Poly(ethylene Glycol)-Induced and Temperature-Dependent Phase Separation in Fluid Binary Phospholipid Membranes

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ABSTRACT Exclusion of the strongly hygroscopic polymer, poly(ethylene glycol) (PEG), from the surface of phosphatidylcholine liposomes results in an osmotic imbalance between the hydration layer of the liposome surface and the bulk polymer solution, thus causing a partial dehydration of the phospholipid polar headgroups. PEG (average molecular weight of 6000 and in concentrations ranging from 5 to 20%, w/w) was added to the outside of large unilamellar liposomes (LUVs). This leads to, in addition to the dehydration of the outer monolayer, an osmotically driven water outflow and shrinkage of liposomes. Under these conditions phase separation of the fluorescent lipid 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (PPDPC) embedded in various phosphatidylcholine matrices was observed, evident as an increase in the excimer-to-monomer fluorescence intensity ratio (I_E/I_M). Enhanced segregation of the fluorescent lipid was seen upon increasing and equal concentrations of PEG both inside and outside of the LUVs, revealing that osmotic gradient across the membrane is not required, and phase separation results from the dehydration of the lipid. Importantly, phase separation of PPDPC could be induced by PEG also in binary mixtures with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), for which temperature-induced phase segregation of the fluorescent lipid below T_m was otherwise not achieved. In the different lipid matrices the segregation of PPDPC caused by PEG was abolished above characteristic temperatures T_0 well above their respective main phase transition temperatures T_m. For 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), DMPC, SOPC, and POPC, T_0 was observed at ~50, 32, 24, and 20°C, respectively. Notably, the observed phase separation of PPDPC cannot be accounted for the 1°C increase in T_m for DMPC or for the increase by 0.5°C for DPPC observed in the presence of 20% (w/w) PEG. At a given PEG concentration maximal increase in I_E/I_M (correlating to the extent of segregation of PPDPC in the different lipid matrices) decreased in the sequence 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) > DPPC > DMPC > SOPC > POPC, whereas no evidence for phase separation in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) LUV was observed (Lehtonen and Kinnunen, 1994, *Biophys. J.* 66: 1981–1990). Our results indicate that PEG-induced dehydration of liposomal membranes provides the driving force for the segregation of the pyrene lipid. In brief, phase separation of PPDPC from the matrix lipid could be attributed to the diminishing effective size of the phosphatidylcholine polar headgroup resulting from its partial dehydration by PEG. This in turn would allow for enhanced van der Waals interactions between the acyl chains of the matrix lipid, which then caused the exclusion of PPDPC due to the perturbing bulky pyrene moiety. Phase separation in DMPC/PPDPC liposomes was abolished by the inclusion of 25 mol % cholesterol and to a lesser extent by epicholesterol.

INTRODUCTION

Phase separation and domain formation in biomembranes have been postulated to be of functional significance to cells (Sackmann, 1980; Kinnunen, 1991; Knoll et al., 1991). Translocation of hydrophobic ions and valinomycin-mediated ion transport, e.g., have been related to a Ca^{2+}-induced phase separation of the negatively charged phosphatidic acid in black lipid membranes (Schmidt et al., 1982; Miller et al., 1985). Likewise, the activity of gramicidin has been reported to correspond to a pH-induced phase separation in the membrane (Mittler-Neher and Knoll, 1990). Phase separation of phospholipids in sarcoplastic reticulum vesicles has been correlated to changes in the conformation as well as function of Ca^{2+}-ATPase (Asturias et al., 1990). Peripheral interactions of proteins such as phospholipase A_2 (Grainger et al., 1990; Burack et al., 1993; Maloney and Grainger, 1993), lipases (Smaby et al., 1994), and

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Abbreviations used in this article: PEG, poly(ethylene glycol) of an approximate molecular weight of 6000; PPDPC, 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine; PPDPG, 1-palmitoyl-2-[6(pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac'-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-rac'-glycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; $[PEG]_{OUT}$, $[glucose]_{OUT}$, the concentration of PEG or glucose outside the liposomes; $[PEG]_{SYM}$, $[glucose]_{SYM}$, the concentration of PEG or glucose symmetrically distributed at both sides of the liposomal membranes; $[PEG]_{IN}$, the concentration of PEG encapsulated in the liposomes; [PEG], the concentration of PEG; $\Delta\Omega$, the magnitude of the transmembrane osmotic gradient; Ω, osmotic pressure produced by osmolyte; I_E, excimer fluorescence intensity at 480 nm; I_M, monomer fluorescence intensity at 394 nm; T_o, temperature above which PEG no longer induces phase separation of PPDPC; T_m, phospholipid main phase transition temperature; C_{MAX}, concentration of PEG-producing aximal increase in I_E/I_M;E_{MAX}, maximal value for I_E/I_M; LUVs, large unilamellar vesicles.

cytochrome c (Mustonen et al., 1987) with membrane surfaces can be modulated by the lateral heterogeneity of phospholipids. Phase separation has been proposed to be of importance also for membrane fusion (Hoekstra, 1982). In addition to model systems there is a wealth of evidence indicating domain formation and lateral heterogeneity of phospholipids also in various biological membranes (e.g., Pagano et al., 1973; Ashley and Brammer, 1984; Gordon-Kamm and Steponkus, 1984; He and Hui, 1985; Orci et al., 1989; Selinsky and Yeagle, 1990; Rodgers and Glaser, 1991; Kinnunen, 1991). Obviously, it is essential that the underlying molecular level mechanisms influencing isothermal distribution of lipids in membranes be elucidated.

Lateral organization in mixed phospholipid membranes is modulated by various stimuli. In binary mixtures containing acidic lipids isothermal phase separation can be induced by charge neutralization of the anionic lipid species. This may be accomplished by Ca^{2+} (Jacobson and Papahadjopoulos, 1975; Parente and Lentz, 1986; Haverstick and Glaser, 1988; Eklund et al., 1988; Silvius, 1990), polycations such as spermine and spermidine (Ikeda et al., 1990), and pH (Tilcock and Cullis, 1981), as well as by basic proteins, e.g., prothrombin (Mayer and Nelsestuen, 1981). Thermally induced phase separation is observed under appropriate immiscibility conditions determined by the acyl chains and polar headgroups (Shimshick and McConnel, 1973; Lentz et al., 1981; Hui, 1981). High pressure as well as electric fields have been shown to induce lateral phase separation (Wattiaux-De Coninck et al., 1977; Klinger and McConnell, 1993). Phase segregation in proper phosphatidylethanolamine and phosphatidylcholine mixtures can be caused by dehydration (Webb et al., 1993) and ethanol (Rowe, 1987).

PEG is a hygroscopic, water-soluble, and chemically relatively inert synthetic polymer commonly used as a cell-cell fusogen in the production of somatic hybrids (Roos et al., 1987). Different cell lines vary in their susceptibility to the fusogenic effects of PEG, the fatty acyl composition of the plasma membrane lipids being an important determinant of this property (Roos and Choppin, 1985). PEG treatment of cells has been demonstrated to induce the formation of large areas lacking intramembranous particles in plasma membrane, thus representing reorganization and phase separation processes. These events seem to be prerequisites for PEG-induced membrane fusion (Roos et al., 1983). Phase separation of proteins due to PEG is probably a physicochemical process, as it is evident in addition to cells also in proteoliposomes after PEG addition (Hui et al., 1985).

Connected to the above, the importance of osmotic forces in regulating a number of physiological membrane processes is at present beginning to be understood. It is of interest that both hypo- as well as hyperosmotic shocks induce changes in plant cell metabolism (Maeda and Thompson, 1986; Einspahr et al., 1988). Cells have specific mechanisms for the restoration of their volume after osmotic shocks, involving the activation of tyrosine kinases (Tilly et al., 1993). Cell volume changes have been suggested to be of importance in the regulation of protein degradation and synthesis (Häussinger and Lang, 1991). The increased activity of ornithine decarboxylase in osmotically swelled cells is at least partially related to an enhanced synthesis of the enzyme protein in the absence of a change in its mRNA production (Poulin and Pegg, 1990). Transcription of specific genes in bacteria as well as in mammalian cardiomyocytes is enhanced by hyperosmotic shock (Csonka, 1989; Brewster et al., 1993). Hypoosmotic swelling induces stimulation of adenyl cyclase (Watson, 1990). Osmotic stress has been shown to participate in the regulation of mechanically activated ion channels (Martinac et al., 1987; Morris and Sigurdson, 1989; Rayner et al., 1992; Oliet and Bourque, 1993). Importantly, the lipid composition of the membrane could modulate the response of transmembrane ion channels to osmotic signals (Martinac et al., 1990). Exocytosis as well as fusion of liposomes with planar bilayers are sensitive to osmotic forces (Cohen et al., 1982; Pollard et al., 1984). The molecular mechanisms underlying the above processes have remained elusive.

Phospholipid analogs covalently labeled with, e.g., pyrenedecanoyl chain are at present widely employed for instance to study the lateral distribution and mobility of phospholipids (for a brief review see Kinnunen et al., 1993). In the absence of possible quantum mechanical phenomena and the formation of superlattices (Kinnunen et al., 1987; Tang and Chong, 1992), excimer formation by pyrene-labeled phospholipids such as PPDPC requires an intermolecular collision, and I_E/I_M thus depends on the local concentration as well as on the lateral mobility of PPDPC (Galla and Sackmann, 1974). Accordingly, I_E/I_M is sensitive to phase separation processes taking place in membranes (Galla and Sackmann, 1974; Galla and Hartmann, 1980; Somerharju et al., 1985; Hresko et al., 1986; Junker and Creutz, 1993). In PPDPC the pyrene fluorophore resides embedded in the hydrocarbon region, and minimal perturbation of interactions such as hydration taking place on the membrane surface is anticipated. Yet, compared with DPPC, PPDPC occupies a slightly larger area in lipid monolayers on an air/water interface (Kinnunen et al., 1985; Somerharju et al., 1985). The rigid pyrene moiety acts as a steric perturbant that disfavors co-localization of PPDPC and DPPC below T_m of the latter lipid when augmented van der Waals interactions between the acyl chains of DPPC cause the substitutional impurity PPDPC to became partially enriched into domains, thus yielding increased I_E/I_M values (Somerharju et al., 1985; Hresko et al., 1986). However, no phase separation of PPDPC embedded in DMPC is observed below the T_m of the latter lipid (Hresko et al., 1986). Likewise, temperature-induced segregation of PPDPC is not seen in unsaturated lipid membranes such as SOPC and POPC.

We have provided evidence for a decrease in the membrane free volume upon osmotic shrinkage and dehydration of DOPC LUV by PEG (Lehtonen and Kinnunen, 1994). These effects are readily explained by the removal of water from the hydration shell of the phosphatidylcholine by PEG, which results in a decrease in the effective size of the lipid

headgroup. Accordingly, this causes an enhanced lateral packing of the membrane lipids (Arnold et al., 1983; Sen and Hui, 1988; Rand and Parsegian, 1989). The present study provides evidence for PEG-induced phase separation of PPDPC in large unilamellar liposomes composed of different saturated (i.e., DHPC, DPPC, and DMPC) as well as unsaturated phosphatidylcholines such as SOPC and POPC. The extent and temperature dependencies of this process are correlated to the structure of the polar headgroup, carbonyl moieties, acyl chain length, and unsaturation of the matrix lipid. The available data and the lack of temperature-induced segregation for PPDPC/DMPC liposomes below T_m of DMPC and in the absence of PEG strongly indicates that this effect is not caused by an increase and broadening of T_m due to PEG. Cholesterol inhibits the lateral phase separation induced by PEG, and this inhibition depends on the proper stereoconfiguration of its hydroxyl group.

MATERIALS AND METHODS

Materials

PPDPC and PPDPG were purchased from K & V Bioware (Espoo, Finland); and DMPC, DPPC, DHPC, DOPC, POPC, SOPC, epicholesterol, and cholesterol were from Sigma Chemical Co. (St. Louis, MO). PEG, with an average molecular weight of 6000, was obtained from Fluka (Bubendorf, Switzerland) and was used without further purification (Yamazaki et al., 1989; Burgess et al., 1991). No impurities were detected in the above lipids upon thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, v/v) as a solvent system and examination of the plates for pyrene fluorescence or after iodine staining.

The concentrations of the nonfluorescent phospholipids were determined by phosphorus assay (Bartlett, 1959) and those of cholesterols by dry weight as well as from compression isotherms measured for monolayers residing on an air/aqueous buffer interface. Surface pressure was measured with the Wilhelmy plate method, and the recorded area/molecule isotherms were correlated with those in the literature (Demel et al., 1972). Concentrations of the pyrene-containing probes were determined spectrophotometrically at 342 nm using 42,000 cm^{-1} as the molar extinction coefficient for pyrene.

Liposome preparation

Lipids were dissolved in chloroform. After mixing of the desired lipid composition the solvent was removed under a stream of nitrogen. The dry lipid mixture was then maintained under reduced pressure overnight and subsequently hydrated in 2 mM TES, 1 mM EDTA and 100 mM NaCl, pH 7.4, at least 10°C above the gel → liquid crystalline transition temperature (T_m) of the phospholipid in question. To obtain liposomes with PEG_{SYM} (i.e., equal [PEG] both inside and outside of the liposomes) lipids were hydrated in buffer containing the indicated concentrations of PEG. The lipid dispersions were subsequently pressed through polycarbonate filters (see below) (at a lipid concentration of 1.5 μmol/500 μl) and then diluted in buffer with equal [PEG] in order to have $[PEG]_{IN} = [PEG]_{OUT}$. In some experiments glucose was used instead of PEG. The final concentration of lipid used in the fluorescence measurements was 30 μM. In all experiments the amount of the fluorescent phospholipid (PPDPC) was maintained constant at 1.0 mol % of total phospholipid. To obtain LUVs the dispersions were extruded through 0.1-μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA) using a LiposoFast low-pressure homogenizer (Avestin, Ottawa, Canada) essentially as described (MacDonald et al., 1991).

Fluorescence measurements

Fluorescence measurements were carried out with an SLM 4800S spectrofluorometer (SLM Instruments Inc., Urbana, IL). Excitation wavelength of 344 nm as well as the pyrene monomer fluorescence at 394 nm were selected by monochromators, and the excimer emission was monitored simultaneously with a 470-nm long pass filter. Slits of 1 and 16 nm were used for the excitation and monomer emission beams, respectively. 2 ml liposome solution (60 nmol of lipid) was placed in a magnetically stirred four-window quartz cuvette in a holder thermostated at the indicated temperatures with a circulating water bath. Weak fluorescence due to PEG was observed both at the monomer and excimer emission wavelengths upon excitation at 344 nm. Because of the low pyrene concentrations used, this background was subtracted from the measured emission intensities before the calculation of the excimer-to-monomer fluorescence ratio (I_E/I_M). No evidence for energy transfer from pyrene to PEG or vice versa was observed. Although ≈15% higher emission intensities have been measured after removal of oxygen from the samples, the relative values remain unaltered. Accordingly, for more facile experimentation the fluorescence data were collected in the presence of atmospheric oxygen (Mustonen and Kinnunen, 1993; Mustonen et al., 1993). To be able to express the numeric data in a more manageable range and to allow for an easier comparison of the different measurements, the I_E/I_M values used in illustrations were normalized so that I_E/I_M for 1 mol % PPDPC in DMPC at 25°C was assigned to unity, and all other values were calculated in relation to this. To obtain I_e/I_m values corrected for instrument response and for normalization the given values should be divided by 11.3.

To avoid scattering due to air bubbles, fluorescence intensities were measured 10 min after the addition of PEG or glucose to LUV solutions. In temperature scans the suspensions were allowed to stabilize for 4 min after reaching the desired temperature. The emission signals were stable for at least 30 min. Accordingly, in keeping with previous studies, aggregation of liposomes should be minimal at the lipid concentrations used (Tilcock and Fisher, 1982). Osmotic shrinkage of small unilamellar vesicles by LiCl (Lerebours et al., 1993) does not produce the content leakage from these vesicles. Likewise, $[PEG]_{OUT}$ (up to 20%, w/w) does not cause the disruption of DOPC LUV (Burgess et al., 1991). For DPPC LUV only minor leakage of contents below $[PEG]_{OUT}$ = 20% (w/w) is evident, whereas at higher $[PEG]_{OUT}$ leakiness is seen (Lentz et al., 1992; Massenburg and Lentz, 1993). Accordingly, leakage of the LUV contents in the course of our experiments is unlikely. Furthermore, PEG concentrations were below those required for fusion (Massenburg and Lentz, 1993). Because of the viscosity of concentrated PEG solutions 20% (w/w) was not exceeded. Maximum concentration of glucose utilized was 1 M. Osmolalities were measured using a freezing point osmometer (Advanced Microosmometer, Model 3 Mo, MA).

RESULTS

Phase separation of PPDPC in DMPC LUV

An excited pyrene monomer may form an excited-state dimer (excimer) with a ground-state pyrene, and the excimer-to-monomer fluorescence intensity ratio (I_E/I_M) depends on the rate of intermolecular collisions between pyrenes (Förster, 1969). Consequently, for a pyrene-containing phospholipid analog such as PPDPC the value for I_E/I_M reflects the lateral mobility (Galla and Sackmann, 1974; Galla et al., 1979) as well as the local concentration of the fluorophore in the membrane (Galla and Hartmann, 1980; Somerharju et al., 1985; Hresko et al., 1986; Eklund et al., 1988). Phase separation of PPDPC occurs in DPPC liposomes below T_m of the matrix lipid and is evident as an increase in I_E/I_M (Somerharju et al., 1985; Hresko et al., 1986). However, no phase separation of PPDPC in DMPC is observed below T_m of the latter lipid at 24°C (Hresko et al., 1986). For this reason it was somewhat surprising that increasing $[PEG]_{OUT}$ at 24°C (i.e., PEG added to the outside of LUV) induced an increase in the I_E/I_M values for 1 mol % PPDPC residing in DMPC (Fig. 1 *A*). This effect was reduced at higher temperatures, and at 38°C practically

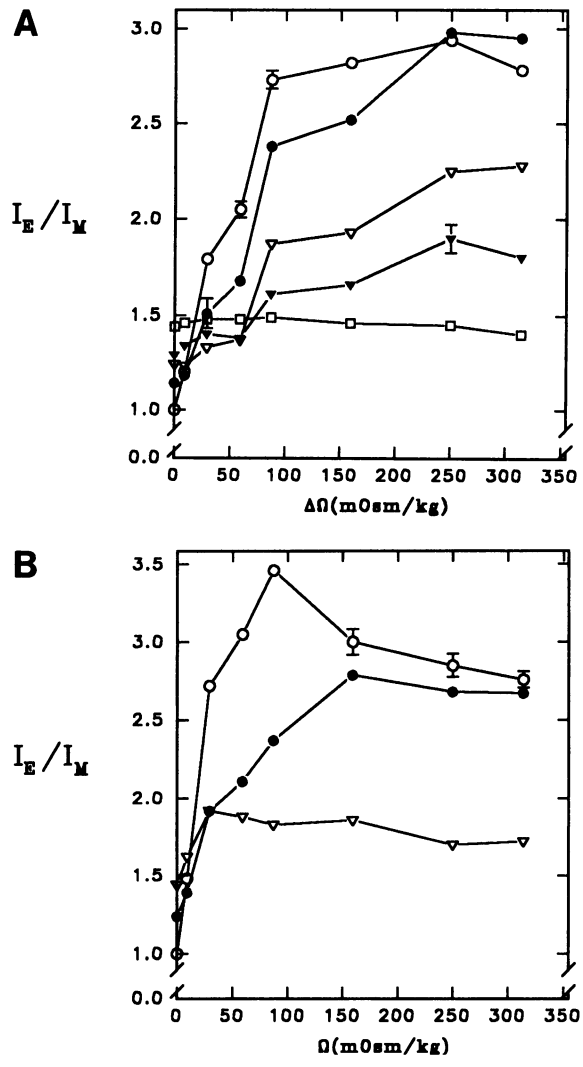

FIGURE 1 (*A*) Changes in I_E/I_M for 1 mol % PPDPC in DMPC LUV upon osmotic shrinkage induced by varying $[PEG]_{OUT}$ at 24 (●–●), 30 (○–○), 33 (▽–▽), 35 (▼–▼), and 38°C (□–□). Error bars indicate the range of measurements for two to six separate experiments. For the sake of clarity error bars overlapping with symbols are not shown. Buffer was 2 mM TES, 1 mM EDTA, and 100 mM NaCl, pH 7.4. (*B*) Same experimental conditions as for (*A*) but varying $[PEG]_{SYM}$ was used instead of $[PEG]_{OUT}$.

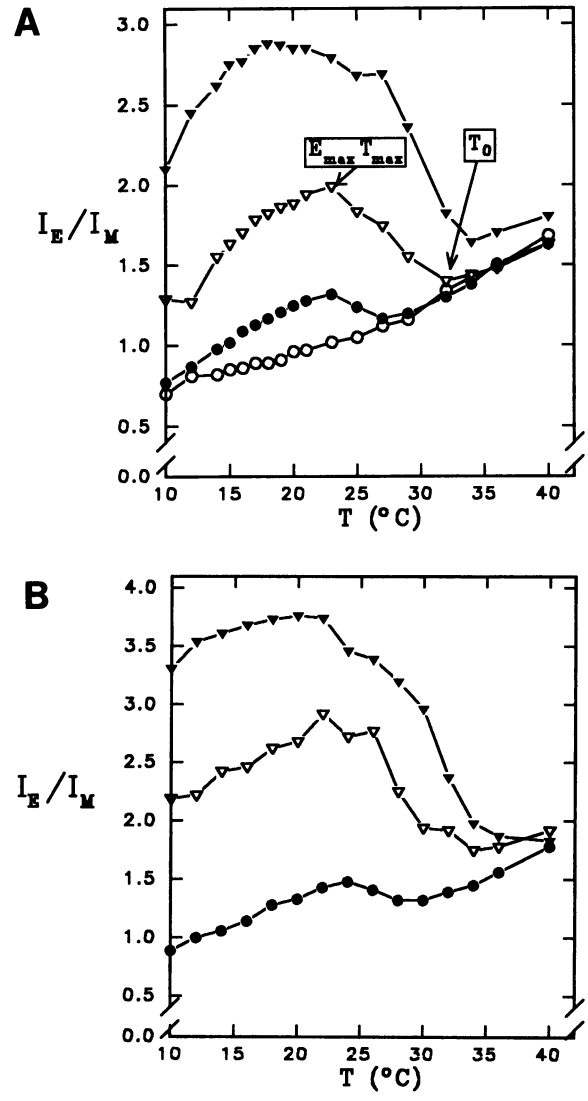

FIGURE 2 (*A*) Changes in I_E/I_M as a function of temperature and accompanying osmotic shrinkage of PPDPC/DMPC LUV due to $[PEG]_{OUT}$. $[PEG]_{OUT}$ was 0 (○–○), 9 (●–●), 29 (▽–▽), and 250 mOsm/kg (▼–▼). Definition of E_{MAX}, T_{MAX}, and T_0 is also illustrated. (*B*) Similar experiment as depicted in (*A*) but using $[PEG]_{SYM}$. Symbols are identical to those used for (*A*). Conditions were otherwise as described for Fig. 1.

no changes in I_E/I_M due to $[PEG]_{OUT}$ (=10% (w/w)) were evident. Upon increasing $[PEG]_{SYM}$, i.e., with equal [PEG] on both sides of the liposomal membranes the increase in I_E/I_M was further augmented; also, the temperature dependency was slightly altered compared with the results with varying $[PEG]_{OUT}$ (Fig. 1 *B*).

The temperature dependency of PEG-induced changes in I_E/I_M was investigated in more detail. The maximum in I_E/I_M (noted as E_{max}) for PPDPC/DMPC LUV caused by PEG_{OUT} was observed in the vicinity of T_m of DMPC at 24°C, Fig. 2 *A*. Interestingly, at 2.5% (w/w) polymer the increase in I_E/I_M was abolished at ~29°C. Above this temperature (here abbreviated as T_0; see Fig. 2 *A* for definition) values for I_E/I_M were indistinguishable from those measured in the absence of PEG and increased progressively with temperature, weakening intermolecular interactions allowing for faster lateral mobility, and increased thermally driven collision frequency between the pyrene moieties of PPDPC (Somerharju et al., 1985; Hresko et al., 1986). The value for T_0 depends on the magnitude of the polymer gradient and is observed at ~34°C at $[PEG]_{OUT}$ = 15% (w/w), corresponding to 250 mOsm/kg. Similar results were obtained with PEG_{SYM}. However, compared to results with PEG_{OUT} T_0 is seen at a higher temperature (≈40°C), and E_{max} at 24°C is slightly increased (Fig. 2 *B*).

For comparison with PEG we also studied the effects exerted by the monomeric osmotically active substance glucose on I_E/I_M values for PPDPC/DMPC LUV. At 270 mOsm/kg of $[glucose]_{OUT}$ a ~10% rise in I_E/I_M was observed (data not shown). Compared with the effect caused by 10% (w/w) PEG

(87 mOsm/kg) this enhancement in I_E/I_M due to glucose is significantly smaller and, in addition, disappears at higher glucose concentrations. Glucose is not excluded from the surface of membrane and has been shown to interact with the polar headgroups of phosphatidylcholine. Therefore, it is not expected to dehydrate the membrane surface as efficiently as PEG does (Crowe et al., 1984, 1988; Rudolph et al., 1990; Goodrich et al., 1991; Rand et al., 1993). In contrast to PEG_{SYM}, $glucose_{SYM}$ has no effects on I_E/I_M for PPDPC (data not shown). Polar headgroup-glucose interactions increase the effective size of the polar headgroup, thus forcing the lipids further apart. This is evident in lipid monolayers where the addition of glucose increases the area/molecule (Crowe et al., 1984), whereas PEG has the opposite effect (Maggio and Lucy, 1978). Therefore, van der Waals interactions between the acyl chains are not augmented upon increasing $[glucose]_{SYM}$.

The effects of PEG on different phospholipids

The polar headgroup

To examine the possibility that the above PEG-induced changes are specific for phosphatidylcholine we conducted at this stage measurements also with DMPG LUV. To exclude effects due to different headgroups PPDPG was employed as the fluorescent probe. As the experiments with DMPC LUV revealed no qualitative differences between PEG_{OUT} and PEG_{SYM} these studies with DMPG were, for the sake of more facile experimentation, performed only using PEG added to the outside of the liposomes. As in the case of PPDPC/DMPC liposomes, phase separation of PPDPG in DMPG was induced by PEG (data not shown). Yet, at $[PEG]_{OUT}$ = 10% (w/w) (87 mOsm/kg), there appeared to be a second maximum in the I_E/I_M at 27°C, which was not seen for DMPC. For comparison with data on the other lipids the values of E_{max}/and T_0 are compiled in Table 1.

TABLE 1 Comparison of the data for PPDPC embedded in different phosphatidylcholine matrices measured at $[PEG]_{OUT}$ = 5% (w/w). T_0 is the critical temperature where the PEG-induced phase separation is abolished and E_{MAX} is the maximum I_E/I_M value for PPDPC in the indicated matrix. See Discussion for details.

Lipids	T_0	E_{MAX}	I%*	T_m‡
DOPC	–	–	–	−21°C
POPC	10°C	0.75	3	4°C
SOPC	14°C	0.97	25	6–13°C
DMPC	32°C	1.8	90	23.6°C
DPPC	44°C	3.5	85¶	41.3°C
DHPC	45°C	4.1	46¶	43.4°C

*I% was calculated as, 100 × [E_{MAX} at 5% (w/w) of PEG]/[I_E/I_M at the same temperature for liposomes in the absence of PEG]. Accordingly, this figure gives the relative effect of PEG on the extent of segregation of PPDPC

‡T_m is phase transition temperature for different lipids; from Silvius, 1982

§No evidence for segregation of PPDPC; from Lehtonen and Kinnunen, 1994

¶In these matrices there is temperature-induced phase separation of PPDPC below the T_m.

The acyl chain length

In contrast to PPDPC/DMPC liposomes there is a thermally induced partial phase separation of PPDPC below the main phase transition temperature of DPPC at ≈42°C (Somerharju et al., 1985; Hresko et al., 1986). The difference by two methylene groups in the acyl chain lengths of DPPC and DMPC also enhanced the PEG-induced exclusion of PPDPC from DPPC, evident as augmented values for I_E/I_M above T_m of the latter lipid. Judged from E_{MAX}, the PEG-induced exclusion of the pyrene lipid from fluid DPPC was more extensive (≈3-fold) than the thermally induced segregation of PPDPC from the gel phase of DPPC in the absence of the polymer. After E_{max} at 29°C for $[PEG]_{OUT}$ corresponding to 59 mOsm/kg, the I_E/I_M values for PPDPC/DPPC LUV declined rapidly and at temperatures exceeding T_0 = 44°C the effect caused by PEG_{OUT} was abolished (Table 1.)

Carbonyl group

To study the role of the ester bond carbonyls in PEG-induced phase separation we used DHPC. This diether lipid is a close structural analog of DPPC and differs from the latter only by the type of linkage of the hydrocarbon chains to the glycerol backbone. As in the case of DPPC, partial segregation of PPDPC was observed below the main transition of DHPC. Compared with PPDPC/DPPC LUV the effects of PEG on PPDPC/DHPC membranes were more pronounced, however, and ≈25% higher I_E/I_M values were observed. For DHPC also the dependency of T_0 on $[PEG]_{OUT}$ differs from DPPC. Accordingly, T_0 was observed at 46°C when $[PEG]_{OUT}$ = 5% (w/w) (29 mOsm/kg) and at 50°C for 10 and 20% (w/w) of PEG (corresponding to 87 and 494 mOsm/kg, respectively) (Table 1).

Acyl chain unsaturation

Introduction of *cis*-double bonds into the acyl chains of phospholipids resulted in a marked reduction in their T_m (Op den Kamp et al., 1975). Accordingly, it was of interest to study whether PEG-induced segregation of PPDPC would be observed also with unsaturated phosphatidylcholines. In DOPC, which contains two unsaturated acyl chains, neither thermal nor PEG-induced phase separation of PPDPC has been observed in the temperature range of 8 to 40°C and only attenuated lateral diffusion of PPDPC is evident due to PEG_{OUT} reducing the membrane free volume (Lehtonen and Kinnunen, 1994).

As with PPDPC/DOPC LUV, there are in the absence of PEG essentially no indications for the phase separation of PPDPC in I_E/I_M versus temperature scans in either POPC or SOPC LUV in the temperature range examined. Naturally, for PPDPC/POPC LUV a high concentration of PEG (20% (w/w), 494 mOsm/kg) and low temperatures (<20°C) are required to induce weak segregation of PPDPC evident as a relatively small increment in I_E/I_M (Table 1). For a phospholipid with an oleoyl chain at the *sn*-2 position an increase in the length of the saturated *sn*-1 acyl chain (i.e., using

SOPC instead of POPC) should enhance the PEG-induced phase separation of PPDPC. This prediction turned out to be correct (Table 1).

Effects of cholesterol

The presence of cholesterol decreased the extent of PEG_{OUT}-induced increase in I_E/I_M of PPDPC/DMPC LUV (Fig. 3 *A*), and at 25 mol % of cholesterol no segregation of PPDPC was observed. Notably, epicholesterol was less efficient in reducing E_{MAX} (Fig. 3 *B*).

To further characterize the differences between the two cholesterol stereoisomers on PEG-induced separation of PPDPC in DMPC the temperature dependency of I_E/I_M was also investigated. Already 2 mol % of cholesterol incorporated in DMPC LUV inhibited effectively the segregation of PPDPC induced by 87 mOsm/kg $[PEG]_{OUT}$. By comparison, 2 mol % of epicholesterol had only a moderate effect (Table 2). At higher cholesterol content the PEG-induced segregation of PPDPC was even more efficiently reduced. Notably, there was segregation due to PEG_{OUT} in PPDPC/DMPC LUV containing 10 mol % of epicholesterol, although the value for E_{MAX} was slightly reduced compared with PPDPC/DMPC LUV (Table 2).

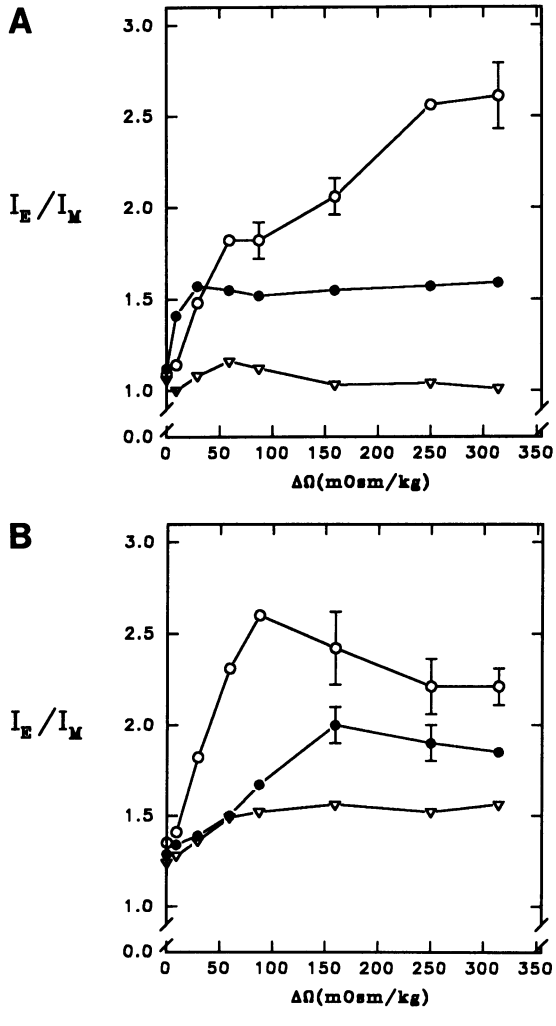

FIGURE 3 (*A*) The effect of cholesterol on $[PEG]_{OUT}$-induced changes I_E/I_M for PPDPC/DMPC LUV. The concentrations of cholesterol were 2 (○–○), 10 (●–●), and 25 mol % (▽–▽). Experimental conditions were as described for Fig. 1. (*B*) Same experimental conditions as for (*A*) but utilizing epicholesterol.

TABLE 2 Comparison of the data for PPDPC embedded in DMPC/cholesterol matrices measured at $[PEG]_{OUT}$ = 10% (w/w). T_0 is the critical temperature where the PEG-induced phase separation is abolished and E_{MAX} is the maximum I_E/I_M value for PPDPC in the indicated matrix. See Discussion for details.

Cho%*	T_0	E_{MAX}	I%‡
β-2%	30°C	1.7	200
β-10%	33°C	1.6	167
α-2%	35°C	2.6	260
α-10%	35°C	2.4	175

*The percent cholesterol in DMPC liposomes. α- and β- indicate the stereoconfiguration of hydroxyl group of cholesterol

‡I% was calculated as 100 × [E_{MAX} at 10% (w/w) of PEG]/[I_E/I_M at the same temperature for liposomes in the absence of PEG]. Accordingly, this figure gives the relative effect of PEG on the extent of segregation of PPDPC

DISCUSSION

The present results demonstrate in a simple model system that dehydration and osmotic shrinkage of phosphatidylcholine liposomes by the strongly water-binding polymer PEG may produce at proper temperatures phase separation of PPDPC even in those binary membranes that otherwise are miscible below T_m of the matrix lipid (e.g., DMPC below 24°C, Hrésko et al., 1986). More strikingly, phase separation caused by PEG is also evident for unsaturated phosphatidylcholines such as SOPC and POPC, whereas for DOPC with two unsaturated chains segregation has not been observed (Lehtonen and Kinnunen, 1994). Dehydration-induced phase separation has been proposed earlier due to theoretical considerations (Bryant and Wolfe, 1989).

Notably, PEG does not induce the formation of nonlamellar lipid phases by phosphatidylcholine liposomes (Tilcock and Fisher, 1982; Arnold et al., 1983). In addition, as segregation of PPDPC caused by PEG_{SYM} (i.e., in the absence of [PEG] gradients across the bilayer) exceeds in magnitude that due to PEG_{OUT}, destabilization of the bilayer as an underlying cause for the effects observed is improbable. Likewise, a parallel lipid flow between the monolayers of the bilayer accompanying osmotic water flow (Boroske et al., 1981) cannot explain the PEG-induced segregation of PPDPC as the latter process becomes enhanced by PEG_{SYM}, i.e., in the absence of net water flow across the bilayer. X-ray diffraction studies on DOPC LUVs have revealed an abrupt change from diffuse to constant diffraction patterns when $[PEG]_{OUT}$ exceeds 10% (w/w). These results were obtained at high lipid concentrations (10 mg/ml) and were interpreted as the stacking of aggregated discoid vesicles (Burgess et al., 1992). Whereas DOPC LUVs have been proposed to become discoidal due to water outflow, one could expect that in the

presence of PEG_{SYM} vesicles should retain their shape, as there is no net water flow through the liposomal membrane. For this reason, it is improbable that segregation of PPDPC is caused by vesicle deformation.

Upon hydration the effective size of the strongly hygroscopic phosphatidylcholine polar headgroup increases and larger acyl chain separation and reduced van der Waals interactions between the chains are evident (Jendrasiak and Hasty, 1974a, b; Finer and Darke, 1974; Klose and Stelzner, 1974; Wilkinson et al., 1977; Keith et al., 1977; Cowley et al., 1978; Rand et al., 1985; Marra and Israelachvili, 1985; Cevc and Marsh, 1985; Wong and Mantsch, 1988; Rand and Parsegian, 1989; Marsh, 1989; Gawrisch et al., 1990). Vice versa, analogously to proteins (Rand, 1992) dehydration of phospholipids allows tighter lateral packing of lipids as the effective size of the polar headgroup is reduced, thus leading to an enhanced acyl chain packing (Rand and Parsegian, 1989). PEG does not bind to phospholipids (Arnold et al., 1990), and its effects should be limited to dehydration (Arnold et al., 1983), which results from the exclusion of the polymer from the surface of liposomes, thus creating an osmotic imbalance between the exclusion layer and the bulk PEG solution. Hydration of phosphatidylcholine is reduced by 30% in the presence of 20% (w/w) PEG (Sen and Hui, 1988). Notably, effects caused by PEG_{SYM} can be attributed solely to dehydration, whereas increasing $[PEG]_{OUT}$ also causes an osmotic shrinkage. Decreased internal volume due to the osmotically driven water efflux across the membrane (Bittman and Blau, 1972; Carruthers and Melchior, 1983; Deamer and Bramhall, 1986) results in an enhanced lateral lipid packing in the membrane (Boroske et al., 1981; Lichtenberg et al., 1982) in a manner similar to the liquid crystalline → gel phase transition.

Nonideal interactions between two different lipids can result in large-scale phase separation or microscopic clustering of the other lipid component depending on the strength of the interaction. Isothermal compression (reduction in area/molecule) may induce phase separation in monolayers (Peters and Beck, 1983; McConnell et al., 1984). Interestingly, although there is no thermally induced phase separation of PPDPC in DMPC liposomes (Hresko et al., 1986) indications for submicroscopic phase separation of PPDPC/DMPC monolayers at high surface pressures have been reported (Merkel, 1993). For monolayers of phosphatidylcholine PEG induces reduction in area/molecule (Maggio and Lucy, 1978).

Osmotic shrinkage and dehydration of LUV due to PEG decreases the membrane free volume, i.e., enhances lateral packing of lipids (Lehtonen and Kinnunen, 1994). The extent of exclusion of PPDPC (i.e., value for E_{MAX}) induced by PEG depends on the properties of lipid matrix and decreases in the sequence DHPC > DPPC > DMPC > SOPC > POPC (Table 2). The demixing of PPDPC results from the excess mixing energy ΔG_M of PPDPC/matrix phosphatidylcholine, which correlates to the perturbation of the matrix lipid packing by PPDPC. The enhanced lateral packing of phospholipids induced by dehydration by PEG also increases ΔG_M. Accordingly, increase in acyl chain length of the (DMPC → DPPC) leads to enhanced chain-chain interactions, and a more efficient exclusion of PPDPC is observed. Similarly, DHPC lacking the ester carbonyl moieties is more efficiently packed than DPPC (Paltauf et al., 1971; Ruocco et al., 1985; Kim et al., 1987), and the exclusion of PPDPC due to PEG is more pronounced in this lipid. The segregation of PPDPC decreases upon the introduction of an unsaturated acyl chain to *sn*-2 position, and no indications for immiscibility are observed for PPDPC/DOPC membranes.

The PEG-induced segregation of PPDPC is abolished above a characteristic temperature T_0 above T_m of the matrix lipid (Table 1). Increment of the acyl chain length by two methylene segments (DMPC → DPPC) or lack of carbonyl groups (DPPC → DHPC) increases T_0. Importantly, T_m of DMPC is increased by 20% (w/w) PEG only up to 25°C (Yamazaki et al., 1992), whereas maximum for T_0 of PPDPC/DMPC LUV at $[PEG]_{OUT}$ = 15% (w/w) is observed at ≈34°C. Likewise, 20% (w/w) PEG causes an increase by 0.5° in the T_m of DPPC (Burgess et al., 1991), whereas T_0 for this lipid is 44°C. Therefore, together with the lack of segregation of PPDPC from DMPC below the T_m of the latter lipid in the absence of PEG, it is clear that T_0 does not merely represent an increased T_m due to PEG. For POPC a weak segregation of PPDPC at $[PEG]_{OUT}$ = 20% (w/w) (corresponding to 494 mOsm/kg) is seen below 20°C. Increasing the length of the *sn*-1 acyl chain (i.e., palmitoyl → stearoyl) further enhances the phase separation process evident as higher values for T_0 as well as E_{MAX}. To conclude, the shorter or more saturated the acyl chains are the larger is the difference between T_m and T_0 of the lipid. This is in accordance with the notion that the weaker van der Waals interactions are between the acyl chains, the more important are the interactions on the headgroup level to the behavior of the lipid (Cevc, 1988).

The molecular events at T_0 remain open at present, yet changes in the headgroup/water interactions should be involved. Evidence for a new phase transition above T_m has been forwarded for osmotically stressed liposomes of DLPC and DMPC (Lis et al., 1982). There are some indications for a change in the conformation and/or organization of DMPC in the temperature range of 30–35°C (Mendelsohn et al., 1981; Sankaram et al., 1992). T_0 could arise from reduction of the steric pressure because of an increase in membrane free volume due to temperature-dependent increment in *trans*-gauche isomerization of the acyl chains, which then results in a gradual fading of the phase separation of PPDPC. However, this view may not be valid, as this process should be evident also in the absence of dehydration due to PEG. Accordingly, one would then expect a smooth, monotonous increase of I_E/I_M measured in the absence of PEG as a function of temperature. This is not the case. I_E/I_M versus temperature measured for PPDPC/DMPC liposomes in the absence of PEG reveals in the region of T_0 between 32 and 35°C a small yet clearly distinguishable deviation from the smoothly ascending I_E/I_M curve (Fig. 4). Water binding of phospholipids depends on temperature and increases gradually after T_m

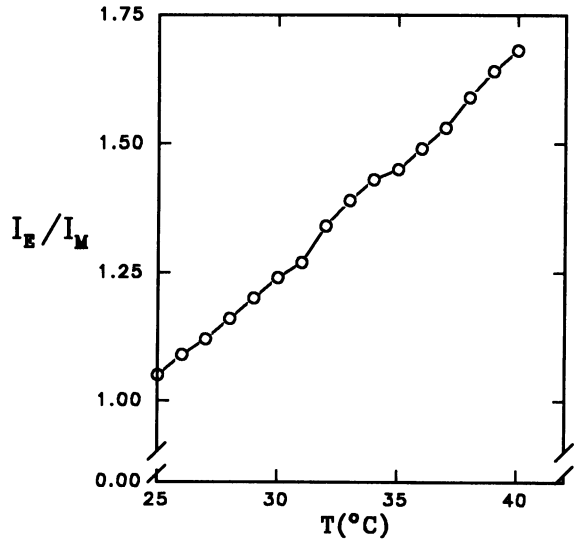

FIGURE 4 I_E/I_M versus temperature for PPDPC/DMPC LUV measured in the absence of PEG.

(Wilkinson et al., 1977). Therefore, we interpreted T_0 to represent the temperature where the affinity of the lipid for water exceeds that of PEG. This is supported by the dependency of T_0 on the T_m of the matrix lipid.

To this end it is of interest that for DMPC monolayers there is a spontaneous formation of surface bilayers at a critical temperature $T^* \approx 29°C$ (Gershfeld and Tajima, 1979; Ginsberg and Gershfeld, 1985; Gershfeld, 1989a). This is also observed for DPPC at $T^* \approx 44°C$ (Tajima and Gershfeld, 1985) and for DMPG at 31.5°C (Gershfeld et al., 1986), thus corresponding reasonably well to the T_0 values of the present study. The change in the organization of these lipids was hypothesized to be related to changes in the hydration of the lipids at T^*; yet, the molecular events have remained elusive (Gershfeld, 1989b; Gershfeld et al., 1993). T^* for lipids extracted from natural membranes was shown to depend on the acyl chain length and the degree of unsaturation (Gershfeld, 1986).

The phase diagram for DMPC/cholesterol liposomes is at present well understood (Ipsen et al., 1987). Although cholesterol is poorly hydrated (Sen and Hui, 1988) its presence increases the hydration of phosphatidylcholine (Jendrasiak and Hasty, 1974a,b; Yeagle et al., 1977; Ter-Minassian-Saraga and Madelmont, 1982). Whereas the cholesterol ring structure effectively rigidifies the upper part of phospholipid acyl chains, a disordering effect on the central region is evident (Stockton and Smith, 1976). We observed cholesterol to abolish in a concentration-dependent manner the PEG-induced increase in the I_E/I_M values for PPDPC in DMPC. It is possible that the steric constraints in the middle regions of the bilayer that cause PPDPC to be segregated are relieved in the presence of cholesterol so that the pyrene moiety can be accommodated within the matrix. Accordingly, this effect of cholesterol would be somewhat analogous to the lack of phase separation of PPDPC in DOPC. Subtle structural features of cholesterol appear to be involved as epicholesterol is less efficient in relieving the osmotically induced phase separation of PPDPC in DMPC.

CONCLUSIONS

We interpret the segregation of PPDPC in different phosphatidylcholine matrices in terms of two kinds of osmotic effects by PEG, as follows. First, an osmotic force is due to the exclusion of the polymer from the surface of liposomes, i.e., an osmotic imbalance between water in the exclusion layer and bulk PEG solution causes partial dehydration of the phospholipid headgroup. Second, transmembrane osmotic gradient can be induced by PEG, which causes the shrinkage of liposomes. For PEG_{OUT} both of these osmotic mechanisms exist, whereas for PEG_{SYM} there is only an osmotic imbalance because of the exclusion layer. Dehydration reduces the effective size of the phospholipid polar headgroup, thus allowing for an enhanced lateral packing of the membrane lipids. Because of both PEG_{OUT} and PEG_{SYM}, the substitutional impurity, PPDPC, becomes excluded from the lipid matrix, as the bulky pyrene moiety does not allow its colocalization within the condensing matrix. The extent of phase separation of PPDPC depends on the lipid matrix decreasing in the order DHPC > DPPC > DMPC > SOPC > POPC, parallel with the attenuation of chain-chain interactions between the matrix lipids. PPDPC does not segregate in DOPC (Lehtonen and Kinnunen, 1994). The segregation of PPDPC disappears at a characteristic temperature T_0 above T_m of the matrix lipid. There is only a 1°C increase in T_m for DMPC and an increase by 0.5°C for DPPC in the presence of 20% (w/w) PEG. Accordingly, the polymer-induced segregation of PPDPC reported here represents fluid-fluid immiscibility. Disappearance of the phase separation at T_0 is probably related to an increasing water-binding affinity of phosphatidylcholine and above T_0 the affinity of phosphatidylcholine for water should exceed that of PEG. Interestingly, values for T_0 for DMPC, DMPG, and DPPC appear to be close to the critical temperatures of these lipids where spontaneous formation of bilayers from monolayers have been observed (Gershfeld, 1989b). The presence of cholesterol abolishes PEG-induced phase separation of PPDPC. This is probably caused by the disordering effect of cholesterol in the middle region of the bilayer, which reduces the steric pressure required to exclude PPDPC upon membrane dehydration by PEG. Finally, our results indicate a novel possibility for an isothermal control of the lateral distribution of lipids in biomembranes.

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