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EFFECT OF POLY(ETHYLENE GLYCOL) ON THE POLARITY OF AQUEOUS SOLUTIONS AND ON THE STRUCTURE OF VESICLE MEMBRANES

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The partitioning of TEMPO into phosphatidylcholine vesicle membranes is reduced upon addition of poly(ethylene glycol). This is caused by reduced polarity of the aqueous phase as well as decreased membrane fluidity in the presence of poly(ethylene glycol). The isotropic hyperfine splitting of TEMPO in aqueous poly(ethylene glycol) solutions was used as a measure of solvent polarity. The alterations of the membrane fluidity were detected by means of two different fatty acid spin labels. The influences of physicochemical properties of an aqueous poly(ethylene glycol) phase on the membrane structure of cells and vesicles are discussed in the light of membrane fusion.

Introduction

Poly(ethylene glycol) is a well-known and often-used fusion agent. Poly(ethylene glycol) causes cell shrinking [1] and cell aggregation [1,2]. After the removal of poly(ethylene glycol), cell fusion takes place [3].

Up to now, the process of the poly(ethylene glycol)-promoted aggregation and fusion of vesicles and cells has not been understood. Alterations in the structure of the membrane such as protein aggregation and defects of the lipid layer are caused by poly(ethylene glycol) [1,2,4–10]. It is assumed that protein-free lipid areas of the membrane are a prerequisite for aggregation and cell fusion [1,6,9,11]. However, the formation of nonlamellar structures in the presence of this fusogen is controversial [4,5,12]. Recently it was suggested that nonbilayer structures are produced by the impurities of commercial-grade poly(ethylene glycol) [12,13].

Two different aspects have to be taken into account in the investigation of aggregation and fusion in the presence of poly(ethylene glycol).

(1) A direct interaction of poly(ethylene glycol) with membrane lipids and proteins [3,14]. Hydrogen bonds and/or hydrophobic interaction forces between poly(ethylene glycol) and hydroxy groups of glycoproteins are discussed [14]. Protein aggregation could be caused in this way. After this a direct interaction of poly(ethylene glycol) with membrane lipids is assumed causing a decrease of the membrane fluidity [3,14].

(2) An indirect influence of poly(ethylene glycol) on the membrane structure might be due to the physicochemical properties of the aqueous poly(ethylene glycol) phase [12]. The molecular structure of poly(ethylene glycol) results in a high

Abbreviations: TEMPO, 1-oxyl-2,2,6,6-tetramethylpiperidine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; I(10,3), 4-(2*n*-undecyl-3-oxyl-4,4-dimethyloxazolidin-2-yl)butyric acid; I(1,14), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; ANS, 8-anilinonaphthalene 1-sulphonate.

binding capacity for water. At poly(ethylene glycol) concentrations higher than 38 wt.%, no free water exists [15]. According to ^{2}H-NMR measurements it was concluded that the amount of water which is bound to the phospholipid headgroups is reduced on the addition of poly(ethylene glycol) [12]. It is known that phospholipids are able to form nonlamellar structures by decreasing the water content [16–18]. Recently, the effect of poly(ethylene glycol) on the polarity of aqueous solutions has been discussed. The partition of molecules between the membrane and the aqueous phase is altered in the presence of poly(ethylene glycol) [12]. Also, a change in the polarity of the surrounding aqueous medium could affect the structure of membrane proteins [19,20].

In the present paper, the partition of small membrane soluble molecules between the membrane and the external phase is investigated in detail using the electron spin resonance (ESR) method. This is compared with the change in polarity of aqueous solutions in the presence of poly(ethylene glycol). Further, the influence of poly(ethylene glycol) on the fluidity of phosphatidylcholine vesicle membranes is studied by means of fatty acid spin labels. We found previously that the lipid phase of intact human erythrocytes is not altered on the addition of poly(ethylene glycol) (Herrmann, A. et al., unpublished data). This is in contradiction to the results of Ohno et al. [3,14] who after a temporary increase found a decrease of the lipid fluidity of ghost membranes by using ANS fluorescence measurements. Phosphatidylcholine vesicle membranes showed only a decrease in membrane fluidity on the addition of poly(ethylene glycol) [3].

Material and Methods

Vesicle preparation and spin labelling

DPPC from Fluka was used without further purification. The samples gave a single spot upon thin-layer chromatography. A thin film of the fatty acid spin label I(10,3) (Reanal, Budapest) or I(1,14) (Syva, Palo Alto) was prepared on the wall of a glass vessel by dissolution in chloroform and evaporating the solvent under a stream of nitrogen gas. Vesicles were prepared in the same glass vessel by sonicating DPPC in distilled water with a Branson sonifier for 10 min at 40 W. The final DPPC concentration was 10 mM. The molar ratio of fatty acid spin labels to DPPC was 1 : 100. TEMPO (Reanal, Budapest) was added to the vesicle suspension after sonication. The final concentrations were 13.9 μM for TEMPO and 20 mg/0.3 ml for DPPC. The aqueous dispersions were mixed with equal volumes of aqueous poly(ethylene glycol) solutions so that the final concentrations of poly(ethylene glycol) were 0, 1, 5, 20, 30 and 40 wt.%. The pH values of the dispersions were unchanged after the addition of poly(ethylene glycol). The isotropic hyperfine splitting, A_{iso}, of TEMPO as a measure of the polarity was recorded in aqueous poly(ethylene glycol) solutions.

Electron spin resonance measurements

ESR spectra were recorded on a Varian E 3 spectrometer with variable temperature equipment. The temperature was measured by a small thermistor inserted in the sample with an accuracy of ± 0.2°C. A flat quartz cell for aqueous solutions was used. The measurements were performed under the following conditions: microwave power, 20 mW; modulation amplitude, 4 G (using label I (10,3)), 1.6 G (using I(1,14)) and 1 G (using TEMPO); scan time, 8 min; time constant, 0.1–0.3 s. The signal of the fatty acid spin label I(1,14), which is dissolved in the aqueous phase, was suppressed by $K_3Fe(CN)_6$ to avoid errors in evaluating the spectra.

Evaluation

The outer hyperfine splitting $2T_{\parallel}$ of the signal of the membrane-bound fatty acid spin label I(10,3) (Fig. 2) and the peak distance, Δh_{-1}, of the high-field line of I(1,14) (Fig. 3) were used as a relative measure of the membrane fluidity [21]. The order parameter, S [22] for I(10,3) was not used because the inner hyperfine splitting $2T_{\perp}$ could not be detected for all spectra.

The partition of TEMPO between the vesicle membranes and the solution was described by the partition coefficient, f_T [23]:

$$f_T = \frac{h_L - k \cdot h_W}{h_L - k \cdot h_W + h_W} \quad (1)$$

where h_L and h_W are the signal heights of the

high-field line of the membrane and the solution signal of TEMPO, respectively [23]. The membrane signal is corrected for the ^{13}C satellite of the aqueous peak by $k \cdot h_W$ ($k = 0.04$ [23]). The values were not corrected for the differences in the linewidths between the membrane signal and that for the solution. The data were fitted by linear regression according to the method of least squares.

The spectrum of TEMPO in aqueous dispersions of DPPC was simulated according to the model of Israelachvili et al. [25] by superimposing two spectra, the signal of the TEMPO molecules in the membrane and in the solvent. The following parameters were used:

(A) Signal of TEMPO in distilled water (values are taken from the measurements): linewidth, 1.2 G; A_{iso} (isotropic hyperfine splitting), 6.9 G; g-factor, 2.0062.

(B) Signal of TEMPO in the membrane. The best fit of the experimental spectrum of TEMPO in aqueous dispersions of DPPC (zero poly(ethylene glycol), 40°C) was achieved with a linewidth of 1.4 G, g-factor of 2.0065 and isotropic hyperfine splitting of 15.7 G, and a portion of 60% of the membrane signal on the whole spectrum measured.

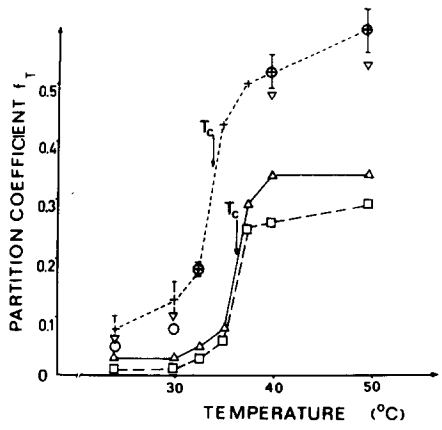

Fig. 1. The partition coefficient f_T of TEMPO between the vesicle membranes of DPPC and the aqueous phase as a function of temperature and poly(ethylene glycol) concentration (+, 0; ○, 1; ▽, 5; △, 20 and □, 30 wt.% poly(ethylene glycol)). The standard deviation is given only for the control (zero poly(ethylene glycol)) for clarity. T_c is the experimentally determined pretransition temperature.

Results

Partition of TEMPO between the vesicle membrane and the aqueous phase

The partition coefficient f_T for TEMPO between vesicle membranes and the aqueous phase as a function of temperature and poly(ethylene glycol) concentration is demonstrated in Fig. 1. This coefficient is a simple measure of the membrane fluidity; increasing the fluidity results in increased partitioning of TEMPO into the membrane. At the phase transition temperature T_c (pretransition [23]) the coefficient changes dramatically. By increasing the poly(ethylene glycol) concentration the coefficient f_T is lowered. No drastic differences between the probes of 0, 1 and 5 wt.% poly(ethylene glycol) occur, whereas the alteration between 0 and 20 wt.% is obvious. In accordance with other studies [12] the transition temperature T_c is shifted to higher temperatures on the addition of poly(ethylene glycol) (zero poly(ethylene glycol): $T_c = 33.8$°C; 20 wt.% poly(ethylene glycol): $T_c =$ 36.2°C). The poly(ethylene glycol) effect on the partition coefficient could be caused by a decrease in the membrane fluidity as well as by a decrease in the polarity of the aqueous phase.

Dependence of the fluidity of the vesicle membranes on the poly(ethylene glycol) concentration

The poly(ethylene glycol) effect on the membrane fluidity was detected by using the fatty acid spin labels I(10,3) and I(1,14) (Fig. 2 and 3). A remarkable feature is the increasing signal of the fatty acid spin label which is dissolved in the aqueous phase by increasing the poly(ethylene glycol) concentration (Fig. 2). The amplitude of this signal trebles upon increasing the poly(ethylene glycol) concentration from zero to 40 wt.%. It was estimated that no more than 5% of the spin label molecules are dissolved in the aqueous phase at 40 wt.% poly(ethylene glycol).

The outer hyperfine splitting $2T_{||}$ of I(10,3) and the peak distance Δh_{-1} of I(1,14) as a function of temperature and poly(ethylene glycol) concentration are presented in Figs. 4 and 5, respectively. Both the outer hyperfine splitting, $2T_{||}$, and the peak distance, Δh_{-1}, depend on the temperature as well as on the poly(ethylene glycol) concentration. Upon increasing the poly(ethylene glycol)

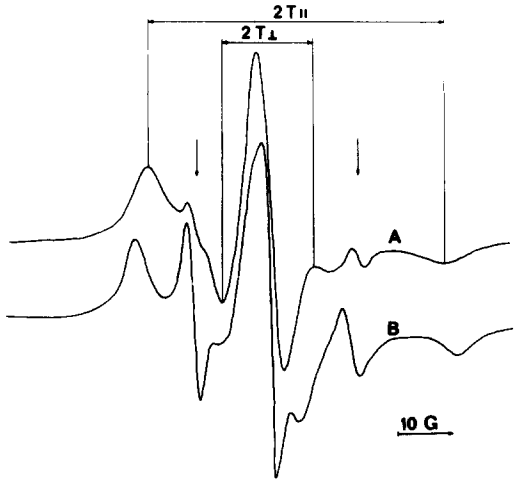

Fig. 2. The spectrum of the fatty acid spin label I(10,3) in DPPC membranes at 21°C (A, zero poly(ethylene glycol); B, 40 wt.% poly(ethylene glycol)). $2T_{\parallel}$ and $2T_{\perp}$ are the outer and the inner hyperfine splitting, respectively. The spectrum of the label in the external aqueous phase (arrows) is superimposed on the signal of the label which is incorporated in the membrane. At 40 wt.% poly(ethylene glycol) the concentration of the label in the aqueous phase (arrows) is enhanced.

concentration, the mobility of the spin labels is reduced. This suggests a decreased membrane fluidity, which is stressed by the narrowing of the linewidths in the presence of poly(ethylene glycol) (Fig. 2).

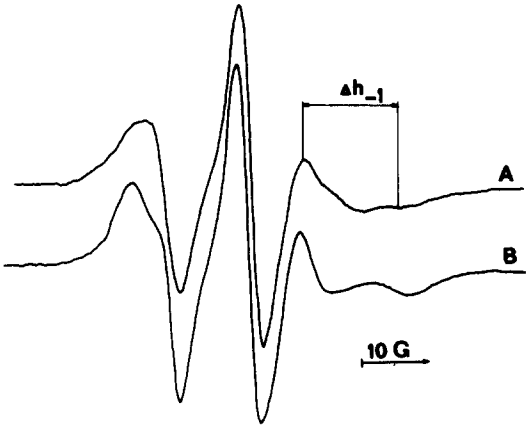

Fig. 3. The spectrum of the fatty acid spin label I(1,14) in DPPC membranes at 21°C (A, zero poly(ethylene glycol); B, 40% wt.% poly(ethylene glycol)). Δh_{-1} is the peak distance of the high-field line. The signal of the label in the aqueous phase was suppressed by $K_3Fe(CN)_6$.

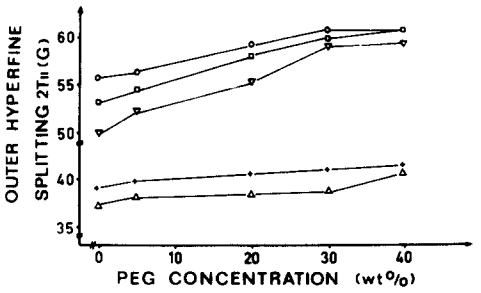

Fig. 4. The outer hyperfine splitting, $2T_{\parallel}$, of I(10,3) as a function of poly(ethylene glycol) (PEG) concentration and temperature (○, 21°C; □, 24°C; ▽, 30°C; +, 40°C and △, 50°C). The absolute error in measuring $2T_{\parallel}$ was smaller than 0.2 G.

McConnel et al. [26] found that the TEMPO solubility in lipid membranes is linearly related to the membrane fluidity, which is described by the order parameter, S, of the fatty acid spin label I(5,10). We detect a similar linear relationship between the peak distance, Δh_{-1}, and the partition coefficient, f_T, for each poly(ethylene glycol) concentration (Fig. 6), suggesting that the peak distance is also an appropriate measure of the membrane fluidity as the order parameter. The correlation coefficient, r, was in all cases about -0.99. The differences between the slopes of the regression lines are highly significant. In view of this, f_T still depends at least on a second variable – the polarity of the aqueous phase.

No linear relationship between the partition coefficient, f_T, and the outer hyperfine splitting, $2T_{\parallel}$, of I(10,3) was found over the whole tempera-

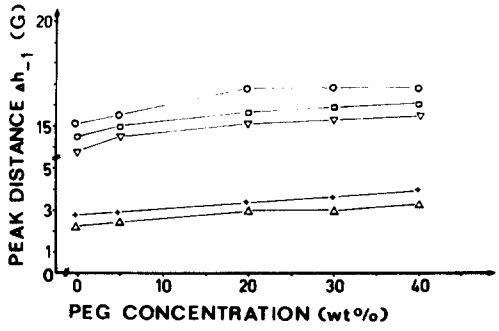

Fig. 5. The peak distance, Δh_{-1}, of the spectrum of I(1,14) as a function of poly(ethylene glycol) (PEG) concentration and temperature (○, 21°C; □, 24°C; ▽, 30°C; +, 40°C and △, 50°C). The absolute error in measuring Δh_{-1} was smaller than 0.2 G.

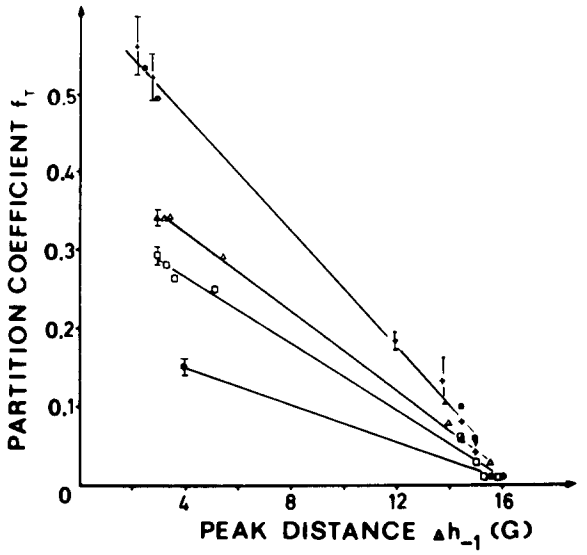

Fig. 6. The partition coefficient, f_T, of TEMPO in aqueous dispersions of DPPC as a function of the peak distance Δh_{-1} of I(1,14) (+, 0; ○, 5; △, 20; □, 30 and ●, 40 wt.% poly(ethylene glycol)). For each poly(ethylene glycol) concentration (values of 0 and 5 wt.% poly(ethylene glycol) were taken together) the regression line is shown. The standard deviation of f_T is presented only for zero poly(ethylene glycol).

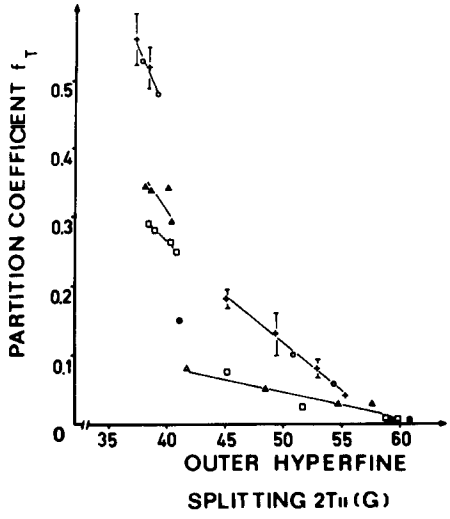

Fig. 7. The partition coefficient, f_T, of TEMPO in aqueous dispersions of DPPC as a function of the outer hyperfine splitting, $2T_{\parallel}$, of I(10,3) (+, 0; ○, 5; △, 20; □, 30 and ●, 40 wt.% poly(ethylene glycol)). The regression lines are shown. For values of $2T_{\parallel}$ lower than 41 G, the values of 0 and 5 wt.% poly(ethylene glycol) and the values of 20 and 30 wt.% poly(ethylene glycol) are taken together. The standard deviation of f_T is presented only for zero poly(ethylene glycol).

ture range measured (Fig. 7). Also no differences between the regression lines of 20 and 30 wt.% poly(ethylene glycol) concentration were found below T_c. This suggests that the outer hyperfine splitting, $2T_{\parallel}$, of I(10,3) is not such a useful parameter as the order parameter of I(5,10) [26] or the peak distance, Δh_{-1}, of I(1,14) (Fig. 6) for describing the alteration in membrane fluidity. This can be explained by the dependence of $2T_{\parallel}$ of I(10,3) on structural as well as dynamic parameters in the temperature region measured [24].

Dependence of the polarity of aqueous solutions on the poly(ethylene glycol) concentration

Briere et al. [27] have shown that the isotropic hyperfine splitting of small paramagnetic molecules is a sensitive measure of the polarity of the solvent. In Fig. 8 the dependence of the isotropic hyperfine splitting, A_{iso}, of TEMPO on the poly(ethylene glycol) concentration in aqueous solutions is shown. A linear relationship $A_{iso} = f$(poly(ethylene glycol) concentration) was found. The regression line is

$$A_{iso} = -0.009c(G) + 16.95(G) \quad (2)$$

(c is the poly(ethylene glycol) concentration in wt.%) with a correlation coefficient, r, of -0.99. According to the study of Briere et al. [27] this

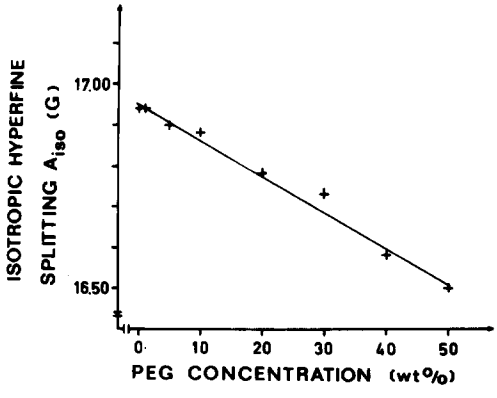

Fig. 8. The isotropic hyperfine splitting, A_{iso}, of TEMPO in aqueous poly(ethylene glycol) solutions as a function of poly(ethylene glycol) concentration at room temperature. A_{iso} was used as a measure of solvent polarity.

suggests that the polarity of the aqueous solution is reduced upon increasing the poly(ethylene glycol) concentration. This could favour the solubility of TEMPO in the aqueous phase.

The decrease in A_{iso} of TEMPO in solutions with poly(ethylene glycol) caused a decrease in the distance between the high-field lines of the membrane and the solvent signal. Therefore, the determination of the partition coefficient could be incorrect. To exclude this possibility, a simulation of the TEMPO signal in aqueous dispersions of DPPC was undertaken (see Material and Methods). Simulation was performed on an experimental spectrum which was measured at zero poly(ethylene glycol). In another simulation, all parameters which were used in the first case were held constant with the exception of A_{iso} of TEMPO in the aqueous phase (16.9 G at zero poly(ethylene glycol)). It was changed to 16.7 G, which corresponds to a value of an aqueous solution with a poly(ethylene glycol) concentration of 30 wt.%. The partition coefficient was determined graphically in the same way as for experimental spectra. The difference of the partition coefficient, f_T, between the both spectra simulated was about 2%. This is very small in comparison to the alteration of f_T observed by increasing the poly(ethylene glycol) concentration.

The partition coefficient, f_T, as a function of the membrane fluidity and the polarity of the solvent

A multiple linear regression of the function $f_T = f(\Delta h_{-1}, A_{iso})$ was carried out using all values measured. The following equation was determined:

$$f_T = 0.492 A_{iso}(1/G) - 0.027 \Delta h_{-1}(1/G) - 7.813 \quad (3)$$

The multiple regression coefficient, R, was 0.96. This suggests a high correlation. Only 9% of the variance of f_T cannot be explained by the dependence on polarity and membrane fluidity which are represented by the isotropic hyperfine splitting, A_{iso}, and the peak distance, Δh_{-1}, respectively. The peak distance as a parameter characterizing the membrane fluidity depends on the temperature as well as on the poly(ethylene glycol) concentration. Holding the linewidth, Δh_{-1}, constant, the dependence of the partition coefficient, f_T, on the polarity (represented by A_{iso}) of the aqueous phase could be calculated using Eqns. 2 and 3. The partition coefficient is reduced by about 35% upon increasing the poly(ethylene glycol) concentration from 0 to 20 wt.%.

Discussion

In the investigation of the mechanism of poly(ethylene glycol)-promoted membrane fusion, direct interaction between poly(ethylene glycol) and the membrane components was mainly discussed. The physicochemical properties of the aqueous poly(ethylene glycol) solutions and their effects on the membrane structure were more-or-less neglected.

Recently, Arnold et al. [12] demonstrated the influence of the dehydrating properties of poly(ethylene glycol) on the water which is bound to the phospholipid headgroups. It was suggested that a change in the polarity of the aqueous phase on the addition of poly(ethylene glycol) can also cause alterations in the membrane structure.

In the present paper, the polarity of aqueous solutions dependent on the poly(ethylene glycol) concentration was investigated. A decrease in the polarity was detected upon increasing the poly(ethylene glycol) concentration. The partition of membrane-soluble molecules between the membrane and the external phase is changed, as demonstrated in the case of TEMPO. It could be shown that this is also a result of the reduced polarity of the aqueous phase. The same behaviour was found for the fatty acid spin labels. Increasing the poly(ethylene glycol) concentration, the fatty acid spin label concentration in the external phase is enhanced. As pointed out above, the amount of the label in the aqueous medium trebles upon increasing the poly(ethylene glycol) concentration from 0 to 40 wt.%. This supports the assumption that the polarity is decreased by poly(ethylene glycol), because the solubilization of fatty acids in distilled water is very low. Saez et al. [28] have pointed out the detergent-like properties of poly(ethylene glycol). They assumed that a partial bilayer solubilization takes place upon the addition of poly(ethylene glycol). These results are important for evaluating fluorescence measurements on membranes in the presence of poly(eth-

ylene glycol). Arnold et al. [12] observed that the effect of poly(ethylene glycol) on the partition of pyrene between the membrane and the aqueous medium becomes important for poly(ethylene glycol) concentrations higher than 10 wt.%. This agrees very well with our results. At 5 wt.% poly(ethylene glycol), no detectable influence of the polarity on the partition coefficient was detected. Using Eqns. 2 and 3, the influence of the polarity on f_T can be estimated. At 5 wt.% poly(ethylene glycol), f_T is reduced by about 8% in comparison to the aqueous dispersion without poly(ethylene glycol). However, at 20 wt.% poly(ethylene glycol) f_T is decreased by more than 30%. Because the release of molecules from the membrane depends on their hydrophobicity, detailed investigations are necessary. The influence of the membrane fluidity on the partition has to be included in such studies.

Arnold et al. [12] found that upon the addition of poly(ethylene glycol) to aqueous solutions of ANS, the fluorescence is enhanced and the maximum is shifted to shorter wavelengths, indicating a lowered polarity. These effects have substantial consequences for the interpretation of methods which use such probe molecules. Especially, a careful reinterpretation of fluorescence studies seems to be necessary.

Recently it was demonstrated that the solubility of proteins is altered in the presence of poly(ethylene glycol) [29]. The melting temperature of the proteins investigated is unchanged [19,29]. No specific interaction between poly(ethylene glycol) and proteins were found [30]. Therefore an influence of the altered polarity was supposed. It could be concluded that membrane proteins can alter the conformation and/or can aggregate upon the addition of poly(ethylene glycol) without any direct interactions between the fusogen and proteins.

The motion of fatty acid spin labels in DPPC vesicle membranes is decreased by poly(ethylene glycol), suggesting a reduced membrane fluidity. This is indicated by the increase of the outer hyperfine splitting of I(10,3) and the linewidth, Δh_{-1}, of I(1,14). In discussing this effect, the physicochemical properties of aqueous poly(ethylene glycol) solutions and a direct interaction of poly(ethylene glycol) with membrane components have to be taken into account.

Possible consequences of the dehydrating properties of poly(ethylene glycol) for the membrane surface have been discussed [12]. Griffith et al. [31] showed that the dehydration of microsomal lipid vesicles reduces the molecular motion of fatty acid spin labels incorporated in such membranes. This might be due to denser packing of the lipids in the membrane. Borochov and Borochov [32] found a decrease in the fluidity of the membranes of liposomes upon increasing the external osmotic pressure. It was suggested that this can be attributed to a decrease of the molar free volume of the lipids. Our results on the influence of poly(ethylene glycol) on the membrane fluidity can be discussed in the same manner. Recently we detected no effect of poly(ethylene glycol) on the membrane fluidity of intact human erythrocytes (Herrmann, A. et al., unpublished data). Also, Rigaud et al. [33] and Nunes [34] demonstrated that the membrane fluidity of intact human erythrocytes is insensitive to the external osmotic pressure. However, Nunes [34] found an irreversible immobilization of membrane proteins of erythrocyte ghosts upon increasing the osmotic pressure of the medium used. We found the same behaviour of the membrane proteins of ghosts on the addition of poly(ethylene glycol). This underlines again that the physicochemical properties of poly(ethylene glycol) could not be neglected in discussing the poly(ethylene glycol)-mediated membrane-fusion processes. A direct interaction of poly(ethylene glycol) with membrane lipids seems to be possible. Assuming an extended poly(ethylene glycol) molecule, the distance between two oxygen atoms is about 6 Å using the values given in Ref. 35. Therefore, it is possible that an interaction of the headgroups and the oxygen atoms of the poly(ethylene glycol) molecule by hydrogen bonds, for instance, could cause restricted motion of phospholipids.

At present we are not able to give detailed information about the influence of physicochemical properties of aqueous poly(ethylene glycol) solutions on the membrane structure. But the experiments presented here and their discussion have demonstrated that this aspect is important in understanding poly(ethylene glycol)-induced membrane fusion.

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