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EFFECT OF POLY(ETHYLENE GLYCOL) ON PHOSPHOLIPID HYDRATION AND POLARITY OF THE EXTERNAL PHASE

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The hydration properties of phosphatidylcholine (PC)/water dispersions on the addition of poly(ethylene glycol) were studied by means of ^{2}H-NMR. The quadrupole splittings and their temperature dependences correspond to measurements of PC/water dispersions at low water content. It is concluded that the bound water is partly extracted by poly(ethylene glycol) but the binding properties of the water in the inner hydration shell of about five water molecules are not changed. The ability of some phospholipid/water dispersions to undergo phase transitions to nonlamellar structures upon dehydration is discussed. Dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine do not form nonlamellar structures on addition of purified poly(ethylene glycol), as was demonstrated by means of ^{31}P-NMR. Poly(ethylene glycol) decreases the polarity of the aqueous phase and the partition of hydrophobic molecules between the membrane and the external phase is changed. This was demonstrated using the excimer fluorescence of pyrene in a ghost suspension. It is suggested that the changes in polarity and hydration on the addition of poly(ethylene glycol) can contribute to the alterations in the membrane surface observed under conditions of membrane contact and fusion.

Introduction

Poly(ethylene glycol) is the most commonly used chemical fusogen for the fusion of different cell species and lines [1–3]. Cells are incubated in concentrated aqueous solutions of poly(ethylene glycol) (about 40 wt.%) for a short time (1–3 min), which leads to cell shrinkage due to the removal of water from cells and changes in the morphology of the membrane (cluster formation of intramembranous particles, defects in the bilayer structure) [3–5]. The aggregation of cells is followed by the fusion of cells after removal of most of the poly(ethylene glycol).

The properties of poly(ethylene glycol) leading to the membrane alterations observed and the physical mechanisms of poly(ethylene glycol) inducing cell fusion were extensively studied [4,6–8]. However, the molecular mechanisms of poly(ethylene glycol)-induced fusion are still not understood. Poly(ethylene glycol) has a structure favourable to the formation of hydrogen bonds with water molecules and no free water exists in an aqueous solution of more than 38 wt.% poly(ethylene glycol) [9,10]. The addition of poly(ethylene glycol) leads to a partial dehydration of phospholipid bilayers, as has been demonstrated by measurements of the lamellar repeat spacings by X-ray diffraction [5].

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine.

By the use of ^{2}H-NMR, a deeper insight into the phospholipid hydration in the presence of poly(ethylene glycol) has been obtained in our study.

However, the effect of poly(ethylene glycol) on the polarity of the aqueous phase has been neglected in all studies. Such properties have been described for proteins in solution [11]. A change in the polarity of the aqueous phase may have a drastic influence on the distribution of hydrophobic and polar residues of membrane components which are exposed to the aqueous phase [12]. We found an altered distribution of apolar molecules between the membrane and the aqueous phase after the addition of poly(ethylene glycol) and discuss consequences of changes in the polarity of the aqueous phase for membrane structure and fusion.

Honda et al. [8,13] demonstrated that purified poly(ethylene glycol) is able to induce only cell aggregation. Cell fusion is caused by contamination by small molecules contained in commercial-grade poly(ethylene glycol). It was concluded that the polymer affects the hydration properties only and in this way enhances the aggregation ability. We were interested in the ability of purified poly(ethylene glycol) to induced nonbilayer arrangements of phospholipids. The formation of structural defects in egg phosphatidylcholine vesicles by poly(ethylene glycol) has been shown earlier using freeze-fracture microscopy and ^{31}P-NMR [5,14,15].

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) from Fluka was used without further purification. The samples gave a single spot in thin-layer chromatography. 1-Anilinonaphthalene-8-sulfonate (ANS) from Ferak and pyrene from Fluka were used for the fluorescence measurements. Erythrocyte ghosts were prepared according to the method described by Dodge et al. [16]. For fluorescence measurements aliquots of a pyrene solution (5 mM in ethanol) were added to a suspension of ghost membranes (1 mg protein per ml) until the ratio of the fluorescence intensities of the excimers to the monomers was large enough (0.7–0.8). The molar ratio of the phospholipid to pyrene was about 200 : 1. This solution was divided and the parts were mixed with equal volumes of poly(ethylene glycol) solutions so that final poly(ethylene glycol) concentrations of 0, 1, 5, 10, 17.5 and 25 wt.% were obtained. After an incubation of 15 min, the membranes were collected by centrifugation (20 000 × *g* for 30 min) and resuspended for fluorescence measurements in poly(ethylene glycol)-free solution (154 mM NaCl, pH 7.4).

The DPPC/water dispersions for NMR measurements were prepared by the addition of the appropriate amounts of ^2H_2O to dry DPPC powder and dispersed by gentle centrifugation. Poly(ethylene glycol) was added to obtain the appropriate concentration. Mixing was effected by vigorous shaking and forward and backward centrifugation at 50°C. NMR measurements were carried out on a Bruker HX 90 NMR spectrometer. The measurements were made at 6.5 MHz for ^{2}H and 36.4 MHz for ^{31}P. ^{31}P NMR spectra were run using a high-powder proton decoupling and a 7 s delay between pulses. Exponential filtering was employed for signal enhancement. For fluorescence measurements, a Perkin-Elmer MPF 44 B spectrometer was used.

Results

^{2}H-NMR measurements and hydration properties of phospholipids

The ^{2}H-NMR spectrum of deuterated water was used to determine the hydration properties of phospholipids and the average orientation of water molecules [17–20]. Characteristic ^{2}H-NMR spectra of phospholipid/water dispersions are given in Fig. 1a for low water content (14.3 water molecules per DPPC molecule) and in Fig. 1c for high water content (46.5 water molecules per DPPC molecule). On the addition of low amounts of water to DPPC (up to 23 water molecules) all the water is arranged between the lamellae. For the first five water molecules added, about the same quadrupole splitting, of the order of 1.2 kHz, is measured in the liquid-crystalline state [19]. These water molecules are attached to the headgroup and form an inner hydration shell. They perform restricted motions and are exchanged between the different binding sites.

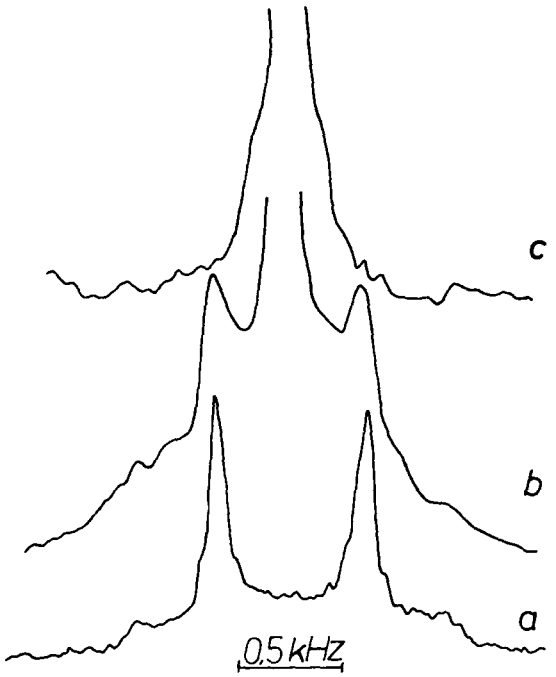

Fig. 1. ^{2}H-NMR spectra of DPPC/^2H_2O dispersions at T = 334 K for samples with (a) 14.3 and (c) 46.5 water molecules per phospholipid molecule. Spectrum (b) was obtained on the addition of 47 wt.% poly(ethylene glycol) (M_r 400). In spectra (b) and (c) the lower part of the signal only is given.

Additional water molecules could be attached to the headgroup, but their effective quadrupole splittings are significantly lower than those of the first five water molecules. The observed signals are the results of a rapid exchange of water molecules between different binding environments [18,19] included the inner hydration shell, so that only one quadrupole splitting is measured with a lowered quadrupole constant compared to the inner hydration shell. It is thought that about 12 water molecules per DPPC molecule are influenced by the phospholipid surface [17]. These water molecules, which comprise the molecules of the inner hydration shell and a thin water layer at the surface, are called bound water by Finer and Darke [17].

11 more water molecules are incorporated between the bilayer with motional characteristics similar to those of free water (trapped water). For more than about 23 water molecules, free water appears characterized by a narrow signal component at the centre of the spectrum. In Fig. 1c the ^{2}H-NMR spectrum of a DPPC/^2H_2O dispersion with 46.5 water molecules per DPPC molecule is given. This spectrum consists of two components. The narrow intensive signal at the centre is assignated to the free water. The broadening of the lower part of the signal is caused by the water incorporated between the lamellae. This second component of the spectrum is broadened by a quadrupole splitting in the order of 300 Hz.

In Fig. 2 the temperature dependence of the measured quadrupole splittings is given for DPPC/^2H_2O dispersions at different water contents (5.0, 8.9 and 14.3 water molecules per phospholipid molecule). At the phase transition temperature the quadrupole splitting has always a minimum and at temperatures below the phase transition temperature the quadrupole splitting decreases if the hydration increases.

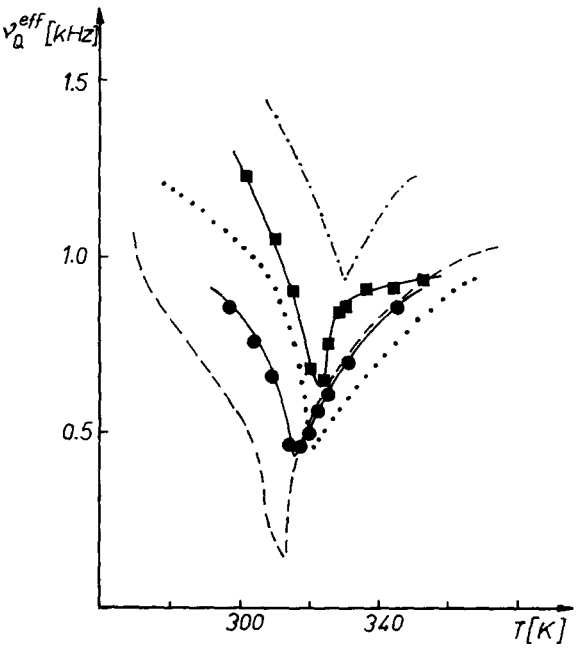

Fig. 2. Temperature dependence of quadrupole splittings ν_Q^{eff} of ^{2}H-NMR spectra of DPPC/^2H_2O dispersions at different water contents and in the presence of poly(ethylene glycol). For greater clarity, the experimental points for the samples without poly(ethylene glycol) are not given in the drawing. These results are given in Ref. 19. Measurements for 5.0 ·–·–·, 8.9 (· · · · · ·) and 14.3 (- - - - - -) water molecules per phospholipid molecule are given. For samples of 46.5 water molecules per phospholipid molecule in the presence of 47 wt.% (●) and 65 wt.% (■) poly(ethylene glycol), the quadrupole splittings derived from the broad component of the spectrum are given.

The further increase in quadrupole splitting with temperature above the phase transition point was explained as being due to increased water binding caused by an increase of area per lipid molecule [17]. The minimum in the quadrupole splitting at the phase transition could be connected with a more isotropic motion of the phospholipid head group [18]. But other processes such as an exchange of water molecules between binding sites of opposite order parameter can also account for the occurrence of the minimum. From the measurement it is concluded that the hydration shells reorient with the individual phospholipid headgroups and do not form a continuous network between adjacent headgroups [18,19] during a period of 10^{-6} s.

The addition of poly(ethylene glycol) to a DPPC/^2H_2O dispersion with excess water leads to an increase of the quadrupole splitting of the second broad signal component (Fig. 1b). The temperature dependence of the quadrupole splitting is given in Fig. 2 for poly(ethylene glycol) contents of 47 wt.% and 65 wt.% together with the measurements of poly(ethylene glycol)-free samples at different water contents. These temperature dependences in the presence of poly(ethylene glycol) have the same behaviour as was measured for samples of low water content. We can conclude that the amount of water incorporated between the phospholipid bilayers was reduced upon the addition of poly(ethylene glycol). A comparison with the results of measurements of samples at low water content given in Fig. 2 and Ref. 19 shows that the amount of incorporated water is decreased from more than 23 water molecules per phospholipid molecule to about 11 water molecules in the presence of 47 wt.% poly(ethylene glycol) and to eight water molecules in the presence of 65 wt.% poly(ethylene glycol). The same behaviour results from the shift of the temperature dependence of the quadrupole splitting which is connected with the temperature of the phase transition. A shift of the phase transition was also observed by differential scanning calorimetry [37].

The finding that the same qualitative behaviour of the temperature dependence of the quadrupole splitting is observed on the addition of poly(ethylene glycol) indicates that the presence of poly(ethylene glycol) does not greatly influence the binding properties of the water in the inner hydration shell of about five water molecules. However, the trapped water and a part of the bound water between the lamellae are reduced to a value lower than 12 water molecules, depending on the amount of poly(ethylene glycol) present, leaving the inner hydration shell unchanged.

The reduction in the interlamellar water is accompanied by a reduction in the lamellar repeat spacings, as was demonstrated by X-ray diffraction studies [5].

^{31}P-NMR measurements and the mobility of the phospholipid headgroup

^{31}P-NMR is sensitive to motional changes of the phospholipid headgroup [21–25] and can be used to monitor changes in the molecular organization of the membrane [26–28].

For DPPC/water dispersions, an asymmetric line-shape characteristic of the lamellar phase is observed [29,30] at all water concentrations of the sample. In Fig. 3 the measured anisotropy of chemical shift is given as a function of the temperature for a sample with excess water (50 wt.%). The phase transition of the sample is indicated by a change of the anisotropy of chemical shifts from about 45 ppm in the liquid-crystalline state to about 56 ppm in the gel state. No appearance of an isotropic signal which would be characteristic of the occurrence of lipidic particles [5,26,28] is observed.

The temperature dependence of the anisotropy of chemical shift for two different poly(ethylene glycol) concentrations is given in Fig. 3. A shift in

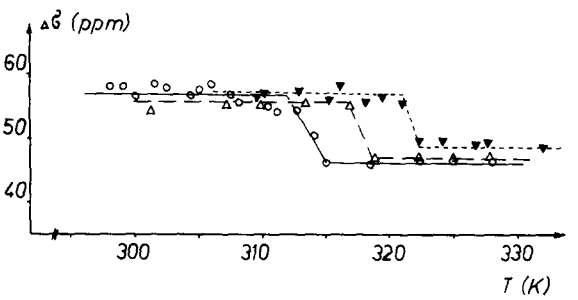

Fig. 3. Temperature dependence of the anisotropy of chemical shift of the ^{31}P-NMR signal of DPPC/water dispersions with 50 wt.% water (○) and on the addition of 60 wt.% (△) and 75 wt.% (▼) poly(ethylene glycol) 6000.

the phase transition is observed, in agreement with the results of ^{2}H-NMR measurements. The values of the anisotropy of chemical shift are only slightly decreased for temperatures below the phase transition point and are not changed at temperatures above the phase transition point. The same behaviour was also observed for DPPC/water dispersions at low contents of water [29,30]. This is further evidence for the dehydrating effect of poly(ethylene glycol) on DPPC/water dispersions. But there is no indication of an interaction of poly(ethylene glycol) with phospholipid headgroups in such a manner that the conformational mobility of the phosphate group is changed because the anisotropy of chemical shift is not altered. Such interactions were observed in sonicated liposome systems using ^{1}H-NMR [31] and fluorescence techniques [52]. This difference could be caused by different sample preparations. In sonicated liposome systems the poly(ethylene glycol) molecules can interact with each bilayer, but in unsonicated multilayer liposome systems the poly(ethylene glycol) molecules have to penetrate into the single water spaces between the bilayers. It seems that poly(ethylene glycol) is excluded from these water layers and has only a dehydrating effect on the multilayer system.

Boni et al. [5] found a symmetric signal in the ^{31}P-NMR spectrum on the addition of poly(ethylene glycol) to egg phophatidylcholine and dimyristoylphosphatidylcholine multilayer liposome systems. The appearance of an isotropic signal which would be characteristic of the occurrence of lipidic particles [26] is not observed in our experiments with the DPPC/water dispersions in the presence of poly(ethylene glycol). For egg phosphatidylcholine/water dispersions prepared by the same procedure as given by Boni et al. [5] there was also no indication for the existence of nonlamellar structures in the ^{31}P-NMR spectrum (experiments not given here). We found from ^{31}P-NMR that egg phosphatidylcholine is able to form nonlamellar phases at water concentrations lower than three water molecules per phospholipid molecule and temperatures higher than 50°C, in agreement with the phase diagram given by Luzzati et al. [33]. It should therefore be impossible to induce nonlamellar phases in egg phosphatidylcholine/water dispersions by simple dehydration of the bilayer system at the poly(ethylene glycol) concentrations used because more than three water molecules are bound per phospholipid molecule. An acceptable explanation of these different results was given by the findings of Honda et al. [8] that commercial-grade poly(ethylene glycol) contains at least two components, one of which has the activity of cell aggregation and the other has the activity of phospholipid bilayer perturbation. The latter is considered to be due to some kinds of impurities. Considering these aspects, it seems difficult to compare the results of different authors because of the differences in the poly(ethylene glycol) used. For our experiments, purified poly(ethylene glycol) prepared according to the method described by Honda et al. [13] was used, and this should contain only the aggregation factor. This could explain that nonbilayer structures connected with the fusion factor are not observed.

Fluorescence measurements and polar properties of the aqueous phase

The solution of ethylene glycol in water leads to a reduction of the dielectric constant to 37.7 for the pure ethylene glycol [32]. Corresponding studies for the polymer have not been published. We have obtained first results confirming a similar effect of poly(ethylene glycol) on the dielectric constant of the aqueous phase, and pertinent work is in progress.

The internal polar properties of a solvent can be studied by means of ANS [34]. Changes in the solvent polarity are detected by shifts of the maxima of fluorescence and changes of the quantum yield. The weak fluorescence of ANS in water is caused by the quenching action of the mobile water dipoles. As demonstrated in Fig. 4 the addition of poly(ethylene glycol) to an aqueous solution of ANS results in an enhancement of ANS fluorescence and the maximum of the fluorescence spectrum is shifted to shorter wavelength. These changes in the fluorescence properties of ANS correspond to alterations of the solvent properties in such a manner that the aqueous phase becomes less polar. These effects of poly(ethylene glycol) on the polar properties were explained as being due to reduced rotational mobility of the water molecules as a result of binding to poly(ethylene glycol) [12]. The decrease in the polarity of the aqueous phase

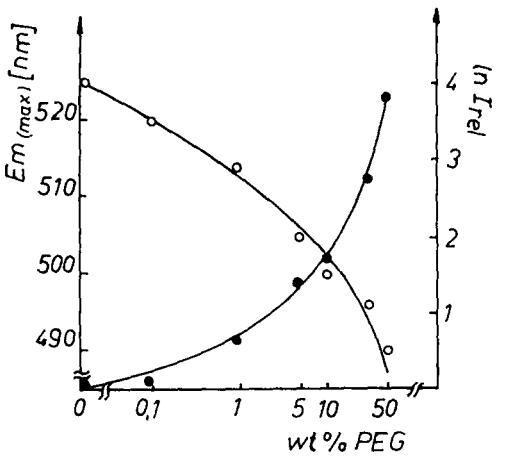

Fig. 4. Quantum yield I_{rel} (relative units) (●) and wavelengths of the maximum $E_{(max)}$ (○) of the fluorescence spectrum of ANS in water as a function of poly(ethylene glycol) (PEG) concentration at an ANS concentration of $1 \cdot 10^{-4}$ M.

should lead to changes in the solubility of hydrophobic molecules and to alterations of the partition of molecules between the membrane and the external phase. This is demonstrated for the probe molecule, pyrene, using the fluorescence properties of this molecule. Pyrene is an apolar molecule with a low solubility in the aqueous phase and enters the lipid phase, forming at higher concentrations excimers which consist of a complex of an excited and a nonexcited molecule [35]. These excimers have appreciable fluorescence well separated from the monomer band. The ratio of the fluorescence intensities of excimers to monomers is proportional to the pyrene concentration in the membrane [35,36].

The excimer signal of pyrene in erythrocyte membranes decreases on the addition of poly(ethylene glycol), whereas the intensity of the monomer signal increases drastically. This behaviour can be explained as the result of the release of pyrene from the membrane on the addition of poly(ethylene glycol) to the external phase. The release of pyrene leads to a dilution of pyrene in the membrane and reduces the probability for the formation of excimers. The increased monomer intensity is partly connected with the reduced quenching by the excimers but on the other side it is due to an increase in the quantum yield of the fluorescence of the released pyrene as a result of the changed solvent polarity.

The release of pyrene is revealed by another experiment. The ghost membranes were separated from the poly(ethylene glycol) solution by centrifugation and resuspended in phosphate buffered solution. In Fig. 5 the ratios of the excimer to monomer fluorescence intensities for the resuspended membranes are given as a function of the poly(ethylene glycol) concentration of the incubation medium. These intensities correspond only to the membrane-bound pyrene. From the experiments it follows that the effect of poly(ethylene glycol) on the release of pyrene from the membrane becomes important for poly(ethylene glycol) concentrations higher than 10 wt.%. The releasing effect is dependent on the hydrophobicity of the molecule used. The excimer-to-monomer ratio is decreased to a lower extent by the use of dodecerylpyrene which has a long alkyl chain (results not given here).

These findings of a change of the partition of molecules between the membrane and the external phase on the addition of poly(ethylene glycol) have important consequences for the application of methods which use probe molecules. We observed a decrease of the fluorescence anisotropy of diphenylhexatriene on the addition of poly(ethyl-

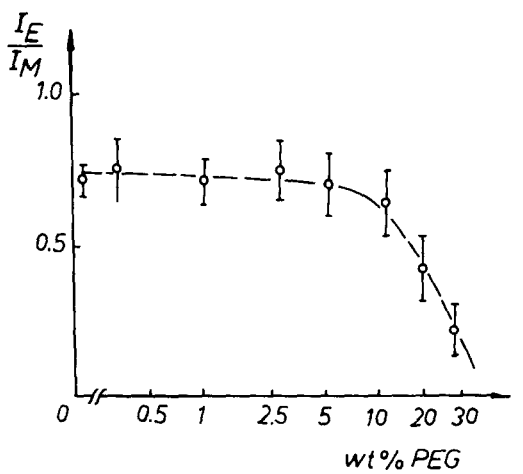

Fig. 5. Ratio of excimer-to-monomer intensity I_E/I_M of pyrene in an erythrocyte membrane suspension as a function of poly(ethylene glycol) (PEG) concentration. After incubation in solutions containing the poly(ethylene glycol) concentrations indicated, the ghost membranes were collected by centrifugation at 20000 × g and resuspended in poly(ethylene glycol)-free solutions. The excimer and monomer intensities were measured at 485 nm and 393 nm, respectively.

ene glycol). But an explanation of these results in terms of an increase in membrane fluidity only is not possible because of the contributions from diphenylhexatriene molecules which have left the membrane. Similar results obtained by other authors [57,52] should be reinterpreted taking into account the changes in the partition of molecules.

Discussion

A commonly accepted model of membrane fusion postulates that prior to fusion membrane proteins have to be excluded from the contact region [1,3,38]. The phospholipid bilayers form close contacts through local dehydration which is followed by transient destabilization of the bilayers [3,39,40,55].

In this paper, a description of the dehydrating effect of poly(ethylene glycol) on the phospholipid bilayer is given. It is demonstrated that the trapped and partly the bound water molecules are extracted from the phospholipid bilayer system by poly(ethylene glycol). The bound water was influenced by poly(ethylene glycol) concentrations higher that 40 wt.%. This correlates with the fusion activity of poly(ethylene glycol) in cellular systems. However, the binding properties of the water in the inner hydration shell of about five water molecules are not changed.

In the systems studied, the dehydration alone cannot induce the formation of nonbilayer arrangements of phospholipids. But it is known that other phospholipids such as cardiolipin [41] or lipid extracts from natural membranes such as mitochondria [42], brain [43] or chloroplasts [44] are able to change the phase from lamellar into nonlamellar arrangements of phospholipids upon decreasing the water content and increasing the temperature. This property of some phospholipids and phospholipid mixtures could partly explain the lability of the bilayer arrangement of membranes in poly(ethylene glycol)-induced fusion processes. There is evidence that the diffusion coefficients are lowered upon decreasing the water content [45,46]. The influence of low water content on phase separation in phospholipid mixtures was shown only for a mixture of sphingomyelin and phosphatidylcholine [54]. But such processes could be of importance for the lateral distribution of membrane components and the induction of nonlamellar arrangements [55]. We can speculate that the dehydration ability of poly(ethylene glycol) contributes to the destabilization as well as to changes of the lateral organization of the membrane.

The influence of poly(ethylene glycol) on the polarity of the aqueous phase described in this paper is another important property which could be of importance for the alterations of the membrane surface observed during the process of membrane-membrane interaction, especially the aggregation of proteins and creation of protein-free areas [6,47]. The pronounced effect of poly(ethylene glycol) on the distribution of hydrophobic molecules as demonstrated for pyrene in this paper indicates the possibility of a perturbation of the membrane up on the addition of poly(ethylene glycol). As discussed by Singer [48], the large differences between the polarity of the membrane and the external phase is an important factor for the stabilization of the membrane structure.

The reduction in the polarity of the external phase can result in rearrangements of polar and hydrophobic groups of membrane components and influences the distribution of molecules and ions between the membrane and the external phase.

There is some other experimental evidence for the occurrence of such processes. A shedding of membrane components upon the addition of poly(ethylene glycol) was observed by McCommon and Van [49] for cellular systems, which demonstrates the effect of poly(ethylene glycol) on the distribution of membrane molecules. The influence of poly(ethylene glycol) on proteins in solution was described in terms of structural changes [11,12] which are not induced by a specific interaction of poly(ethylene glycol) with the proteins. These changes in the protein structure lead to aggregation [50] and crystallization [51] processes of proteins in solution. By means of ESR spectroscopy on spin-labelled membrane proteins, we found changes in the conformational mobility of proteins in the erythrocyte membrane (Herrmann, A. et al., unpublished data) which may be connected with an influence of poly(ethylene glycol on the protein structure or the aggregation of proteins.

At present we are not able to discuss the conse-

quences of a lowered polarity of the external phase for the stability of the phospholipid arrangement. There is only some experience about the influence of organic components on the phase behaviour of surfactants [53]. The polarity effect could be of importance for mixtures of neutral and polar phopholipids. However, the dehydration and polarity effects of poly(ethylene glycol) have to be considered in their influence on the membrane structure together with the effects of the contaminations of commercial grade poly(ethylene glycol) which are considered to be necessary for the fusion of membranes [2,8,13,56].

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