kenworthy, A. K., S. A. Simon, and T. J. McIntosh. 1995b. Structure and phase behaviour of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). *Biophys. J.* 68:1903–1920.
- Lasic, D. D. 1982. A molecular model for vesicle formation. *Biochim. Biophys. Acta.* 692:501–502.
- Lasic, D., and F. Martin. 1995. *Stealth Liposomes*. CRC Press, Boca Raton, FL.
- Liu, F., and D. Liu. 1996. Serum independent liposome uptake by mouse liver. *Biochim. Biophys. Acta.* 1278:5–11.
- Needham, D., K. Hristova, T. J. McIntosh, M. Dewhirst, N. Wu, and D. Lasic. 1992. Polymer-grafted liposomes: physical basis for the “stealth” property. *J. Liposome Res.* 2:411–430.
- Needham, D., and R. S. Nunn. 1990. Elastic deformation and failure of lipid membranes containing cholesterol. *Biophys. J.* 58:997–1009.
- Patel, H. M. 1992. Serum opsonins and liposomes: their interaction and opsonophagocytosis. *Crit. Rev. Ther. Drug Carrier Syst.* 9:39–90.
- Silvander, M., G. Karlsson, and K. Edwards. 1996. Vesicle solubilization by alkyl sulfate surfactants: a cryo-TEM study of the vesicle to micelle transition. *J. Colloid Interface Sci.* 179:104–113.
- Vinson, P. K., Y. Talmon, and A. Walter. 1989. Vesicle-micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-Transmission electron microscopy. *Biophys. J.* 56:669–681.
- Walter, A., P. K. Vinson, A. Kaplun, and Y. Talmon. 1991. Intermediate structures in the cholate-phosphatidylcholine vesicle-micelle transition. *Biophys. J.* 60:1315–1325.
- Woodle, M. C. 1995. Sterically stabilized liposome therapeutics. *Adv. Drug Delivery Rev.* 16:249–265.
- Woodle, M. C., and D. D. Lasic. 1992. Sterically stabilized liposomes. *Biochim. Biophys. Acta.* 1113:171–199.
- Woodle, M. C., K. K. Matthay, M. S. Newman, J. E. Hidyat, L. R. Collins, C. Redemann, F. J. Martin, and D. Papahadjopoulos. 1992. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. *Biochim. Biophys. Acta.* 1105:193–200.