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# Effect of Poly(ethylene glycol) on the $Ca^{2+}$-Induced Fusion of Didodecyl Phosphate Vesicles†

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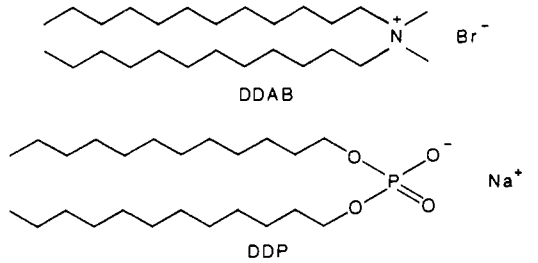
ABSTRACT: This paper reports a study of the effect of the dehydrating agent poly(ethylene glycol) (PEG) on didodecyl phosphate (DDP) bilayers and on the fusion activity of DDP vesicles as a function of the molecular weight of PEG. PEG 8K in a concentration of 10 wt % does not induce fusion. However, $Ca^{2+}$-induced fusion is promoted as reflected by a lowering of the $Ca^{2+}$ threshold concentration. This effect can most likely be attributed to the dehydrating capacity of the polymer. Interestingly, low concentrations (0.1 wt %) of PEG 20K induce a moderate fusion capacity. At higher concentrations (0.5 wt %) fusion is inhibited, irrespective of the presence of $Ca^{2+}$. These molecular weight dependent effects can be rationalized by taking into account that the clouding temperature differs for PEGs of different molecular weights. In the case of PEG 20K a microscopic phase separation will occur at the bilayer–water interface because PEG–PEG interactions and presumably PEG–DDP interactions are favored over PEG–water interactions. As a consequence, the DDP vesicle surface becomes covered with PEG 20K, resulting in a steric stabilization of the vesicles. This will impede or prevent, depending on the polymer concentration, the vesicles from approaching each other sufficiently close for fusion to occur.

Ion-induced fusion of bilayer vesicles formed from the synthetic amphiphiles didodecyldimethylammonium bromide (DDAB)[1] and didodecyl phosphate (DDP) is triggered by a




perturbation of the bilayer–water interface (Rupert et al., 1985, 1986, 1987). The stability of the water–bilayer interface, and in a broader sence that of the bilayer itself, is affected by the headgroup structure, counterion binding, and, in particular, by headgroup hydration (Portis et al., 1979; Rand, 1981; Rupert et al., 1987). For fusion to occur, dehydration of the headgroup is required to reduce the strongly repulsive hydration forces, which would otherwise prevent the bilayers from coming into close, i.e., fusion-susceptible, contact (Portis et al., 1979; Rand, 1981; Evans et al., 1986; Wilschut & Hoekstra, 1986; Hoekstra & Wilschut, 1988). Furthermore, headgroup hydration is also of paramount importance for the bilayer-to-hexagonal $H_{II}$ phase transition (Brown et al., 1986; Rupert et al., 1987). It is anticipated, therefore, that manipulation of the hydration of the headgroup of the amphiphiles

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[1] Abbreviations: DDP, didodecyl phosphate; PEG, poly(ethylene glycol); *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; DDAB, didodecyldimethylammonium bromide; PC, phosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; IMI, inverted micellar intermediate; LCST, lower critical solution temperature.

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with a dehydrating agent could lead to additional information concerning those properties of the bilayer–water interface which are important in determining intermembrane contact.

Dehydration of phospholipid headgroups occurs upon addition of poly(ethylene glycol) (PEG) to a phospholipid vesicle suspension (Hoekstra, 1982; Klose et al., 1985; Boni et al., 1984; Arnold et al., 1983; MacDonald, 1986). The dehydrating capacity of PEG results from the strong binding of water molecules to the polymer via hydrogen-bond interactions (Tilcock et al., 1982), and it has been argued that two to three waters bind per monomer unit (Tilcock et al., 1982; Kjellander et al., 1981; De Vringer et al., 1986). The hydrogen-bonding characteristics of another 12 water molecules are also influenced by the same monomer unit (Baran et al., 1972). It has been calculated that all water molecules experience the presence of PEG at polymer concentrations above ca. 13 wt % and at ca. 40 wt % of PEG all water molecules are bound to the polymer. At a concentration of ca. 30 wt %, PEG is capable of inducing fusion between phosphatidylcholine (PC) vesicles, and it has been proposed that these fusogenic properties originate from a polymer-induced disruption of the hydration layer of the PC bilayer (Morgan et al., 1983; Boni et al., 1984; MacDonald, 1986; Parente et al., 1986). However, one should also take into account that above 20 wt % PEG the polarity of the medium decreases significantly (Herrmann et al., 1983; Arnold et al., 1985). This may facilitate an exchange of amphiphile between the vesicles, and hence, the participation of a fusion process may well be overestimated. It is also not clear whether or not PEG binds to the vesicle surface (Boni et al., 1984; MacDonald, 1986; Parente et al., 1986; Ohno et al., 1981). However, binding of the polymer seems to be no prerequisite for the induction of fusion (MacDonald, 1986).

Polymer–vesicle interactions are of appreciable interest since they may, to some extent, mimic the interactions between proteins and biological membranes. Often charged polymers such as polyacids and poly($\alpha$-amino acids) have been used, and their interactions with vesicles can lead to phase transitions in the bilayer (Carrier et al., 1985; De Kruijff et al., 1985) or even to a complete loss of the bilayer structure (Kunitake et al., 1978; Seki et al., 1984).

In the present study it will be shown that PEG with a $M_r$ of 8000 facilitates $Ca^{2+}$-induced fusion of didodecyl phosphate (DDP) vesicles. Most likely this should be attributed to an effect of PEG on the hydration of the headgroup. By contrast, when PEG of $M_r$ 20 000 is used, the membrane fusion process is almost completely inhibited. We submit that this peculiar molecular weight dependent effect of PEG can be rationalized in terms of a difference in clouding temperature experienced by the PEG molecules which are near the bilayer–water interface. A clouding phenomenon may then lead to an increased binding of PEG 20K to the bilayer which ultimately leads to a steric stabilization of the DDP vesicles.

## Experimental Procedures

*Materials.* Didodecyl phosphoric acid was obtained from Alpha Chemicals, mp 59.1–60.2 °C. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Polar Lipids Inc., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Sigma, and calcium and magnesium chloride were from Merck. All chemicals were used without further purification.

Poly(ethylene glycol) with $M_r$ of 8000 (PEG 8K, previously sold as having a $M_r$ of 6000) and 20 000 (PEG 20K) were obtained from Sigma. Since it has been reported (Honda et al., 1981; Smith et al., 1982; Saez et al., 1982) that some PEGs but not Sigma PEG 8K (Smith et al., 1982) might be contaminated with impurities that can trigger vesicle fusion, the 20K polymer was purified by precipitating 15 g of PEG 20K, dissolved in 100 mL of chloroform, under vigorous stirring into 2 L of distilled petroleum ether (bp 40–60 °C). The precipitate was dried in vacuo, dissolved in aqua bidest, and dialyzed for 7 days against a continuous flow of aqua bidest until the conductivity of the aqueous polymer solution inside the dialysis bag was less than 10 $\mu\Omega$. The polymer was freeze-dried and subsequently dried in vacuo (50 °C).

*Vesicle Preparation.* Sodium didodecyl phosphate (DDP) vesicles were prepared by the ethanol injection method (Kremer et al., 1977); 10 mg of didodecyl phosphoric acid was dissolved in 100 $\mu$L of ethanol containing 0.30 M NaOH (slightly more than equimolar with respect to didodecyl phosphoric acid). With a preheated Hamilton microsyringe, 50 $\mu$L of this solution was injected into 2 mL of 5 mM HEPES/5 mM sodium acetate buffer (pH 7.4) thermostated at 55 °C to yield vesicles with a diameter of ca. 90 nm.

The vesicle–polymer solutions were prepared by adding an aliquot of a vesicle stock solution to the polymer solution of the desired polymer concentration. In the dilution experiments the vesicles were first incubated in a 0.5 wt % PEG 20K solution. After various time intervals, samples of 50 $\mu$L were taken and added to 2 mL of a 0.1 wt % PEG 20K solution.

*Vesicle Aggregation.* Aggregation of DDP vesicles, induced by $Ca^{2+}$ at 40 °C, was measured by monitoring continuously the turbidity at 350 nm with a Perkin-Elmer Lambda 5 spectrophotometer, equipped with a thermostated cell holder and a stirring device. The initial rate of aggregation is defined as the first derivative (at $t = 0$) of the curve describing the change in turbidity as a function of time.

*Fusion Measurements.* Vesicle fusion was monitored continuously with the resonance energy transfer (RET) fusion assay (Struck et al., 1981; Hoekstra, 1982) as described previously (Rupert et al., 1985, 1986, 1988). Vesicles containing 0.8 mol % each of *N*-NBD-PE and *N*-Rh-PE were prepared as described above. Fusion measurements were carried out in HEPES–sodium acetate buffer (pH 7.4) with equimolar amounts of labeled and nonlabeled DDP vesicles. The total amphiphile concentration was 58 $\mu$M. After equilibration at the desired temperature (see legends to the figures) fusion was initiated by injecting a $CaCl_2$ solution into the vesicle–PEG suspension or, in the case of the $Ca^{2+}$ concentration dependent fusion measurements in the DDP–PEG 8K system, by injecting the vesicle solution into a solution containing both PEG 8K and $Ca^{2+}$, both at the appropriate concentrations. NBD fluorescence ($\lambda_{ex}$ = 475 nm, $\lambda_{em}$ = 530 nm) was monitored on a Perkin-Elmer MPF43 spectrophotometer, equipped with a thermostated cell holder and a magnetic stirring device. The fluorescence scale was calibrated such that residual NBD fluorescence of the vesicles is taken as the zero level and the value after the addition of cetyltrimethylammonium bromide (final concentration 1% w/v) corrected for the sample dilution is taken as 100% (infinite dilution). The extent of fusion was determined after the fluorescence reached a plateau value that did not further increase over a time interval of at least 30 min.

*$^{31}P$ NMR Measurements.* $^1H$-Decoupled $^{31}P$ NMR measurements were performed in 10-mm tubes at 81 MHz on a Nicolet NT200 instrument equipped with a temperature controller and a deuterium lock. The vesicle suspensions contained 20 vol % of $D_2O$ to lock the signal. Chemical shifts

(ppm) were determined relative to the external reference hexachlorocyclotriphosphazene in $CDCl_3$ (+19.9 ppm downfield from that of 85% $H_3PO_4$). Accumulated free induction decays were obtained from 400 transients with an interpulse time of 3.2 s and a pulse time of 19 $\mu$s, which corresponds to a pulse angle of ca. 90°.

*Electron Microscopy.* The two-droplet procedure was followed for negative staining with 1% (w/v) uranyl acetate. Carbon-coated Formvar grids, pretreated by glow discharge in air, were used as supporting matrix. The samples were examined with a Philips EM300 electron microscope, operating at 80 kV.

*Differential Scanning Calorimetry.* DSC measurements were performed on multilamellar DDP dispersions with a Perkin-Elmer DSC-2 apparatus and sealed aluminium pans. The pans contained ca. 10 mg of the vesicle suspension. Heating (10 °C $min^{-1}$) and cooling (5 °C $min^{-1}$) scans were recorded between 5 and 90 °C. The temperature scale was calibrated with *o*-terphenyl (mp 56.2 °C). The reproducibility of the peak top temperatures was within 1 °C for different multilamellar DDP vesicle suspensions.

*Viscometric Measurements.* Viscometric experiments were carried out on a Scott-Gerate AVS 400 viscosometer and an Ubbelohde type 1 (flow time of water 73.6 ± 0.1 s) at 40.0 °C.

## Results and Discussion

*Effect of Poly(ethylene glycols) of Different Molecular Weights on $Ca^{2+}$-Induced Fusion of DDP Vesicles.* By use of the resonance energy transfer (RET) fusion assay, based on the fluorescent probes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) (Rupert et al., 1985, 1988), the $Ca^{2+}$-induced fusion of DDP vesicles was monitored in the presence of PEGs with $M_r$ of 8000 (8K) and 20000 (20K). The results of these measurements, expressed in terms of the initial fusion rate and the extent of fusion, are presented in Figure 1. For comparison, the results obtained for $Ca^{2+}$-induced fusion of DDP vesicles in the absence of PEG (Rupert et al., 1987b) are also included. The results show that in the presence of 10 wt % PEG 8K the $Ca^{2+}$ threshold concentration is lowered from 1.7 to 1.0 mM. In addition, in the presence of PEG the initial fusion rates at the lower $Ca^{2+}$ concentrations increase more rapidly with increasing $Ca^{2+}$ concentration than the rates in the absence of PEG. Evidently, PEG 8K facilitates fusion, which most probably results from a (partial) dehydration of the headgroups. Note that, at 10 wt %, PEG 8K does not induce fusion by itself, which is in accord with the observation that $Ca^{2+}$ is necessary to induce fusion of phosphatidylserine (PS) vesicles at this relatively low polymer concentration (Hoekstra, 1982). That the reduction of the $Ca^{2+}$ threshold concentration for fusion of DDP vesicles is less than that seen for PS vesicles can most likely be attributed to the difference in vesicle diameter in both systems [ca. 90 nm (Rupert et al., 1987, 1988) versus 25 nm (sonicated vesicles), respectively]. Such a difference in size can significantly affect the fusion susceptibility of phospholipid vesicles and is presumably related to additional stress in sonicated vesicles because of the highly curved vesicle surface (MacDonald, 1986).

The effect of PEG 20K on the fusion of DDP vesicles is in marked contrast to that of PEG 8K. It appears that PEG 20K at a concentration of only 0.1 wt % is capable of inducing fusion in the absence of $Ca^{2+}$ (Figure 1). Although the initial fusion rate is fairly low ($4.3 \times 10^{-2}$% $s^{-1}$), a considerable extent of fusion (ca. 18%, not shown) can take place eventually.

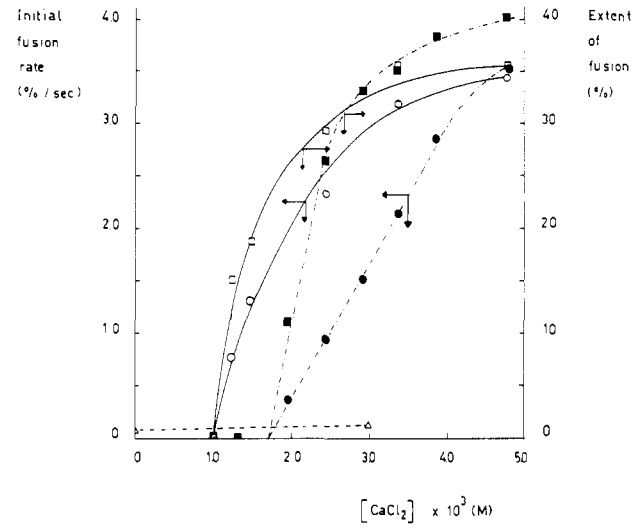


FIGURE 1: Effect of PEG on the $Ca^{2+}$-induced fusion of DDP vesicles. The initial fusion rate (○, ●) and the extent of fusion (□, ■) of DDP vesicles were determined as a function of the $Ca^{2+}$ concentration, in the presence (open symbols) or absence (filled symbols) of 10 wt % PEG 8K. The effect of PEG 20K (0.1 wt %) on the initial fusion rate (△) at 0 and $2.9 \times 10^{-3}$ M $CaCl_2$ is also shown. The incubation temperature was 40 °C, and the ratio of labeled to nonlabeled vesicles was 1:1. Total DDP concentration was $5.8 \times 10^{-5}$ M, and the pH of the buffer was 7.4.

However, at higher polymer concentrations this polymer-induced fusion becomes increasingly suppressed with respect to both the initial rate and the extent of fusion. At 0.5 wt % PEG 20K the initial fusion rate is essentially negligible (see below). Another striking difference between both PEGs became apparent by their distinct effect on the $Ca^{2+}$-induced fusion. In the presence of only 0.1 wt % PEG 20K and 2.9 mM $Ca^{2+}$, the initial fusion rate is ca. 40-fold lower than that observed at 2.9 mM $Ca^{2+}$ and 10 wt % PEG 8K (Figure 1). By contrast, the final extents of fusion, at these conditions, differ only slightly (33 versus 31%, respectively), indicating that PEG 20K at this low concentration mainly influences the fusion kinetics rather than the fusion susceptibility of the vesicles as such. At PEG 20K concentrations as low as ca. $5 \times 10^{-4}$ wt %, the inhibitory effect of the polymer on $Ca^{2+}$-induced fusion of DDP vesicles was no longer apparent (not shown). This concentration of the polymer corresponds to a monomer equivalent concentration of ca. 120 $\mu$M, which is only a factor of 2 higher than the concentration of DDP. Similarly as observed for PEG 20K induced fusion per se, polymer concentrations above 0.1 wt % suppressed the $Ca^{2+}$-induced fusion of DDP vesicles. In the presence of 0.5 wt % PEG 20K $Ca^{2+}$-induced fusion (2.9 mM $Ca^{2+}$) could not be detected by the RET fusion assay. Interestingly, upon dilution of the system, the apparent inhibition of fusion was relieved. The extent of relief, after an incubation at a relatively high PEG 20K concentration followed by dilution to 0.1 wt %, was, however, dependent on the incubation time at that high PEG concentration (Figure 2). The longer the incubation time, the lower the initial fusion rate upon dilution. At present it is not entirely clear whether this effect of the incubation time on the extent of inhibition solely results from the polymer or that a $Ca^{2+}$-induced isothermal phase transition (Rupert et al., 1987b; Hoekstra, 1982; Papahadjopoulos et al., 1977) is also involved. Such a phase transition could reduce the fusion susceptibility of DDP vesicles (Rupert et al., 1987b) and might occur during the incubation time, when the vesicles do not yet fuse. Whatever the precise mechanism underlying this phenomenon, the above results clearly demonstrate a distinct, *molecular weight dependent*

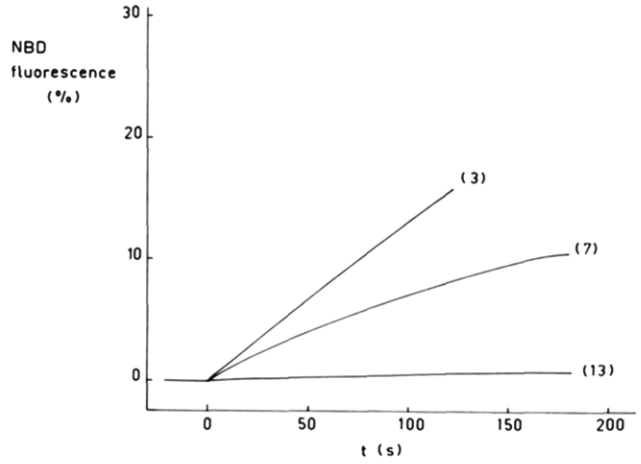


FIGURE 2: Effect of preincubation time with 0.5 wt % PEG and 2.9 $\times 10^{-3}$ M $CaCl_2$ on the $Ca^{2+}$-induced fusion of DDP vesicles. After various incubation times of DDP vesicles in 0.5 wt % PEG 20K and 2.9 $\times 10^{-3}$ M $Ca^{2+}$, during which no relief of energy transfer was observed, the vesicle–PEG suspension was diluted 40-fold (at $t = 0$) with a 0.1 wt % PEG solution, and the subsequent increase in NBD fluorescence was monitored. The number between parentheses refers to the incubation time (in minutes) in 0.5 wt % PEG 20K. The incubation temperature was 40 °C. The ratio labeled to nonlabeled vesicles was 1:1. The final concentrations of DDP and $Ca^{2+}$ were 5.8 $\times 10^{-5}$ and 7.3 $\times 10^{-5}$ M, respectively. The pH was 7.4.

interference of PEG with membrane fusion. Before the potential differences introduced in the bilayer or at bilayer–water interface by the different PEGs were characterized, it was considered important to confirm, by electron microscopy, that the dilution of the fluorescent probes reflected fusion rather than an exchange process.

*Electron Microscopic Investigation of the Effect of Poly(ethylene glycol) on the Fusion of DDP Vesicles.* Electron micrographs of DDP vesicles, that had been incubated in an aqueous buffer solution containing 10 wt % PEG 8K for ca. 20 min, are shown in Figure 3a. The appearance of the vesicles is identical with that seen in the absence of polymer [cf. Rupert et al. (1987a)], but the visualization is hampered by the presence of relatively large amounts of polymer which reduces the contrast. Addition of $Ca^{2+}$ converts the vesicles into long tubular structures (Figure 3b) which, as described in detail elsewhere (Rupert et al., 1987a), possess a hexagonal $H_{II}$ packing.

A different result is obtained when $Ca^{2+}$ is added to DDP vesicles in the presence of 0.1 wt % PEG 20K (Figure 3c). Now tubular structures are also formed, but they are much smaller in diameter and shorter in length than those shown in Figure 3b. Another intriguing observation is that small tubular structures can grow from relatively small vesicles and, furthermore, that, occasionally, clustering of the tubes (Figure 3c, right lower corner) occurs. This phenomenon was not observed when the transformation was triggered solely by $Ca^{2+}$, or in the presence of PEG 8K. This indicates that clustering is specifically related to the presence of PEG 20K. It is tempting to suggest that PEG 20K exerts its clustering effect by bridging adjacent tubes after initial binding to the membrane surface.

Formation of hexagonal $H_{II}$ tubes results from the merging of a sufficient number of unstable, inverted micellar intermediates (IMI's; Siegel, 1986a; Rupert et al., 1987a). Since the formation of IMI's requires interbilayer contact (Siegel, 1986a,b), it appears that, on the basis of the size of the tubes, PEG 20K causes a rapid collapse of relatively small, fused vesicles. Furthermore, it is possible that tube formation in this case may also arise from PEG-induced membrane folding, if

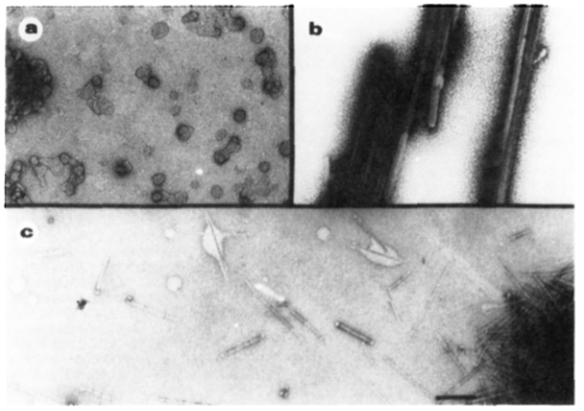


FIGURE 3: Electron micrographs of negatively stained DDP vesicles, incubated in 10 wt % PEG 8K (at pH 7.4) (a), of the hexagonal $H_{II}$ tubes obtained after addition of $Ca^{2+}$ (b), and (c) of the hexagonal $H_{II}$ tubes obtained after the addition of $Ca^{2+}$ to DDP vesicles in 0.1 wt % PEG 20K. The concentration of DDP was 2.9 $\times 10^{-3}$ M and that of $CaCl_2$ (if present) 4.0 $\times 10^{-3}$ M. The bar indicates 300 nm.

the possibility is taken into account that PEG 20K binds to the vesicle surface.

Since PEG 20K can induce fusion by itself (see above), one would anticipate that for the formation of the IMI's the presence of $Ca^{2+}$ may not be a prerequisite. Indeed, tubular structures, similar to those shown in Figure 3c, and some fused vesicles were also observed in a DDP–PEG 20K sample in the absence of $Ca^{2+}$ (not shown). However, the number of tubes seen in the $Ca^{2+}$-free system was much less than that in the presence of $Ca^{2+}$. Hence, it is suggested that $Ca^{2+}$ and PEG 20K display a synergistic effect on IMI formation. To gain further insight into the distinct effects of PEG 8K and 20K on DDP vesicles and, particularly, to examine more closely the possibility of binding of PEG 20K to the DDP vesicles, the influence of the polymers on the DDP bilayer structure per se was investigated.

*Molecular Weight Dependent Influence of Poly(ethylene glycol) on the DDP Bilayer Structure.* It has been shown previously (Rupert et al., 1987b) that the fusion activity of DDP vesicles is completely suppressed when the bilayer is in the gel state. It is, therefore, important to know whether the phase behavior of the bilayer is significantly affected by the presence of PEG 20K. In accordance with previous data on phospholipid vesicles (Boni et al., 1984; Arnold et al., 1983; Tilcock et al., 1982; Herrmann et al., 1983), differential scanning calorimetry (DSC) measurements showed that the transition temperatures of multilamellar DDP vesicles, suspended in a buffer containing only 2 wt % PEG 20K, are not significantly different from those observed in the absence of the polymer (34, 40, and 46 °C), indicating that the phase behavior is not affected by the polymer at the employed concentration. Thus, the reduced fusion rate of DDP vesicles upon increasing the PEG 20K concentration from 0.1 to 0.5 wt % is evidently not due to a PEG-induced thermotropic phase transition.

To examine more closely the influence of PEG on the bilayer packing, $^{31}P$ NMR measurements were carried out. The results of these experiments demonstrate that PEG 8K and 20K exert different effects on the DDP vesicles. As shown in Table I, the presence of 0.5 wt % PEG 8K induces an *increase* in the peak height of the $^{31}P$ NMR signal as a result of decreased line broadening. This effect may originate from an increased mobility of the DDP molecule. A slight PEG 8K induced dehydration and/or an increase in counterion binding will reduce the effective headgroup area. This, in turn, may lead to an increased mobility of the DDP headgroup (or the com-

Table I: Influence of PEG on the Relative Intensity ($I_{rel}$) and the Upfield Shift ($\Delta\delta_{^{31}P}$) of the $^{31}P$ NMR Signal for DDP Vesicles[a]

| conditions | $I_{rel}$ (%) | $\Delta\delta_{^{31}P}$ (ppm) |
|---|---|---|
| 4.6 mM DDP | 56 | 0.00 |
| +0.5 wt % PEG 8K | 100 | −0.01 |
| +10 wt % PEG 8K | 25 | 0.00 |
| +0.5 wt % PEG 20K | 31 | 0.10 |
| +0.5 wt % PEG 20K + 0.25 mM $Ca^{2+}$ | *b* | 0.20, 0.55 |
| +0.5 wt % PEG 20K + 0.60 mM $Ca^{2+}$ | *c* | *c* |

[a] At 40 °C, pH 7.4. [b] Splitting of the signal. [c] Not detectable due to strong line broadening.

plete molecule) and a concomitant decrease of the contribution of chemical shift anisotropy to the line width (Cullis et al., 1976, 1977; Cullis, 1976). For phospholipid systems, both dehydration and alterations in counterion binding as a result of addition of PEG have been reported (Klose et al., 1985; Boni et al., 1984; Arnold et al., 1983; Hoekstra, 1982; Maggio et al., 1978).

Addition of 10 wt % PEG 8K to DDP vesicles leads to a *decrease* in signal intensity (Table I) and a strong increase in line width. At this PEG concentration the viscosity of the medium is significantly enhanced, which results in a slower tumbling of the DDP vesicles and thus in an increase in the contribution of the chemical shift anisotropy to the line width. A similar observation has been made for phospholipid vesicles (Boni et al., 1984; McLaughlin et al., 1975).

Interestingly, in the presence of 0.5 wt % PEG 20K, the $^{31}P$ NMR signal decreased by about a factor of 2, as opposed to the increase seen in the presence of 0.5 wt % PEG 8K. This decrease does not result from a viscosity effect because an incubation of the vesicles in 7.5 wt % glycerol, having the same specific viscosity as 0.5 wt % PEG 20K ($n_{spec}$ = 0.166), only results in a minor decrease in intensity (or an increase in line width). Apparently, PEG 20K reduces the mobility of the DDP molecules in the lateral plane of the bilayer. We note that we have shown previously (Rupert et al., 1988) that a decrease in mobility, which is reflected in a 2-fold decrease in intensity of the $^{31}P$ NMR signal, barely affects the fusion process. Taken together, the ability of PEG 20K (i) to inhibit fusion with increasing concentration and incubation time, (ii) to reduce the mobility of DDP molecules in the lateral plane of the bilayer, and (iii) to induce the formation of *small* tubular structures can be reconciled best with the view that the effects of PEG 20K are caused by binding of the polymer to the vesicle surface.

The observed upfield shift of the $^{31}P$ NMR signal by 0.10 ppm (Table I) indicates that the PEG 20K–DDP interaction is accompanied by a significant dehydration of the headgroups [cf. Rupert et al. (1987b)]. Assuming that PEG 20K binds to the surface of the vesicle, it is emphasized that at least part of the DDP headgroups remain accessible to $Ca^{2+}$. This can be inferred from the observation that 0.25 mM $Ca^{2+}$ induces a splitting of the $^{31}P$ NMR signal. Moreover, when the upfield shift of the system consisting of DDP (4.6 mM), PEG (0.5 wt %), and $Ca^{2+}$ (0.25 mM) is corrected for the upfield shift induced by PEG 20K per se, values of 0.10 and 0.45 ppm are obtained for the headgroups in inner and outer leaflets, respectively, which are identical (Rupert et al., 1987b) with those observed for the $Ca^{2+}$ system in the absence of PEG. The additivity of the effects of PEG 20K and $Ca^{2+}$ on the intensity of the $^{31}P$ NMR signal may also explain the strong line broadening of the $^{31}P$ resonance at 0.6 mM $Ca^{2+}$ (Table I).

The above results imply that the binding of PEG 20K does not significantly affect the "cis"-complexation constant, i.e., the binding constant for the complexation of $Ca^{2+}$ with two

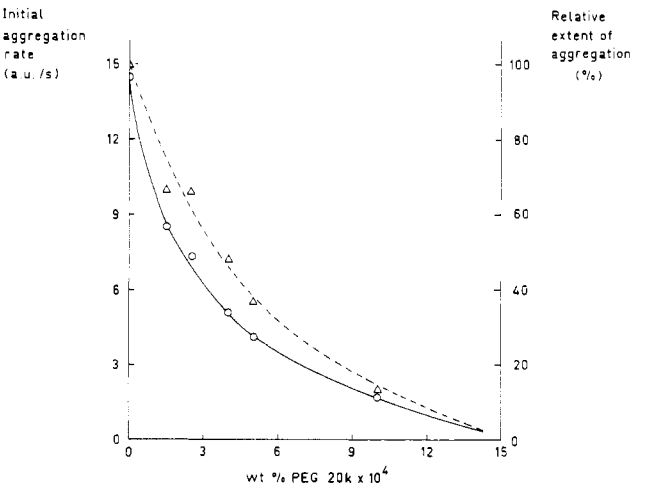


FIGURE 4: Initial rate of aggregation (○) and relative extent of aggregation (△) for the $Ca^{2+}$-induced aggregation of DDP vesicles as a function of the concentration of PEG 20K. The relative extent of aggregation is set equal to the maximal turbidity at $t = \infty$ relative to that in the absence of polymer. The concentrations of DDP and $CaCl_2$ were 58 μM and 3.85 mM, respectively. The pH was 7.4 and the incubation temperature 40 °C.

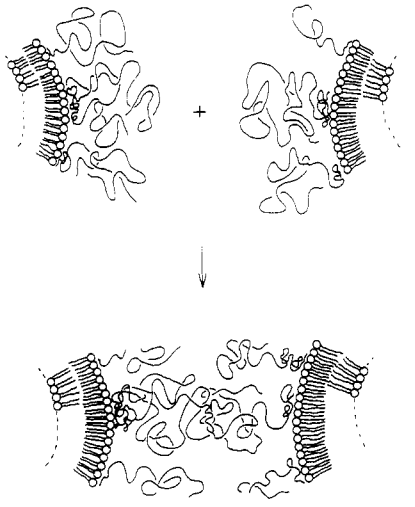

FIGURE 5: Schematic representation of the binding of PEG 20K to the DDP vesicles and the unfavorable overlap of the polymer coils when the vesicles approach each other. The overlap appears to be responsible for the steric stabilization (see text).

headgroups in the lateral plane of the bilayer, and hence, the binding of $Ca^{2+}$ to the DDP vesicles (Rupert et al., 1988).

Measurements of the $Ca^{2+}$-induced *aggregation* of DDP vesicles in terms of the initial aggregation rate and the relative extent of aggregation as a function of the concentration of PEG 20K (Figure 4) show that $Ca^{2+}$ is not able to induce aggregation above ca. $1.5 \times 10^{-3}$ wt % PEG 20K. This is in accord with the fusion experiments which showed that $Ca^{2+}$-induced fusion is restored below $1.0 \times 10^{-3}$ wt % PEG 20K. Thus, the fusion is prevented by PEG 20K because the vesicles cannot become closely apposed to form a fusion-susceptible contact site. Since binding of $Ca^{2+}$ is not significantly affected (see above) and, thus, charge neutralization can take place normally, there must be a steric barrier for aggregation and fusion (Figure 5). Apparently, the PEG 20K coils located at the bilayer–water interface act as an efficient steric (kinetic) barrier to vesicle fusion. Steric stabilization is well-known in colloid science (Hunter, 1987; Napper, 1970), and it has been shown, by direct force measurements, to be very effective (Luckham et al., 1987; Klein, 1986). Furthermore, the unperturbed root mean square end-to-end distance ($r_0$) of PEG

20K is 11.0 nm (Boucher et al., 1978), and since $r_0^{1/2} = (6S_0)^{1/2}$ (Flory, 1978), the corresponding radius of gyration ($S_0$) equals 4.5 nm. Thus, the polymer coil is significantly smaller than the DDP vesicles (diameter ca. 90 nm; Rupert et al., 1987) but large enough to prevent aggregation in a primary minimum (Nir et al., 1983).

Recently, it has been reported that the incorporation of nonionic amphiphilic molecules with a large poly(ethylene glycol) headgroup in liposomal bilayers may act as a steric stabilizer of the liposomes (Rydhag et al., 1984). Moreover, in this study, stabilization was observed at an ethylene glycol to phospholipid ratio of ca. 2:1, which agrees well with the results described above.

At 0.1 wt % PEG 20K, the fusion of DDP vesicles is not completely suppressed, either in the presence or in the absence of $Ca^{2+}$. Apparently, at this polymer concentration the vesicles can still approach each other, although the steric barrier is quite pronounced, as reflected by the strong inhibition of the initial rate of fusion. However, the extent of the fusion is comparable to that reached in the presence of PEG 8K, implying that PEG 20K forms in this case a steric barrier that affects mainly the fusion kinetics and not the susceptibility of the bilayer to fusion. A complete steric barrier that cannot be overcome arises upon increasing the PEG 20K concentration to 0.5 wt %. Presumably, at this polymer concentration an efficient coating of the vesicle surface takes place. The coating process is apparently a time-dependent event (Figure 2). Upon dilution of DDP vesicles to 0.1 wt % PEG 20K, after an incubation in 0.5 wt % PEG 20K, fusion is initiated. However, the longer the incubation time in 0.5 wt % PEG, the more the initial rate of fusion decreases (see above). It appears, therefore, that the amount of reversibly bound PEG 20K is dependent on the incubation time.

The above analysis in terms of steric stabilization does not readily afford an explanation for the different effects induced by PEG 8K and 20K. It seems that PEG 20K binds much stronger to the DDP vesicles than PEG 8K. However, the difference is larger than could be reasonably expected from an increase in molecular weight by a factor of 2.5. Therefore, an additional factor most likely governs the difference in binding efficiency.

*Microscopic Phase Separation in the DDP–Poly(ethylene glycol) Systems.* In water, poly(ethylene glycol) displays a lower critical solution temperature (LCST). Above the LCST, a phase separation occurs with formation of a concentrated polymer phase (e.g., 20–40% PEG) and a dilute polymer phase (<1% PEG). This temperature (also known as the *clouding temperature*) depends on the molecular weight of the polymer and is 389 and 376 K for PEG 8K and 20K, respectively (Saeki et al., 1976). In addition, the temperature above which phase separation takes place also depends on the presence of salts, anions being more effective than cations in lowering the LCST (Boucher et al., 1978; Napper, 1970; Bailey et al., 1959; Florin et al., 1984; Atamar, 1987). In particular, $H_2PO_4^-$ and $Na^+$ effectively lower the clouding temperature (Atamar, 1987), and it has been reported that for PEG 20K in 1 M $NaH_2PO_4$ the clouding temperature is lowered from 103 to ca. 42 °C (Saeki et al., 1976; Atamar, 1987). Taking into account that (i) the DDP bilayer–water interface is comparable to a concentrated $(RO)_2PO_2Na$ electrolyte solution with a concentration of approximately 1.0–1.5 M, that (ii) $(RO)_2PO_2Na$ is indeed a strong structure maker (Tamaki et al., 1987), and that (iii) all the experiments were performed at 40 °C, it appears that this temperature is indeed near the clouding temperature. Therefore, it is tentatively suggested that PEG 20K undergoes a phase separation, implying that the concentration at the bilayer–water interface, relative to that in the bulk phase, will be substantially increased. This process is accompanied by a removal of hydration water from the bilayer headgroups, in accord with the observed upfield shift of the $^{31}P$ NMR resonance (see above). We submit that this clouding phenomenon facilitates the direct interaction of the polymer with the vesicle surface. Such interactions may, for example, involve hydrogen-bond interactions via a water molecule between the phosphate headgroup and an ether oxygen of PEG or the interaction of a polymer segment with the $CH_2O$–P segment of DDP. This latter type of interaction would resemble the PEG–PEG interactions that occur in the absence of DDP vesicles above the clouding temperature (Napper, 1970; Florin et al., 1984; Atamar, 1987). Since the cis-complexation constant is not significantly affected by the presence of PEG 20K, large parts of the vesicle surface must remain freely accessible to $Ca^{2+}$. This implies that PEG 20K is only locally bound to the vesicle surface. On the other hand, PEG 20K prevents (or impedes) close approach of the vesicles (see above), indicating that the whole surface is effectively shielded by the polymer. The experimental data can be rationalized by assuming that the outer parts of the polymer coils (with respect to the vesicle surface) extend into bulk water, which is a good solvent for PEG, and are thus expanded (see Figure 5). Such a type of PEG binding would prevent vesicles from coming into close contact, while $Ca^{2+}$ can readily interact with the DDP headgroups. For PEG 8K, the clouding temperature is ca. 13 °C higher than that for PEG 20K and, therefore, will be probably centered around 55 °C. Thus, it is reasonable to assume that for PEG 8K no phase separation will occur; i.e., the binding of PEG 8K will be essentially negligible.

Further support for the importance of a microphase separation was obtained by monitoring the $Ca^{2+}$-induced fusion of DDP vesicles in the presence of PEG 8K and 20K as a function of the temperature. Previously, we observed (Rupert et al., 1987b) that in the absence of polymer a smooth but steep increase in the fusion activity takes place above ca. 31 °C. In the presence of PEG 20K the fusion activity increases above ca. 32 °C (Figure 6), but it appears that the curves in Figure 6 represent two opposing effects, an increase in fusion with temperature due to increased bilayer fluidity which is counteracted above ca 34 °C as a result of clouding and concomitant steric stabilization. The overall effect is that the fusion activity increases much less with temperature than it does in the absence of PEG 20K. In the presence of PEG 8K, a reduced initial fusion rate is found above ca. 45 °C, whereas in the absence of this polymer the fusion rate still increases above this temperature and then drops beyond 46 °C, most likely as the result of a phase transition of the DDP bilayer at this temperature (Figure 7). Thus, the data suggest that the clouding temperature for PEG 8K in the presence of DDP vesicles is ca. 45 °C, which is 12 °C higher than that for PEG 20K, in good agreement with the earlier estimate, as described above.

In a broader context, the present results may, at least in part, provide an explanation as to the superior properties of PEG 8K over PEG 20K for the induction of cell–cell fusion, a frequently used technique in the production of monoclonal antibodies (Westerwoudt, 1977). It is furthermore interesting to note that preliminary experiments revealed a molecular weight dependent effect of PEG on $Ca^{2+}$-induced fusion of PS and PS–PE vesicles (unpublished observations), similarly as observed for the fusion of DDP vesicles.

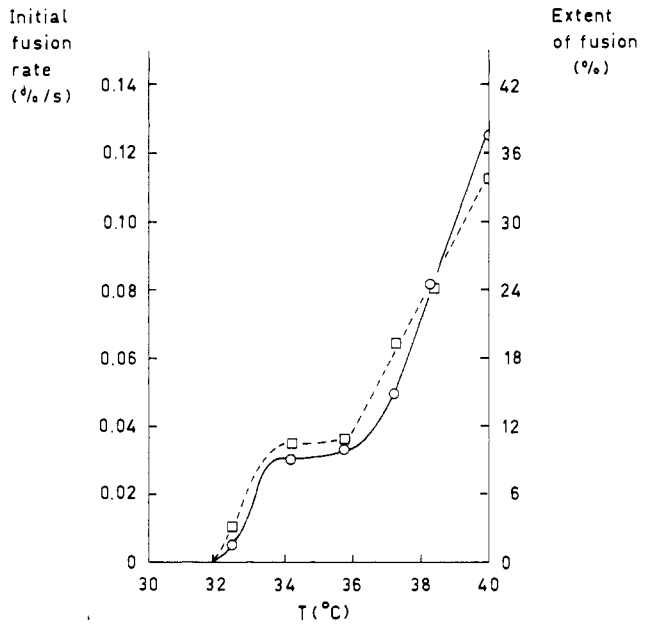


FIGURE 6: Initial fusion rate (○) and extent of fusion (□) for the $Ca^{2+}$-induced fusion of DDP vesicles in the presence of 0.3 wt % PEG 20K as a function of the temperature. The ratio of labeled to nonlabeled vesicles was 1:1. The concentration of DDP was 60 μM and that of $CaCl_2$ 3.85 mM, while the incubations were carried out at pH 7.4.

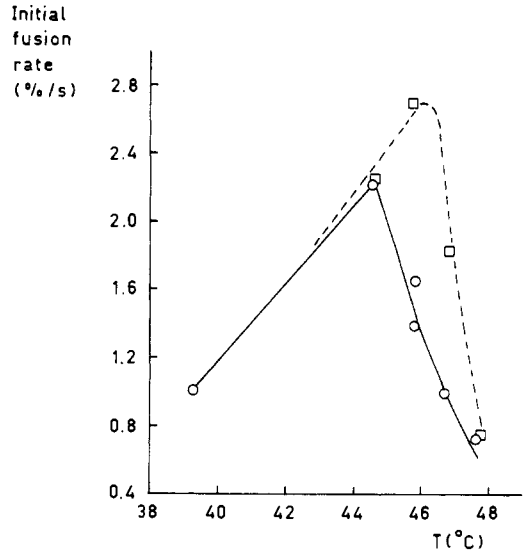


FIGURE 7: Initial fusion rate for the $Ca^{2+}$-induced fusion of DDP vesicles in the absence (□) and presence (○) of 10 wt % PEG 8K as a function of the temperature. The ratio of labeled to nonlabeled vesicles was 1:1. Concentrations of DDP and $CaCl_2$ were as those indicated in the legend of Figure 6. The pH was 7.4.

## ACKNOWLEDGMENTS

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**Registry No.** PEG, 25322-68-3; DDP, 7057-92-3; Ca, 7440-70-2.

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# Fourier Transform Infrared Spectroscopy of $^{13}C{=}O$-Labeled Phospholipids Hydrogen Bonding to Carbonyl Groups†

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ABSTRACT: Fourier transform infrared spectroscopy has been used to characterize the carbonyl stretching vibration of DMPC, DMPE, DMPG, and DMPA, all labeled with $^{13}C$ at the carbonyl group of the *sn*-2 chain. Due to the vibrational isotope effect, the $^{13}C{=}O$ and the $^{12}C{=}O$ vibrational bands are separated by ca. 40–43 $cm^{-1}$. This frequency difference does not change when the labeling is reversed with the $^{13}C{=}O$ group at the *sn*-1 chain. For lipids in organic solvents possible conformational differences between the *sn*-1 and *sn*-2 ester groups have no effect on the vibrational frequency of the C=O groups. In aqueous dispersion unlabeled phospholipids always show a superposition of two bands for the C=O vibration located at ca. 1740 and 1727 $cm^{-1}$. These two bands have previously been assigned to the *sn*-1 and *sn*-2 C=O groups. FT-IR spectra of $^{13}C$-labeled phospholipids show that the vibrational bands of both, the *sn*-1 as well as the *sn*-2 C=O group, are clearly superpositions of at least two underlying components of different frequency and intensity. Band frequencies were determined by Fourier self-deconvolution and second-derivative spectroscopy. The difference between the component bands is ca. 11–17 $cm^{-1}$. Again, the conformational effect as shown by reversed labeling is negligible with only 1–2 $cm^{-1}$. The splitting of the C=O vibrational bands in $H_2O$ and $D_2O$ is caused by hydrogen bonding of water molecules to both C=O groups as shown by a comparison with spectra of model ester compounds in different solvents. To extract quantitative information about changes in hydration, band profiles were simulated with Gaussian–Lorentzian functions. The chemical nature of the head group and its electronic charge have distinctive effects on the extent of hydration of the carbonyl groups. In the gel and liquid-crystalline phase of DMPC the *sn*-2 C=O group is more hydrated than the *sn*-1 C=O. This is accord with the conformation determined by X-ray analysis. In DMPG the *sn*-1 C=O group seems to be more accessible to water, indicating a different conformation of the glycerol backbone.

In the last years Fourier transform infrared spectroscopy has been used with great success to elucidate the conformational properties of phospholipids in different lamellar phases and the interaction of phospholipids with ions, cholesterol, and membrane proteins (Cameron et al., 1979; Parker, 1983; Amey & Chapman, 1984; Casal & Mantsch, 1984; Mendelsohn & Mantsch, 1986; Mantsch et al., 1986). Most of these studies have focused on the analysis of the vibrational bands of the $CH_2$ groups and the change of their intensity and frequency with temperature. Other vibrational bands of phospholipids were not studied in such detail. It was noted relatively early that the C=O stretching bands of phospholipids in aqueous dispersion displayed an asymmetry which changed with the phase state of the lipid, with the degree of hydration, or with

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