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^{1}H-NMR STUDY OF THE EFFECT OF SYNTHETIC POLYMERS ON THE FLUIDITY, TRANSITION TEMPERATURE AND FUSION OF DIPALMITOYL PHOSPHATIDYLCHOLINE SMALL VESICLES

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Summary

The interaction of water-soluble polymers with dipalmitoyl phosphatidylcholine small vesicles and the effect on vesicle fusion were studied by means of ^{1}H-NMR spectrometry. The motion of dipalmitoyl phosphatidylcholine molecules decreased on interaction with the polymers and was detected as a change in the signal intensity. The interaction behavior of polymers is very sensitive to the chemical structure of the applied polymers. Poly(styrene sulfonic acid) and poly(ethylene glycol) decreased the motion of the choline methyl group, predominantly through coulombic and hydrophobic interaction forces, respectively. For example, in the case of the poly(styrene sulfonic acid)-containing system, the signal intensity of the choline methyl group was decreased about 15% while those of the hydrophobic methylene and terminal methyl groups were scarcely decreased by the addition of polymer to a final concentration of $4.0 \cdot 10^{-2}$ unit mol/l. These polymers are considered to interact with the surface of the vesicle membrane. On the other hand, poly(L-glutamic acid) and poly(*N*-vinyl-2-pyrrolidone) decreased the signal intensities of not only the choline methyl group, but also those of the hydrophobic methylene and terminal methyl groups. This result suggests that part of these polymers might be incorporated into the hydrophobic region of the vesicle membrane.

Addition of the non-ionic polymers inhibited vesicle fusion considerably. This effect was explained by the stabilization of dipalmitoyl phosphatidylcholine vesicles by complexation with these polymers.

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Introduction

In recent years, some synthetic polymers were found to be particularly useful for the induction of membrane fusion. Poly(ethylene glycol) (molecular weight 6000) was reported to be an excellent fusogenic agent of plant protoplasts [1] or hen erythrocytes [2,3]. Though the fusogenic activity of poly(ethylene glycol) was investigated, there were few discussions on the mechanism by which membrane fusion is induced by synthetic polymers [2,4,5]. It was suggested that membrane fusion required the interaction of cells with a high concentration of polymer solution (more than 40 wt%). The polymer was considered to give rise to cluster formation on the membrane. An excess of polymers should interact with the exposed lipid layers. It is important to clarify the effect of the polymer-lipid layer interaction on membrane fusion. This interaction is considered to prevent membrane fusion because the fusion occurs initially at a small localized area of the cell surface (cytoplasmic connection) after the removal of most of the poly(ethylene glycol).

The interaction of polymers with vesicle membranes has been studied as a model system in order to clarify the protein-lipid interaction in biological membranes. In particular, mainly the interaction between synthetic polypeptides and phospholipid bilayers have hitherto been studied. Hammes and Schullery [6] studied the interactions between phospholipid vesicles and various polypeptides. They observed the obvious interaction between poly(L-lysine) and phosphatidylserine. The interaction between dipalmitoyl phosphatidylcholine (DPPC) and poly(L-glutamic acid) was also studied by Yu et al. [7] and Chang and Chan [8]. This interaction was clearly observed by using ESR and NMR techniques. The details have already been reported by Chang and Chan [8].

As the use of NMR gives much information on vesicle membranes [9—16], we studied the interactions of some synthetic water-soluble polymers with phospholipid vesicles by means of NMR spectroscopy. The effects of the polymers on the molecular motion of phospholipid and the fusion of vesicles are discussed. This study might be applicable to the elucidation of the mechanisms of biological membrane fusion.

Materials and Methods

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Sigma. The purity of this phospholipid was checked by thin-layer chromatography (TLC). Samples which gave only one spot on the TLC plate were used without further purification. A dispersion of DPPC in ^2H_2O was sonicated in a water bath at 20°C for 30 min at 45 W with a sonicator (UR-200P; Tomy Seiko Co.). The single-walled small vesicle dispersion was kept at 50°C for 60 min to anneal the vesicles. ^2H_2O and $Eu(NO_3)_3 \cdot 6\ ^2H_2O$ were purchased from Merck. 20 mM Eu^{3+} (^2H_2O solution) was added to the vesicle dispersions to a 4.8 mM final concentration in order to shift the choline methyl signal of DPPC in the outward facing membrane.

Poly(styrene sulfonic acid) (sodium salt) (molecular weight 50 000) was obtained by the polymerization of sodium styrene-sulfonate in H_2O at 70°C for

3 h under an N_2 atmosphere with $K_2S_2O_8$ as an initiator. The polymer was reprecipitated from acetone and dried in vacuo. Poly(methacrylic acid) was obtained by the polymerization of methacrylic acid in H_2O at 50°C for 5 h under an N_2 atmosphere with $K_2S_2O_8$. Poly(methacrylic acid) (molecular weight 40 000) was purified by using the same procedure as previously reported [17]. Poly(L-glutamic acid) (sodium salt) (molecular weight 50 000) was prepared and purified by using the same method as previously reported [18].

Poly(ethylene glycol) (molecular weight 7500) was purchased from Wako Pure Chemical Co. Ltd. A $CHCl_3$ solution of poly(ethylene glycol) was poured slowly into an excess of diethyl ether. Reprecipitated poly(ethylene glycol) was washed with diethyl ether three times and dried in vacuo. Poly(*N*-vinyl-2-pyrrolidone) was obtained by the polymerization of *N*-vinyl-2-pyrrolidone in degassed CH_3OH at 50°C for 6 h with azobis(isobutyronitrile) as an initiator. The reaction mixture was poured into an excess of diethyl ether. Then the washed polymer was dried in vacuo. Its average molecular weight is 40 000 which was determined from the viscosity measurement.

Apparatus

^{1}H-NMR spectra were measured by means of a JEOL-100 MHz NMR spectrometer with a temperature control apparatus. Chemical shifts were measured relative to an external capillary of 1% $(CH_3)_4Si$ in C^2HCl_3.

Scattered-light intensities of vesicle dispersions were measured by means of an LS-601 type light-scattering spectrometer (Union Giken) with an He-Ne laser (632.8 nm) as light source. The associated (apparent) molecular weight of DPPC vesicles in ^2H_2O was measured in parallel with the NMR measurements at 50°C.

Results

The NMR spectrum of sonicated DPPC small vesicle dispersions consists of three peaks as assigned to the choline methyl, methylene and terminal methyl protons at 3.20, 1.27 and 0.85 ppm, respectively, at temperatures above the phase transition temperature. The changes of the intensities or linewidths of peaks are used as indices which reflect the molecular motion of phospholipids [19]. The gel-to-liquid crystalline phase transition temperature and the approximate size of vesicles could also be estimated from the NMR spectrum [14,20]. The sonicated DPPC vesicles studied in this work have an average radius of 160 Å as calculated from the results of NMR and light-scattering measurements.

Effect of water-soluble polymers on the motion of DPPC molecules

The motional change of DPPC molecules is detectable at the molecular level by measuring the intensities of the individual signals. The effect of poly(styrene sulfonic acid) (molecular weight 50 000) on the molecular motion (or fluidity) of DPPC vesicles was studied initially. The NMR spectra of 2.0 wt% of DPPC vesicles with different concentrations of poly(styrene sulfonic acid) are depicted in Fig. 1. Each peak of the DPPC vesicles was decreased by the addition of poly(styrene sulfonic acid). The decrease in signal intensity was also observed by adding other water-soluble polymers such as poly(L-glutamic acid),

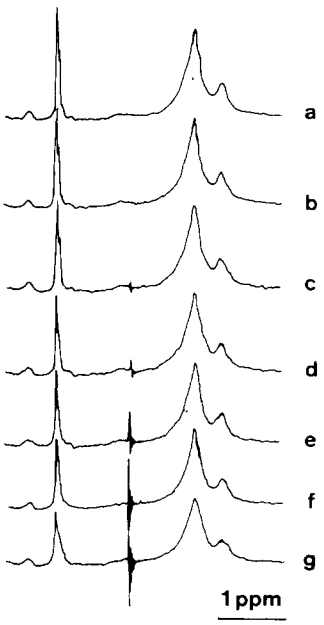

Fig. 1. Effect of poly(styrene sulfonic acid) on the ^{1}H-NMR spectra of DPPC vesicles in ^2H_2O at 50°C. 2.5 wt.% of ^2H_2O solutions of DPPC vesicles were initially prepared by sonication at 20°C for 30 min. The vesicle solution was kept at 50°C for 60 min, then 0.1 unit mol/l of poly(styrene sulfonic acid) solution was added stepwise at 50°C. The NMR spectra were measured 10 min after mixing at 50°C. The final concentrations of poly(styrene sulfonic acid) were (in unit mol/l): a, 0; b, 4.2 10^{-3}; c, $1.2 \cdot 10^{-2}$; d, $1.0 \cdot 10^{-2}$; e, $2.5 \cdot 10^{-2}$; f, $3.4 \cdot 10^{-2}$ and g, $5.6 \cdot 10^{-2}$.

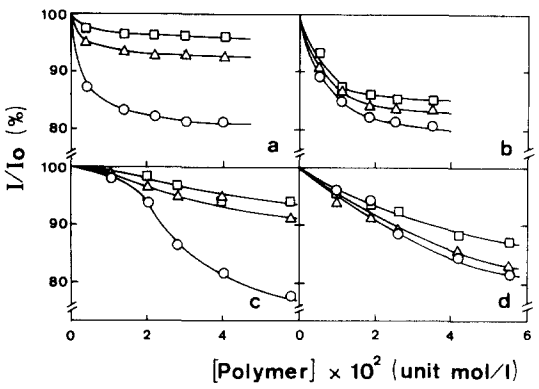

Fig. 2. Effect of water-soluble polymers on the changes of motion of DPPC molecules at 50°C. I/I_0 is the relative intensity ratio of signals in the presence (I) and absence (I_0) of polymers. The decrease in the intensity due to the dilution of the DPPC vesicle solution by the addition of polymer solution was corrected. Added water-soluble polymers were: a, poly(styrene sulfonic acid); b, poly(L-glutamic acid); c, poly(ethylene glycol) and d, poly(*N*-vinyl-2-pyrrolidone). The initial concentration of DPPC was 2.0 wt.%. (○) Choline methyl protons, (□) methylene protons, (△) terminal methyl protons.

poly(ethylene glycol) and poly(*N*-vinyl-2-pyrrolidone). The changes of the ratio of the initial height of each peak (I_0) to that of the decreased peak (I) caused by the addition of polymers were plotted against the final concentration of added polymer (Fig. 2). I_0 and I represent the intensities of each peak of the DPPC vesicles in the absence and presence of polymers, respectively. The ratio (I/I_0) is considered as one of the parameters which indicate the extent of molecular motion of lipids, i.e., membrane fluidity.

It is apparent that poly(styrene sulfonic acid) interacted with the ionic (hydrophilic) groups of DPPC molecules because the addition of poly(styrene sulfonic acid) decreased the signal intensity mainly of the choline methyl protons about 15% at polymer concentrations greater than $1 \cdot 10^{-2}$ unit mol/l (see Fig. 2a). This means that the interaction of poly(styrene sulfonic acid) with DPPC molecules was taking place on the surface of membranes mainly through coulombic forces. The effect of poly(L-glutamic acid) was different from the result obtained with the poly(styrene sulfonic acid)-containing system, i.e., choline methyl, hydrophobic methylene and terminal methyl peaks were all decreased

to the same extent (Fig. 2b). It was suggested that poly(L-glutamic acid) interacted with DPPC on the surface of vesicles through coulombic forces and that the partial incorporation of poly(L-glutamic acid) chains in the hydrophobic region of DPPC vesicles also occurred. Chang and Chan [8] have already reported that poly(L-glutamic acid) might be partially incorporated into the hydrophobic region of DPPC vesicles (as detected by the conformational change of poly(L-glutamic acid). In contrast to these polymers, poly(methacrylic acid) interacted strongly with DPPC vesicles and the aggregation of the vesicles occurred immediately, so that the effect of this polymer could not be evaluated using NMR.

On the other hand, though poly(ethylene glycol) has no ionic sites itself, it also interacted with phospholipid vesicles (Fig. 2c). Though the interaction force is not so strong, the relative intensity of each peak decreased as in the case of the poly(styrene sulfonic acid)-containing system, so it was supposed that poly(ethylene glycol) interacted with the surface of DPPC vesicles predominantly through a kind of hydrophobic interaction. Some units of poly(ethylene glycol) chains might replace water molecules mainly in the vicinity of the hydrophobic interaction. Some units of poly(ethylene glycol) chains might replace water molecules also in the vicinity of the hydrophobic hydrocarbons closest to the hydrophilic part of the DPPC molecules. Cabane [21] has already studied the interaction of poly(ethylene glycol) with sodium dodecyl sulfate micelles, and the same interaction mechanism was discussed by using the results of ^{1}H- ^{13}C- and ^{23}Na-NMR spectrometry. Poly(*N*-vinyl-2-pyrrolidone), another type of non-ionic polymer, also interacted with DPPC vesicles and decreased the molecular motion of lipids (Fig. 2d).

The molecular motion of polymers should also decrease through the interaction with lipid bilayers. Poly(ethylene glycol) with a high molecular weight shows only one sharp NMR signal as assigned to the methylene protons at 3.62

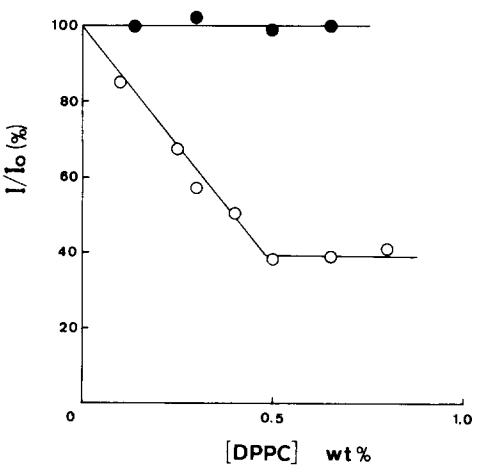

Fig. 3. Changes of signal intensity of methylene protons (3.62 ppm) of poly(ethylene glycol) by the addition of DPPC vesicle $^{2}H_2O$ solutions at 25°C (○) and 50°C (●). 2.0 wt.% of DPPC vesicle solution was added stepwise to 0.02 unit mol/l of a poly(ethylene glycol) $^{2}H_2O$ solution. The signal intensity (I) was also corrected in the same manner as that in Fig. 2.

ppm, and an intensity change of the signal was investigated in the presence of DPPC vesicles. The signal intensity of poly(ethylene glycol) was little affected by temperatures at about 20—60°C. Addition of DPPC vesicles made the signal intensity of poly(ethylene glycol) decrease and saturate at 25°C (below T_c) (Fig. 3). On the other hand, it was not affected at 50°C (above T_c). However, poly(ethylene glycol) interacted with DPPC vesicles through a kind of hydrophobic interaction; its molecular motion might be slightly suppressed because of a larger mobility of DPPC molecules at temperatures above T_c.

Effect of polymers on the transition temperature of small DPPC vesicles

It was expected from the foregoing results that some water-soluble polymers would affect the gel-to-liquid crystalline phase transition of the vesicle membrane. The temperature dependence of the signal intensity of protons in hydrophobic regions (methylene and terminal methyl) was compared in the absence and presence of these polymers. As noted above, the transition temperature (T_c) was defined as the temperature at which the peaks of the methylene and terminal methyl disappeared. A small DPPC vesicle dispersion showed the T_c at 39°C. It was observed that T_c was increased about 3°C by the addition of poly(ethylene glycol) to a final concentration of $4.0 \cdot 10^{-2}$ unit mol/l. Although the addition of the same amount of poly(*N*-vinyl-2-pyrrolidone) and poly(styrene sulfonic acid) also increased the T_c about 2 and 1°C, respectively, the effect of poly(L-glutamic acid) on the T_c of small DPPC vesicles was not observed. The increase in T_c was explained by the suppression of the molecular motion of lipids through the interaction with polymers.

Determination of vesicle radius from NMR measurements

In general, the NMR peak due to the choline methyl group showed a shoulder on the higher magnetic field side. This shoulder was assigned to the choline methyl group on the inward facing vesicle membrane [10,22,23]. As the shift was caused by the effect of the curvatures of vesicles [24], this shift or the relative intensity of these two peaks should provide a measure of the vesicle radius. Addition of Eu^{3+} made the peak of the outward facing choline methyl group shift upfield. Although a suitable concentration of Eu^{3+} made these two peaks separate completely, an excess of Eu^{3+} decreased all peak intensities due to the paramagnetic properties of Eu^{3+}. Therefore, a suitable concentration of Eu^{3+} was determined as 4.8 mM (final concentration). From the intensity ratio of these split peaks, the vesicle radius was calculated approximately by using the following equations:

$$N = N_E + N_I = (4\pi/S) \cdot [r_E^2 + (r_E - \Delta r)^2] \quad (1)$$

where r_E is the radius to the outer membrane, and Δr is the thickness of the bilayer membrane. S is the free surface area per polar head group of phospholipid. N_E and N_I are the numbers of phopholipid molecules present on the outward and inward facing membrane, respectively. N is the total numbers of phospholipid molecules per vesicle. The values of Δr and S of DPPC vesicles were 37 Å and 58 Å^2, respectively, above the phase transition temperature [25]. I_E and I_I are the intensities of the choline methyl group present on the outward and inward facing membranes, respectively. The ratio (N_E/N_I) is equal

to the ratio of the radius squared (r_E^2/r_I^2). Furthermore, the ratio of NMR signal intensities of split choline methyl peaks (I_E/I_I) is also equal to r_E^2/r_I^2. The intensity ratio is expressed as a function of r_E (Eqn. 2).

$$I_E/I_I = r_E^2/(r_E - \Delta r)^2 = 1 + [(2\, r_E \Delta r - \Delta r^2)/(r_E - \Delta r)^2] \quad (2)$$

From Eqn. 2, the radius of a DPPC vesicle could be assessed approximately by measuring the intensity ratio of split choline methyl peaks. However, there were considerable experimental errors for the larger vesicle (more than 500 Å in radius). The results from light-scattering measurements supported the average radii of DPPC vesicles determined from the NMR results.

Effect of polymers on the fusion of vesicles

Since vesicle curvature has a profound influence on the molecular packing in a vesicle, sonicated small vesicles might be less stable than larger or multilamellar vesicles. Vesicle-vesicle fusion takes place in order to dissipate an excess of surface energy [26,27]. In general, freshly prepared small vesicles tend to undergo vesicle-vesicle fusion at temperatures below the transition temperature due to defects in membrane structures. This vesicle fusion was observed as a decrease in the intensity ratio of choline methyl signals split by Eu^{3+}. Freshly prepared DPPC vesicle dispersions were incubated at 5°C for a suitable period of time, then these were heated to 50°C and Eu^{3+} was added. NMR spectra

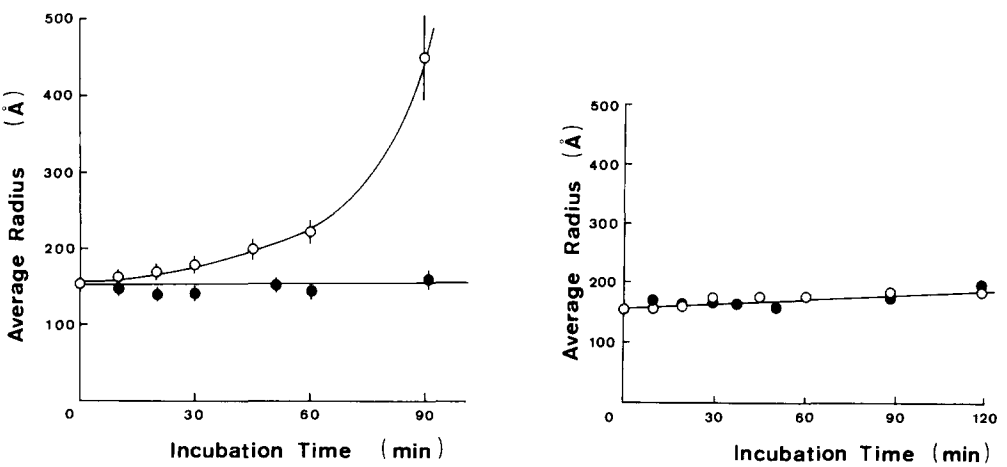

Fig. 4. Changes of the average radius of DPPC vesicles due to fusion. DPPC vesicle solutions (2.5 wt.%) were sonicated for 30 min at 5°C and immediately incubated from 10 to 90 min to induce vesicle fusion at 5°C (○) and 50°C (●) (annealing was not performed). Incubated sample solutions were quickly heated to 50°C and maintained at this temperature for 10 min. Then a ^2H_2O solution of $Eu(NO_3)_3$ was added (4.8 mM final concentration). The NMR spectra, especially I_I and I_E, were measured at 50°C. The average radius was calculated from Eqn. 2.

Fig. 5. Effect of non-ionic polymers on the fusion of DPPC vesicles. 2.0 wt.% DPPC vesicle solutions were sonicated at 5°C, then mixed with ^2H_2O solutions of poly(ethylene glycol) (○) or poly(*N*-vinyl-2-pyrrolidone) (●) at 5°C. The mixed solutions were incubated individually at 5°C from 10 to 120 min. Incubated sample solutions were heated to 50°C quickly and kept for 10 min. Then a ^2H_2O solution of $Eu(NO_3)_3$ was added (4.8 mM final concentration). The final concentrations of DPPC and polymers were 1.6 wt.% and $4.0 \cdot 10^{-2}$ unit mol/l, respectively. The decrease in signal intensity due to the dilution of the vesicle solution and the interaction with polymers was corrected by using the previous results (see Fig. 2).

were measured 10 min after mixing with Eu^{3+} at 50°C. The peak intensity ratio (I_E/I_I) was about 1.80 at first, then this decreased exponentially with incubation time at temperatures below T_c. For example, the radius of a freshly prepared small DPPC vesicle was calculated to be about 160 Å (I_E/I_I = 1.80), and this increased to 450 Å (I_E/I_I = 1.18) on incubation for 90 min at 5°C (Fig. 4). In contrast, DPPC vesicles showed no marked intensity change at temperatures above T_c.

It was demonstrated that small amounts of vesicles were fused by incubation at the lower temperature in the presence of polymers. Fig. 5 shows the effect of poly(ethylene glycol) or poly(*N*-vinyl-2-pyrrolidone) on the fusion of DPPC vesicles at 5°C. Addition of these polymers at a final concentration of $4.0 \cdot 10^{-2}$ unit mol/l decreased the motion of DPPC molecules by about 20% on the surface of the membrane (Fig. 2c and d). No marked vesicle fusion was observed. It is obvious that the water-soluble polymers inhibit the fusion between vesicles. The small DPPC vesicles might be stabilized by complexation with such non-ionic polymers as poly(ethylene glycol) or poly(*N*-vinyl-2-pyrrolidone).

Discussion

It was observed that the signal intensities of DPPC were decreased by adding water-soluble polymers. There is a small possibility that the viscosity change of the DPPC solution which is raised by the added polymers may lower the extent of vesicle tumbling. However, the changes of their intensities depend on the chemical structure of added polymers (Fig. 2). That is, the decrease in signal intensities may reflect mainly the interaction between added polymers and DPPC molecules. Water-soluble polymers can interact with DPPC vesicles through secondary binding forces. It is conceivable that polyelectrolytes interact with the surface of the vesicles through coulombic forces and that the motion of the choline methyl group of DPPC molecules should be decreased by the interaction. The poly(styrene sulfonic acid)-containing system is a typical example. Poly(styrene sulfonic acid) interacted predominantly with the hydrophilic part (choline group) of DPPC molecules (Fig. 2a). Though poly(L-glutamic acid) is also an anionic polyelectrolyte, the motion of the hydrophobic part of DPPC molecules also decreased on the addition of this polymer (Fig. 2b). These different actions of polyanions might be caused by differences in the solubility of polymers in organic solvents. Although poly(styrene sulfonic acid) is more hydrophobic than poly(L-glutamic acid), the former is insoluble in almost all organic solvents. The hydrophobic part of the vesicle membrane is considered as a non-polar organic solvent system. A polymer which is insoluble in such organic solvents might not be incorporated into the hydrophobic part of vesicle membranes. However, although hydrophobic polymers formed mixed-micelle systems with some amphiphilic molecules, spontaneous incorporation into micelles or vesicles does not occur so easily. On the whole, the mechanisms of the interaction of polymers with lipids are affected by factors such as the density of the ionic site, the solubility of polymers in non-polar solvents, etc. In other words, the mechanisms are strongly influenced by the chemical structures of the polymers. These relationships will be summarized in the near future.

The motion of lipid molecules was suppressed and the gel-to-liquid crystalline transition temperature increased by the interaction of water-soluble polymers. Indeed, the phase transition temperature increased about 1—3°C on addition of these polymers to dispersions of DPPC small vesicles. Tilcock and Fisher [28] have reported that a concentrated poly(ethylene glycol) solution lowered the free-water content. Though the transition temperature of DPPC vesicles increased dramatically with the decrease in free-water content [28], the transition temperature was observed to increase slightly on addition of relatively diluted polymer solutions. The increase in the transition temperature indicates the suppression of the mobility of lipid molecules.

The added polymers are expected to affect vesicle fusion. The addition of polymers inhibited the fusion of vesicles (Fig. 5). As poly(ethylene glycol) is known to decrease the surface potential of DPPC vesicles [29] and vesicle fusion takes place to dissipate the surface potential, it is expected that vesicle fusion is inhibited by the addition of polymers. Poly(*N*-vinyl-2-pyrrolidone) showed the same effect as poly(ethylene glycol) (Fig. 5).

It is well known that poly(ethylene glycol) can be used to fuse several cell lines. The cell fusion is carried out by treating the cells with a concentrated (more than 40 wt%) poly(ethylene glycol) solution for a short period of time (less than 5 min). Poly(ethylene glycol) induces cluster formation of membrane proteins and aggregation of cells [4]. It may also interact with the lipid layers which were exposed to the water phase by cluster formation. In fact, cell fusion was not observed by simply mixing the cells with the concentrated poly(ethylene glycol) solution. As Knutton [4] pointed out, cell fusion requires the removal of most of the poly(ethylene glycol) from the surface of the cell membrane. An excess of poly(ethylene glycol) might interact with the exposed lipid layer. Namely, cell fusion was performed after washing the polymer-treated cells to exclude the excess of polymers. This fact indicates that the formation of a polymer-lipid complex inhibits cell fusion. This is in agreement with the results obtained in this study.

General Conclusions

The results of the present study may have the following implications.

(1) The strength and mechanism of the interaction of polymers with DPPC vesicles depend on the chemical structure of the polymers, and more particularly, on ionic site density, hydrophobicity and solubility. And this may be adaptable to other lipid systems.

(2) The fusion of small DPPC vesicles was inhibited by complexation with polymers.

(3) The previous conclusion (2) also suggested that the fusion of cell membrane required the removal of most of the polymers from the lipid layer which was exposed to the water phase.

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