Effects of Poly(ethylene glycol) (PEG) Chain Length of PEG-Lipid on the Permeability of Liposomal Bilayer Membranes

Kaname Hashizaki,*[,a] Hiroyuki Taguchi,[a] Chika Itoh,[b] Hideki Sakai,[c] Masahiko Abe,[c] Yoshihiro Saito,[a] and Naotake Ogawa[a]

[a] College of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan: [b] DDS Development Division, NOF Co.; 3–3 Chidori-cho, Kawasaki, Kanagawa 210–0865, Japan: and [c] Faculty of Science and Technology, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba 278–8510, Japan.
Received February 14, 2003; accepted April 19, 2003

The effects of poly(ethylene glycol) (PEG) chain length of PEG-lipid on the membrane characteristics of liposomes were investigated by differential scanning calorimetry (DSC), freeze-fracture electron microscopy (FFEM), fluorescence polarization measurement and permeability measurement using carboxyfluorescein (CF). PEG-liposomes were prepared from mixtures of dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylethanolamines with covalently attached PEG molecular weights of 1000, 2000, 3000 and 5000 (DSPE-PEG). DSC and FFEM results showed that the addition of DSPE-PEG to DPPC in the preparation of liposomes caused the lateral phase separation both in the gel and liquid-crystalline states. The fluidity in the hydrocarbon region of liposomal bilayer membranes was not significantly changed by the addition of DSPE-PEG, while that in the interfacial region was markedly increased. From these results, it was anticipated that the CF leakage from PEG-liposomes is accelerated compared with DPPC liposomes. However, CF leakage from liposomes containing DSPE-PEG with a 0.060 mol fraction was depressed compared with regular liposomes, and the leakage decreased with increasing PEG chain length. Furthermore, the CF leakage from liposomes containing DSPE-PEG with a 0.145 mol fraction was slightly increased compared with that of liposomes containing DSPE-PEG with a 0.060 mol fraction. It is suggested that the solute permeability from the PEG-liposomes was affected by not only properties of the liposomal bilayer membranes such as phase transition temperature, phase separation and membrane fluidity, but also the PEG chain of the liposomal surface.

Key words liposome; poly(ethylene glycol); permeability; freeze-fracture electron microscopy; fluorescence polarization; carboxyfluorescein

For many years, various types of particulate drug carriers have been investigated with the aim of perfecting the drug delivery system (DDS) which localizes drugs to their desired *in vivo* target tissue while minimizing their side effects. Liposomal drug delivery systems have been widely researched as a sustained release system, in addition to being a targeted drug delivery system.[1)] Liposomes are ideal drug carriers being biodegradable and of minimal toxicity, however, there are drawbacks to their use *in vivo* such as the lack of stability in blood and short blood circulation time. Despite lipid compositions in liposomes closely resembling those of cell membranes, liposomes after their intravenous administration are rapidly removed from the circulation primarily by Kupffer cells of the liver and fixed macrophages of the spleen. A prolonged residence of the drug-entrapped liposomes in the circulation is important for their sustained release. Thus, it is important to develop modified liposomes that are able to avoid uptake by the reticuloendothelial system (RES) and extend the circulation half-life *in vivo*. Many studies have reported that the liposomes conjugated with amphipathic polyethylene glycol (PEG), PEG-liposomes, significantly increase the blood circulation half-life of the liposomes compared with those without PEG.[2—5)] The increased circulation half-life of PEG-liposomes has been attributed to the steric repulsive barrier by the covalently attached PEG around the liposomes.[6,7)]

In addition, it is necessary to control a release of the drug from the liposomes for the more effective treatment using the PEG-liposomes. The lipid composition of the membranes and the PEG chain length of PEG-lipid are major factors that govern the aggregate structure and phase behavior.[11—13)] In general, the physicochemical properties of liposomal bilayer membranes, such as surface charge,[8)] membrane fluidity,[9)] phase transition and separation,[10)] are known to affect the permeability of the drug. Therefore, it can be anticipated that the difference of the PEG chain length of the PEG-lipid affects the permeability of the drug from the liposomes.

In this study, to elucidate the permeation mechanism in PEG-liposomes, we investigated the effects of the PEG chain length of the PEG-lipid on the phase conditions of liposomes and temperature dependency of CF permeability from liposomes.

Experimental

Materials Distearoyl-*N*-monomethoxy poly(ethylene glycol)-succinyl-phosphatidylethanolamines (DSPE-PEG) were from NOF Co., Ltd. (Tokyo, Japan). Their weight-average molecular weights of poly(ethylene glycol) were approximately 1000, 2000, 3000 and 5000. L-α-Dipalmitoyl phosphatidylcholine (DPPC, 99.6% pure) was from NOF Co., Ltd. 5-(6)-Carboxyfluorescein (CF, 99% pure) was purchased from Molecular Probes, Inc. (OR, U.S.A.) and was used without further purification. 1,6-Diphenyl-1,3,5-hexatriene (DPH, 98% pure) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH, 95% pure) were purchased from Sigma Chemical Co. (MO, U.S.A.) and were used without further purification. Phosphate-buffered saline (PBS), whose composition was NaCl, KCl, Na_2HPO_4 and KH_2PO_4, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PBS was dissolved in water for injection (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and the isotonic solution whose pH was 7.4 was obtained. All other chemicals were commercial products of reagent grade.

Preparation of PEG-Liposomes Two kinds of PEG-liposomes were prepared using DPPC and 0.060 or 0.145 molar fractions of DSPE-PEG. DPPC and DSPE-PEG were dissolved in chloroform in a test tube. The solvent was then removed by blowing nitrogen gas into the test tube, and the

* To whom correspondence should be addressed. e-mail: khashiza@pha.nihon-u.ac.jp

residual solvent was further dried overnight at room temperature in a desiccator under vacuum. PBS was added to this lipid film and warmed (55—60 °C) above the phase transition temperature of DPPC (41 °C) for 30 min. The test tube was then shaken vigorously in a vortex mixer, and the multilamellar vesicles (MLV) were obtained. The large unilamellar vesicles (LUV) were obtained from the MLV suspension extruded five times through two-stacked polycarbonate membrane filter (pore size: 200 nm) using an Extruder (Lipex Biomembranes Inc., British Columbia, Canada).

Differential Scanning Calorimetry (DSC) The phase transition temperature of liposomal bilayer membranes was measured with a differential scanning calorimeter (8230, Rigaku Co., Tokyo, Japan). The liposome solution (25 mg) was put in a sampling vessel, made of stainless steel (resistance pressure: 50 atm, vessel size: 3×5 (ϕ) mm), and then the vessel was sealed. The measurement conditions were 1 °C min^{-1} for the scanning rate, 30—50 °C scanning range and 0.1 m cal s^{-1} for the sensitivity.

Freeze-Fracture Electron Microscopy Samples for electron microscopy were prepared by freeze-fracture replication. A small volume of liposome (MLV) solution was placed on a sample holder, and it was frozen immediately in liquid nitrogen at −190 °C. The frozen samples thus prepared were transferred to a freeze-replica apparatus (FR-7000A, Hitachi Science Systems Co., Ibaraki, Japan) and fractured at −150 °C and $<10^{-5}$ Torr. The fractured surfaces were immediately replicated by evaporating a platinum-carbon mixture from an electrode at an angle of 45° to the fracture surface, followed by a carbon film coating at normal incidence to increase the mechanical stability of the replica. It was cleaned using acetone and distilled water, and then picked up on 300 mesh copper electron microscope grids. The replicas thus prepared were observed using a transmission electron microscope (JEM-1200EX II, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV electrons. Images were recorded on an electron-microscopic film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Fluorescence Polarization Measurements The microscopic-state of liposomal bilayer membranes was determined from the fluorescence polarization (P) measurement using the fluorescent probes. DPH and TMA-DPH were dissolved in tetrahydrofuran and ethanol,[14] respectively. The fluorescent probe was added to the dilute liposome solution and incubated for 1 h at 55 °C. The liposome solution was diluted so that the fluorescence polarization was not affected by the scattering signals from liposomes. The molar ratio of lipid to probe was 300 : 1 (DPH) or 600 : 1 (TMA-DPH). Fluorescence polarization (P) was calculated according to the following equation:

$$P=(I_P-GI_V)/(I_P+GI_V) \quad (1)$$

where I_P and I_V are the fluorescence intensities of the emitted light polarized parallel and vertically to the exciting light, respectively, and G is the grating correction factor.[15] I_P and I_V were measured with a spectrofluorometer (FP-777, JASCO Co., Tokyo, Japan). DPH was excited at 350 nm and fluorescence emission was detected at 450 nm. When TMA-DPH was used, the excitation and emission wavelengths were 360 and 430 nm, respectively.

Permeability Measurements To study the solute leakage from liposomes, CF as a solute marker was encapsulated into liposomes, and the leakage of CF from the liposomes was measured using a dialysis technique similarly to that of Johnson *et al.*[16] and Nikolova *et al.*[17] Liposomes were prepared as described above, except that 50 mM CF was included to monitor the CF leakage. The liposomes encapsulating CF were separated from unencapsulated CF by gel chromatography on Sephadex G-50 column (Amersham Pharmacia Biotech, Uppsala, Sweden) with PBS as an eluant. Liposome solution (1 ml) was placed in a dialysis bag (Spectra/Por Membrane No.7, Spectrum Laboratories Inc., CA, U.S.A.), and it was dialyzed at 4 °C to perfectly remove the unencapsulated CF. Beforehand, 100 ml of PBS for dialysis fluid was placed in a screw-cap bottle and kept at the desired temperature. The dialysis bag enclosing the liposome solution was transferred to the screw-cap bottle, and the dialysis fluid was stirred with a magnetic stirrer. Aliquots of the dialysis fluid were withdrawn at appropriate time intervals, and then the fluorescence intensity was measured with a spectrofluorometer. The wavelengths of excitation and emission were 490 and 520 nm, respectively. The samples were returned to the bottle after each measurement to avoid a change in the dialysis fluid volume. After the measurement was completed, the dialysis bag was cut open in the bottle, and the total fluorescence intensity of CF was determined after the addition of a 10% Triton X-100 solution.

Results and Discussion

Phase Behavior of Liposomal Bilayer Membranes Lipid bilayer membranes are composed of the lamellar gel state and liquid-crystalline state below and above the main phase transition temperature, respectively. The phase transition temperature of phospholipid is known to depend on the length of the hydrocarbon chain, the existence of the unsaturated bond and the kind of hydrophilic group.[18,19] Thus, the phase transition temperature of the DPPC bilayer membranes containing DSPE-PEG was measured.

Figure 1 shows the DSC curves for DPPC liposomes containing DSPE-PEG. The sharp DSC peak of DPPC in the absence of DSPE-PEG was changed into a broad peak having a shoulder in the presence of DSPE-PEG. From this result, it was suggested that the addition of DSPE-PEG to DPPC bilayer membranes brings about a lateral phase separation. Bedu-Addo *et al.*[12] suggested that the occurrence of the phase separation in the PEG-liposomes due to the property of the PEG chain of the PEG-lipid, that is, the phase separation of the PEG-liposome, is generated by the PEG chain-chain entanglement due to the van der Waals forces, and also inter- and intra-chain hydrogen bonds which act in the PEG chains. Furthermore, the main phase transition temperature of DPPC bilayer membranes was shifted to a higher temperature by the addition of DSPE-PEG except for DSPE-PEG5000. The shift became significant as the mole fraction of DSPE-PEG increased.

To ascertain the lateral phase separation in the liposomal bilayer membranes suggested from the change in DSC peaks, electron microscopy combining a freeze-fracture technique was carried out. Figure 2 shows the electron micrographs of freeze-fracture replicas of DPPC liposomes both in the absence and in the presence of DSPE-PEG2000, where MLV were quenched from 37 °C or 50 °C. The freeze-fracture electron micrograph of DPPC liposomes quenched from 37 °C showed a banded texture corresponding to the $P_{\beta'}$ phase alone (Fig. 2a) and that quenched from 50 °C showed a jumbled texture corresponding to the L_α phase alone (Fig. 2b). In DPPC liposomal bilayer membranes, the pretransition and the main phase transition temperature were 35 and 41 °C, respectively, and the pretransition corresponded to the transformation from $L_{\beta'}$ to $P_{\beta'}$ and the main transition corresponded to that from $P_{\beta'}$ to L_α. Therefore, DPPC liposomes showed the single-phase structure both in the gel and liquid-crystalline states. The micrographs of DPPC liposomes con-

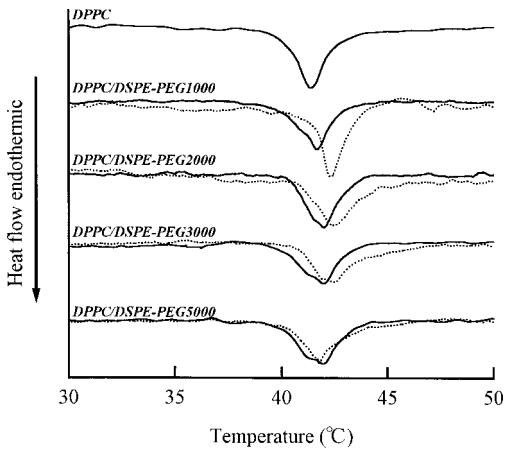

Fig. 1. Effects of DSPE-PEG on the Phase Transition Temperature of DPPC Bilayer Membranes

—: $X_{DSPE-PEG}=0.060$, ---: $X_{DSPE-PEG}=0.145$.

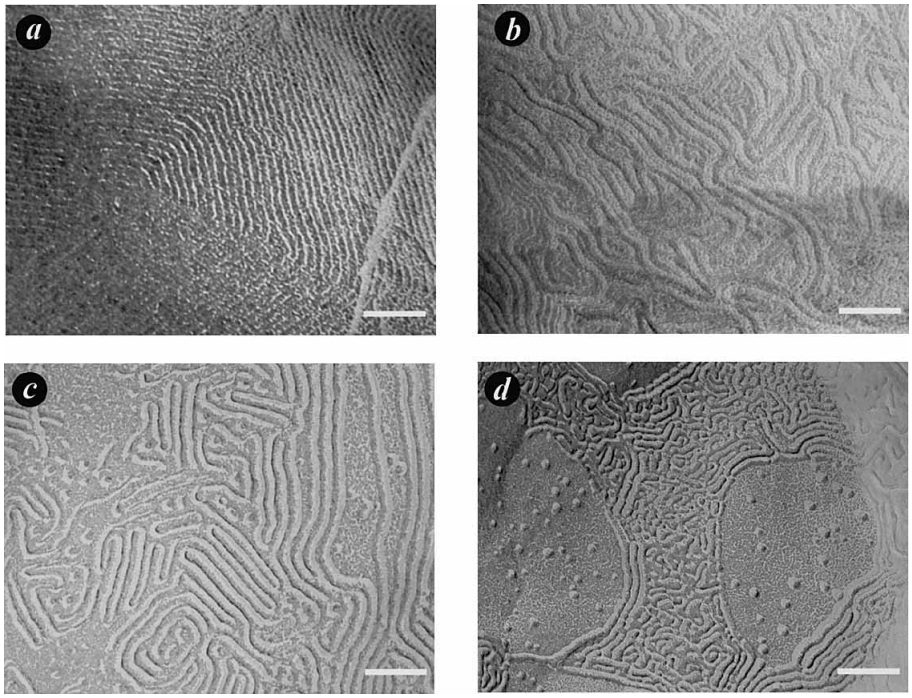

Fig. 2. Freeze-Fracture Electron Micrographs of Liposomes

(a) DPPC quenched from 37 °C, (b) DPPC quenched from 50°C, (c) DPPC/DSPE-PEG2000 quenched from 37 °C, (d) DPPC/DSPE-PEG2000 quenched from 50 °C. The mole fraction of DSPE-PEG2000 is 0.145. The length of white bar is 100 nm.

taining DSPE-PEG2000 quenched from 37 °C showed the coexistence of a planar texture in addition to the banded texture (Fig. 2c) and that quenched from 50 °C exhibited a jumbled texture and planar texture (Fig. 2d). In addition, it was observed that the form of the planar textural domain changed from an irregular shape to an elliptical shape with an increase in temperature as shown in Figs. 2c and d. The mobility of the lipid molecule in the bilayer membrane significantly increased by the change from the gel to liquid-crystalline states, and it appeared to rearrange the circular (elliptical) domains which showed thermodynamically and geometrically stable shapes. These results suggested that the addition of DSPE-PEG to DPPC liposomes caused a lateral phase separation both in the gel and liquid-crystalline states.

Fluorescence Polarization of Liposomal Bilayer Membranes The DSC measurement provides thermodynamic information of the phase changes at the phase transition temperature, while the fluorescence polarization measurement individually examines the phase conditions below and above the phase transition temperature. The fluorescent probe DPH is used to investigate changes in fluidity and packing in the hydrocarbon region of liposomal bilayer membrane, while TMA-DPH is often used to obtain information in the hydrophobic-hydrophilic interfacial region of the liposomal bilayer membrane.

The temperature dependence of fluorescence polarization values of DPH and TMA-DPH are shown in Figs. 3 and 4, respectively. The fluorescence polarization of DPH and TMA-DPH in the DPPC bilayer membranes without DSPE-PEG suddenly decreased at approximately 41 °C, which agrees with the main phase transition temperature of DPPC by the DSC measurement. The main phase transition temperature was changed by the addition of DSPE-PEG as shown in Fig. 1. However, no significant changes in fluorescence polarization of DPH for liposomal bilayer membranes was found both in the gel and liquid-crystalline states by the addition of DSPE-PEG (Fig. 3). On the other hand, the fluorescence polarization of TMA-DPH in PEG-liposomes was significantly decreased compared with that of liposomes without DSPE-PEG both in the gel and liquid-crystalline states as shown in Fig. 4. The above results show that the addition of DSPE-PEG affects not fluidity in the hydrocarbon region of the liposomal bilayer membrane but fluidity in the interfacial region of the liposomal bilayer membrane. It can be explained that the fluidity in the interfacial region of the liposomal bilayer membrane increased after the addition of the PEG-lipid because the interaction near the hydrophilic group of the DPPC molecules were weakened by the bulky PEG chain of DSPE-PEG. A similar result was reported in a recent ESR study by Belsito *et al.*[20] They reported that the addition of DPPE-PEG2000 to DPPC in the preparation of liposomes reduced the fluidity in the hydrocarbon region of liposomal bilayer membranes, while enhancing the fluidity in the interfacial region in the gel state relative to liposomes prepared without PEG-lipid. Although the result in their hydrocarbon chain region was different from the present result, it is suggested that the reason for this was the difference in the hydrocarbon chain length of the PEG-lipid used for the preparation of liposomes.

CF Leakage from the Liposomes Figure 5 shows the time-dependence of CF leakage from liposomes at 30, 37 and 50 °C as examples. The CF leakage from the liposomes at 30 and 37 °C was slight, while the CF leakage at 50 °C suddenly increased. To evaluate the CF leakage from the liposomes in further detail, the rate constant of CF leakage from liposomes was calculated according to the following equation:

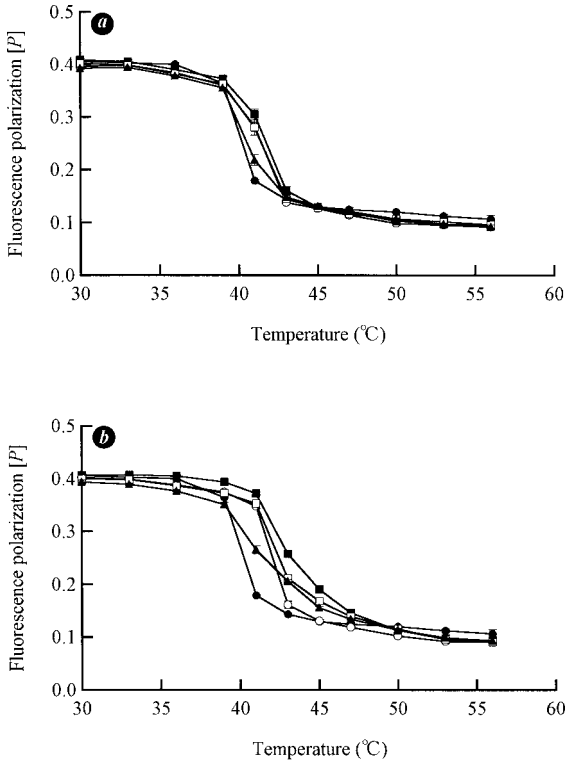

Fig. 3. Relation between Fluorescence Polarization of DPH and Temperature for DPPC Bilayer Membranes Containing DSPE-PEG

The mole fractions of DSPE-PEG are (a) 0.060 and (b) 0.145. ●: DPPC, ○: DPPC/DSPE-PEG1000, ■: DPPC/DSPE-PEG2000, □: DPPC/DSPE-PEG3000, ▲: DPPC/DSPE-PEG5000. Each point with a vertical bar represents the mean±S.E. of 3 experiments.

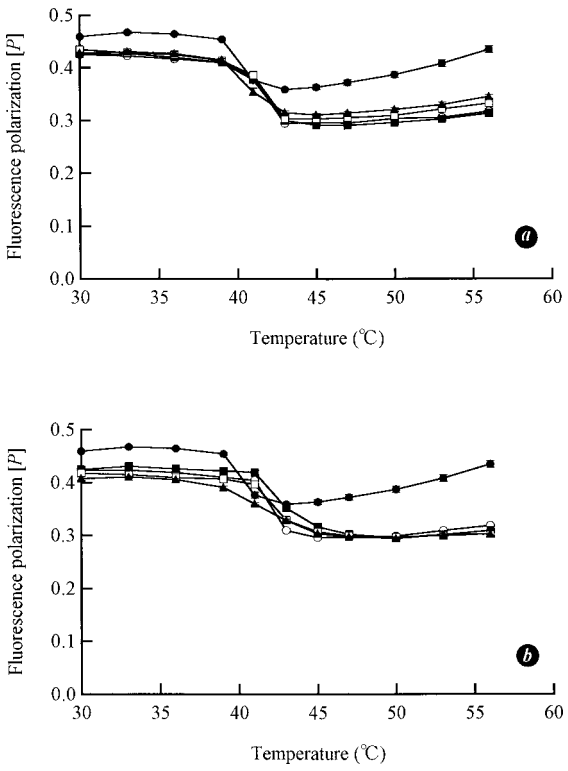

Fig. 4. Relation between Fluorescence Polarization of TMA-DPH and Temperature for DPPC Bilayer Membranes Containing DSPE-PEG

The mole fractions of DSPE-PEG are (a) 0.060 and (b) 0.145. The symbols correspond to those in Fig. 3. Each point with vertical bar represents the mean±S.E. of 3 experiments.

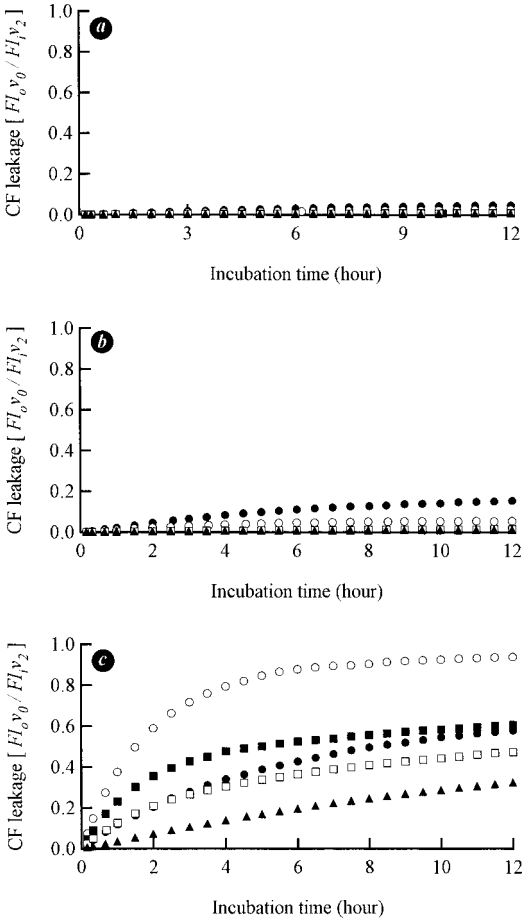

Fig. 5. Time-Dependence of CF Leakage from Liposomes Containing DSPE-PEG at 30 °C (a), 37 °C (b) and 50 °C (c)

The mole fraction of DSPE-PEG is 0.145. The symbols correspond to those in Fig. 3.

$$\frac{FI_o \cdot v_0}{FI_i \cdot v_2} = 1 - \frac{1}{K-L}\{(1-\alpha)Ke^{-Lt} - (L-\alpha K)e^{-Kt}\} \quad (2)$$

where FI_o and FI_i are the fluorescence intensities outside and inside the dialysis bag, respectively, v_0 is the total volume of the system, v_2 is the external solution (dialysis fluid) volume, K and L are the rate constants of CF leakage from the dialysis bag and liposome, respectively, α is the fraction of CF leaked out of the dialysis bag from the liposomes before the start of the experiment. K in Eq. 2 can be determined according to the following equation using a CF solution instead of the liposome solution.

$$\frac{FI_o \cdot v_0}{FI_i \cdot v_2} = 1 - e^{-Kt} \quad (3)$$

In general, when the solute leakage from the liposomes is discussed, differences in the liposomal sizes, such as surface areas and inner volumes of liposomes, should be taken into consideration.[18] In liposomes consisting of multiple lipids, it is difficult to estimate the liposomal size and the bilayer thickness in the intricate phase-separated state. Therefore, in the present study, the CF leakage is discussed in terms of the rate constant.

Figure 6 shows the plots according to Eq. 2 for CF leakage from liposomes at each temperature together with the calculated curves that gave the best fit of the experimental data,

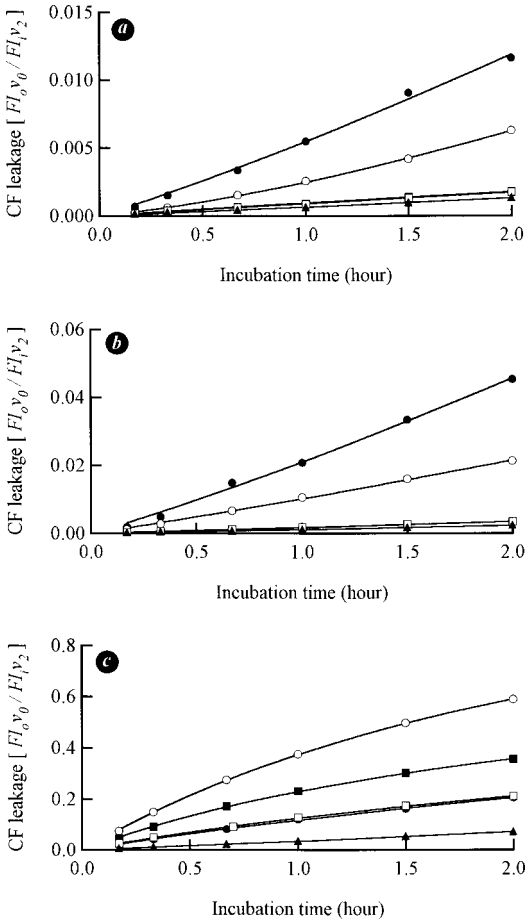

Fig. 6. Best-Fit Curves for CF Leakage from Liposomes Containing DSPE-PEG Based on Eq. 2 at 30 °C (a), 37 °C (b) and 50 °C (c)

The symbols correspond to those in Fig. 3.

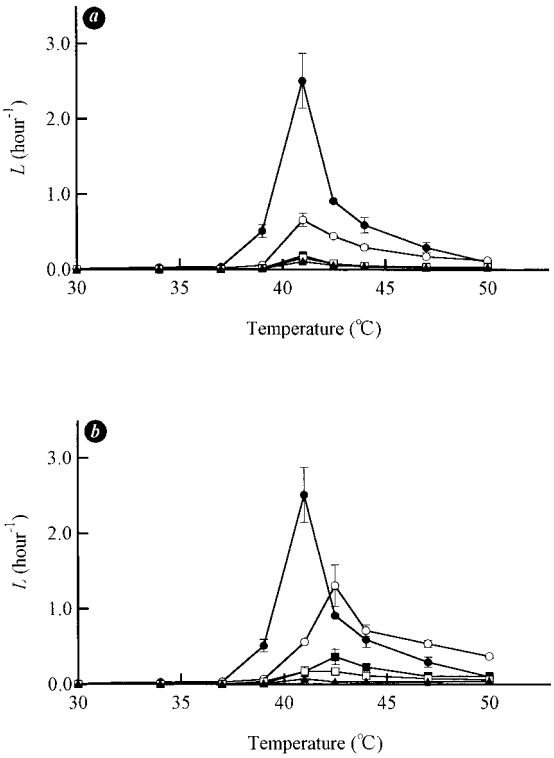

Fig. 7. Relation between Rate Constant (L) of CF Leakage from DPPC/DSPE-PEG Liposomes and Temperature

The mole fractions of DSPE-PEG are (a) 0.060 and (b) 0.145. The symbols correspond to those in Fig. 3. Each point with a vertical bar represents the mean±S.E. of 3—5 experiments.

and the resulting values of L are plotted as a function of temperature shown in Fig. 7.

Below about 37 °C, the L value for liposomes without DSPE-PEG gradually increased with an increase in the temperature. At a temperature (41 °C) corresponding to the main phase transition temperature of DPPC, the leakage of CF became significantly higher, and it rapidly decreased with increasing temperature. As shown in Fig. 7, the PEG-liposomes also showed the maximum L value near the temperatures of their main phase transition as shown in Fig. 1. The solute leakage from liposomes rapidly increases near the main phase transition temperature, and this phenomenon is responsible for the reduction of the stability of the boundary regions in liposomal bilayer membranes owing to the formation of the discontinuity of the gel phase and liquid-crystalline phase.

It was confirmed that the addition of DSPE-PEG to DPPC in the preparation of liposomes caused the phase separation both in the gel and liquid-crystalline states from the results of TEM observations and DSC measurements, moreover, the fluidity near the interfacial region of PEG-liposomes significantly increased. Consequently, it is anticipated that the leakage of CF from the liposomes is accelerated by the addition of DSPE-PEG. However, the L value of liposomes containing DSPE-PEG with a 0.060 mol fraction was smaller than that of liposomes without DSPE-PEG both in the gel and liquid-crystalline states. Therefore, it was not confirmed that the acceleration of the CF leakage was due to the phase separation. Furthermore, the L value of PEG-liposomes decreased with increasing PEG chain length of DSPE-PEG. This shows that the CF leakage is depressed by the addition of PEG-lipids. The PEG chains of DSPE-PEG are exposed from the liposomal surfaces when DSPE-PEG forms liposomal bilayer membranes with DPPC.[3] The exposed PEG chains shield the CF leakage from the liposomes, and the shielding effect of DSPE-PEG increases with increasing PEG chain length of DSPE-PEG. Therefore, in this mole fraction studied, it is suggested that the shielding effect by the PEG chain is superior to the accelerating effect by the phase separation.

The CF leakage from liposomes containing DSPE-PEG with the 0.145 mol fraction was slightly increased compared with that of liposomes containing DSPE-PEG with a 0.060 mol fraction. From this finding, it would be surmised that the phase separation in bilayer membrane is progressed by the addition of a large amount of DSPE-PEG to DPPC liposomes. Moreover, the CF leakage from PEG-liposomes increased in the liquid-crystalline state, because the boundary of the phase separation became unstable with increasing fluidity of the liposomal bilayer membrane. In contrast to the present results, Nicholas *et al.*[21] reported that the permeability coefficient of the glucose from the DPPC liposomes increased by the addition of DSPE-PEG5000. Sriwongsitanont and Ueno[22] also observed that the permeability of calcein from EggPC liposomes increased by the addition of DSPE-PEG5000. It is difficult at present to explain this disagreement, but it may be caused by differences in the experimental

methods and conditions, such as the kind of lipids and the nature of the markers.

From the above results, it is suggested that the solute leakage from PEG-liposomes originates from a balance of the accelerating effect by the phase separation of the liposomal bilayer membrane and the shielding effect by PEG chains of the liposomal surface.

Conclusions

We investigated the effects of PEG chain length of PEG-lipid on the physicochemical properties of liposomal bilayer membranes such as the phase condition and solute leakage. The electron micrographs of freeze-fracture replicas of DPPC liposomes containing DSPE-PEG showed a lateral phase separation both in the gel and liquid-crystalline states. In addition, the hydrocarbon region and the interfacial region were widely different from each other in the effect of the PEG chains on membrane fluidity. Furthermore, it was suggested that the solute permeability from the PEG-liposomes was affected not only by properties of liposomal bilayer membranes such as phase transition temperature, membrane fluidity and phase separation, but also by the PEG chain of the liposomal surface. Knowledge of the experimental findings of the present study becomes a fundamental guide in the selection of PEG-lipid for preparation of PEG-liposomes.

Acknowledgements This work was partially supported by a Research Grant for Assistants provided by Nihon University Research Grant for 2001, Japan.

References

1) Gregoriadis G., "Liposomes as Drug Carriers. Recent Trend and Progress," J. Wileyand Sons, New York, 1988.
2) Klibanov A. L., Maruyama K., Torchilin V. P., Huang L., *FEBS Lett.*, **268**, 235—237 (1990).
3) Klibanov A. L., Maruyama K., Beckerleg A. M., Torchilin V. P., Huang L., *Biochim. Biophys. Acta*, **1062**, 142—148 (1991).
4) Allen T. M., Hansen C., Martin F., Redemann C., Yau-Young A., *Biochim. Biophys. Acta*, **1066**, 29—36 (1991).
5) Woodle M. C., Lasic D. D., *Biochim. Biophys. Acta*, **1113**, 171—199 (1992).
6) Lasic D. D., Martin F. J., Gabizon A., Huang S. K., Papahadjopoulos D., *Biochim. Biophys. Acta*, **1070**, 187—192 (1991).
7) Needham D., McIntosh T. J., Lasic D. D., *Biochim. Biophys. Acta*, **1108**, 40—48 (1992).
8) Sada E., Katoh S., Terashima M., Kawahara H., Katoh M., *J. Pharm. Sci.*, **79**, 232—235 (1990).
9) Inoue K., *Biochim. Biophys. Acta*, **339**, 390—402 (1974).
10) Blok M. C., Van der Netu-Kok E. C. M., Van Deenen L. L. M., De Gier J., *Biochim. Biophys. Acta*, **406**, 187—196 (1975).
11) Kenworthy A. K., Simon S. A., McIntosh T. J., *Biophys. J.*, **68**, 1903—1920 (1995).
12) Bedu-Addo F. K., Tang P., Xu Y., Huang L., *Pherm. Res.*, **13**, 710—717 (1996).
13) Edwards K., Johnssom M., Karlsson G., Silvandar M., *Biophys. J.*, **73**, 258—266 (1997).
14) Engelke M., Bojarski P., Bloß R., Diehl H., *Biophys. Chem.*, **90**, 157—173 (2001).
15) Inoue T., Muraoka Y., Fukushima K., Shimozawa R., *Chem. Phys. Lipids*, **46**, 107—115 (1988).
16) Johnson S. M., Bangham A. D., *Biochim. Biophys. Acta*, **193**, 82—91 (1969).
17) Nikolova A. N., Jones M. N., *Biochim. Biophys. Acta*, **1304**, 120—128 (1996).
18) Nojima S., Sunamoto J., Inoue K., "The Liposomes," Nankodo, Tokyo, 1988.
19) Yamauchi H., Shimazaki I., Hashizaki K., Sakai H., Yokoyama S., Abe M., *J. Jpn. Oil Chem. Soc.*, **48**, 25—32 (1999).
20) Belsito S., Bartucci R., Sportelli L., *Biophys. Chem.*, **75**, 33—43 (1998).
21) Nicholas A. R., Scott M. J., Kennedy N. I., Jones M. N., *Biochim. Biophys. Acta*, **1463**, 167—178 (2000).
22) Sriwongsitanont S., Ueno M., *Chem. Pharm. Bull.*, **50**, 1238—1244 (2002).