

Physicochemical Properties of PEG-Grafted Liposomes

Supaporn SRIWONGSITANONT and Masaharu UENO*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930-0194, Japan. Received April 19, 2002; accepted June 15, 2002

Egg phosphatidylcholine (EggPC) or dimyristoylphosphatidylcholine (DMPC) liposomes containing polyethylene glycol (PEG)-lipids covering a range of 0–30 mol% have been prepared by Extrusion method. The physicochemical properties including size evolution and calcein permeation were evaluated to investigate the effect of PEG-lipids on bilayer structure. The results from quasielastic light scattering (QELS), freeze-fracture microscopy, and gel exclusion chromatography revealed that presence of low concentration of PEG-lipid results in decreasing of vesicle size and further increase in the PEG-lipid concentrations lead to a transition from the lamellar membranes to micelles. The permeability for calcein increased with increase in concentration of distearoylphosphatidylethanolamine (DSPE)-PEG. On the other hand, the permeability decreased with low amount of cholesterol-PEG (below 20% cholesterol-PEG) and increased with high amount of it. The maximum concentration of PEG-lipid that may be incorporated without alteration of the liposome structure depends on the composition of the bilayer. The concentration of DSPE-PEG2000 incorporated into vesicles without damaging vesicle structures were <20 mol% for EggPC and <10% for DMPC.

Key words liposome; PEG-lipid; gel exclusion chromatography; quasielastic light scattering; freeze-fracture microscopy; permeability

After the discovery of liposomes, their application for drug delivery was expected as described in many reviews.^{1–5)} Liposomes were used as vehicles for drug delivery because of their biocompatibility and the possibility of incorporating both water-soluble and hydrophobic materials. However, liposomes possess a big disadvantage like other colloidal particles that is after systemic application, liposomes are usually rapidly cleared from the circulation by the reticuloendothelial system (RES), accumulating mostly in the liver and spleen within a few minutes or a few hours.^{6–9)} In order to overcome above disadvantages, many attempts have been made. One venture is to use liposomes as a material for chemoembolization therapy against cancer instead of conventional drug carrier in blood.^{10–13)} In this method liposomes work to cut off blood stream near cancer and locally release anti-cancer drug without drug diffusion to whole body. Another method is to modify liposomes to avoid RES. In 1987, Allen and coworkers reported that the inclusion of monosialoganglioside G_{M1} in the bilayer can reduce considerably the rate and extent of uptake of liposomes into the RES.¹⁴⁾ More recently, incorporation of polyethylene glycols (PEG) covalently bonded to the polar headgroup of phospholipid, such as distearoylphosphatidylethanolamine-PEG (DSPE-PEG), has been shown to markedly increase their blood circulation time,^{15–19)} and the activity of PEG-lipid to prolong the circulation time was greater than that of G_{M1} on the molar basis.¹⁵⁾ The particular efficiency of surface-bonded PEG chains has been explained by the steric repulsive barrier around the liposomes provided by the covalently bonded PEG.^{18,20–22)} Both the range and magnitude of the steric barrier are strong functions of PEG-lipid concentration and PEG-size, and comparably high PEG-lipid concentrations are required for efficient steric barrier formation.²³⁾ The effects of PEG-PE on structure and phase behavior in systems containing liposomes with varying bilayer compositions have been investigated using such as X-ray and NMR.^{24–27)}

In present study we use gel exclusion chromatography, quasielastic light scattering, and freeze-fracture electron mi-

croscopy to investigate the effect of PEG-lipids on the bilayer structure and the amount of DSPE-PEG that can be incorporated without appreciably modifying the bilayer structure. Gel exclusion chromatography is an inexpensive, quick, and convenient method for the fractionation and size analysis of liposomes.^{28,29)} The permeability of the vesicles containing various concentration of PEG-lipids was also investigated by measuring the release of calcein from the vesicle.

We focus on PEG-lipid possessing different molecular weight (900, 2000, 5000). These particular PEG-lipids, which were covalently bonded to cholesterol or DSPE, have previously been used in studies to determine the effects of polymer lipids on the circulation time of liposomes.^{17,20)} In our study we have used Egg phosphatidylcholine (EggPC) and dimyristoylphosphatidylcholine (DMPC) as a host lipid in liposomes.

Experimental

Materials Egg yolk lecithin (EggPC; purity of PC=98.8%), Dimyristoylphosphatidylcholine (DMPC; purity of PC=99.8%), DSPE-PEG2000 and DSPE-PEG5000 were purchased from Nihon Yushi (Tokyo, Japan). Cholesterol-PEG900 was purchased from Fluka Chemie AG (Buchs, Switzerland). Calcein and Tris-hydroxymethyl aminomethane (Tris) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other commercially available reagents used were of analytical grade.

Methods. Preparation of Liposomes A known composition of phospholipids (EggPC or DMPC) and PEG-lipids (cholesterol-PEG900, DSPE-PEG2000 or DSPE-PEG5000) was dissolved together in chloroform and methanol mixture (2:1, v/v). The solvent was removed under a stream of nitrogen. The dry lipid mixture was then evaporated under reduced pressure for more than 6 h to yield a thin film. The film was subsequently dispersed in 20 mM Tris buffer (150 mM NaCl, 20 mM Tris, pH 7.4). The lipid mixtures were subjected to freeze-thaw cycles (freezing in liquid nitrogen and thawing in warm (40 °C) water) five times. Large unilamellar liposomes were prepared by extruding the lipid mixture through two stacked 600 nm-defined and 200 nm-defined pore polycarbonate filters (Nucleopore, Costar Co., U.S.A.) 10 times using an extruder (Lipex Biomembranes Inc.). The extrudings were performed at room temperature for Egg PC liposomes and at 35 °C for DMPC liposomes.

Freeze-Fracture Electron Microscopy Samples were rapidly frozen at liquid nitrogen temperature (–196 °C). The samples were fractured at –120 °C with a Freeze replica apparatus (FR-7000B, Hitachi, Japan). After fracturing the sample, an electric discharge was applied to deposit Pt/C, then

* To whom correspondence should be addressed. e-mail: mueno@ms.toyama-mpu.ac.jp

C on the surface of the fractured samples at an angle of 45° and 90°. The replicas were removed from holders by submersion in solution of commercial bleaches and distilled water. The cleaned replicas were mounted on 300-mesh Ni grids, dried, and examined with an electron microscope (JEM200 CX, JEOL, Tokyo, Japan).

Gel Exclusion Chromatography The vesicle suspensions were prepared in 20 μ M of calcein (a low molecular weight fluorescent probe) solution by extrusion and then separated by gel exclusion chromatography through a Sephacryl® S-1000 Superfine (Pharmacia Biotech, Sweden) column (48 \times 1 cm). The sample suspensions were passed through the column, whose gel was presaturated with the phospholipids.

Determination of Phospholipid Concentration Total phospholipid concentration (phosphatidylcholine+PEG lipid) was determined as phosphorous based on the method of Ames.³⁰⁾

Calcein Permeation The measurement of calcein permeation from vesicles was carried out at 25 °C in 100 mM calcein/20 mM Tris buffer (pH 7.4; 388 mOsm), in which the fluorescence intensity of calcein is self-quenched. The vesicles were separated from untrapped calcein by gel permeation chromatography using Sephadex G-75 gel (0.5 \times 10 cm column) equilibrated with an isotonic buffer, glucose/20 mM Tris buffer (pH 7.4). The separated vesicles were rapidly diluted (1:200) into the well-stirred isotonic buffer. Calcein permeation was detected as an increase in fluorescence intensity.

RESULTS

Particle Sizes The mean diameters of the vesicles containing various concentrations of PEG-lipids were determined. Figure 1 shows the variation of the mean diameter with PEG-lipid concentration for the aggregate containing either EggPC (Fig. 1a) or DMPC (Fig. 1b). As shown in Fig. 1b, the particle sizes of vesicles decrease with increasing

PEG-lipid content. The particle size of DMPC liposomes containing DSPE-PEG2000 decreases significantly when DSPE-PEG2000 content increases. The mean diameter of vesicles was 179 nm for DMPC vesicles without DSPE-PEG2000 and 94 nm for DMPC with 30 mol% DSPE-PEG2000. Samples containing DSPE-PEG5000 or cholesterol-PEG900 exhibit a similar behavior, but beyond 10 mol% of DSPE-PEG5000 or cholesterol-PEG900 concentration, the diameter decrease very slightly. Decrease in size with increasing PEG-lipid concentration is also observed in EggPC liposomes containing DSPE-PEG2000, as shown in Fig. 1a. Nevertheless, amount of decrease in diameter of EggPC/DSPE-PEG2000 liposomes is lower than that of DMPC/DSPE-PEG2000 liposomes. The mean diameters decrease from 197 nm (0 mol% DSPE-PEG2000) to 150 nm when 30 mol% of DSPE-PEG2000 was added. For Egg PC containing DSPE-PEG5000 or cholesterol-PEG900, liposomal diameters are nearly constant.

Freeze-Fracture Electron Microscope Structural features of DMPC/DSPE-PEG2000 vesicles were revealed by freeze-fracture electron microscope. Figure 2 shows freeze-fracture electron micrographs of DMPC/DSPE-PEG2000 LUV. Figure 2a shows freeze-fracture electron micrograph of DMPC LUV with 5 mol% of DSPE-PEG2000. Spheroidal unilamellar vesicles with diameter mainly in the range from 130 to 170 nm are observed. Increasing DSPE-PEG2000 concentration to 30 mol%, vesicle structures almost disappear. The irregular structures are observed (Fig. 2b).

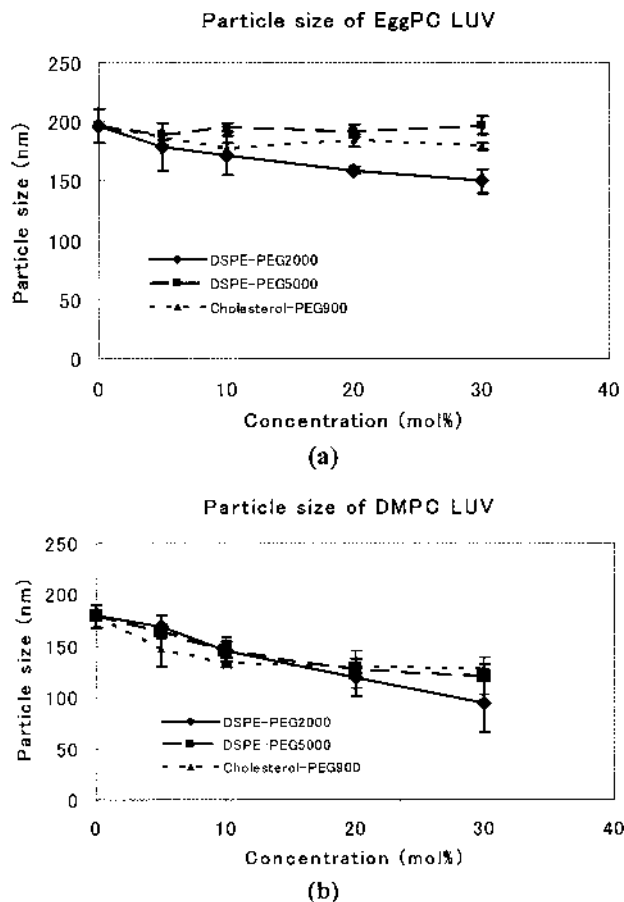


Fig. 1. The Diameter of the (a) EggPC LUVs and (b) DMPC LUVs as a Function of PEG-Lipid Concentration

◆, DSPE-PEG2000; ■, DSPE-PEG5000; ▲, cholesterol-PEG900. The values are mean \pm S.D. of three different experiments.

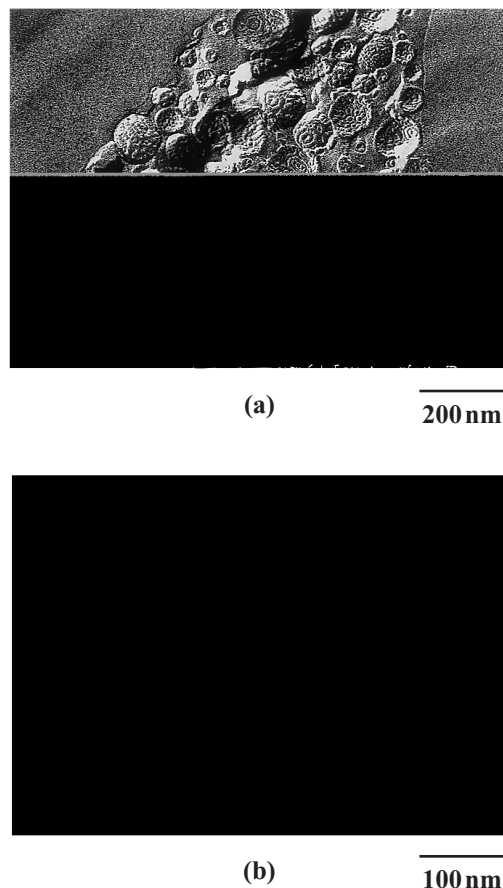


Fig. 2. Freeze-Fracture Electron Micrographs of DMPC/DSPE-PEG2000 LUV at DSPE-PEG2000 Concentration of 5% (a), and 30% (b)

Figure 3 shows freeze-fracture electron micrograph of EggPC LUV containing 30% DSPE-PEG2000. This micrograph reveals that vesicles with diameter ranging from 70 to 120 nm are still present.

Gel Chromatography The chromatograms (fluorescence intensity and phospholipid concentration *versus* elution volume (V_e) curve) for Egg PC and DMPC are shown in Figs. 4 and 5, respectively. The figures are representatives of several experiments. Fluorescence detection curve has revealed two elution peaks. The first peak position coincides with that of the chromatogram of phospholipid concentration, so, this corresponds to the calcein-containing vesicles. The second peak is attributed to free calcein molecules.

As shown in Figs. 5d and e the intensity of the first peak for DMPC vesicles dramatically decrease when the concentration of DSPE-PEG is greater than 20%. The elution patterns indicate that the fluorescence intensities are very weak, whereas phospholipid concentrations are high. This suggests that addition of DSPE-PEG2000 more than 20% to DMPC



100 nm

Fig. 3. Freeze-Fracture Electron Micrograph of EggPC/30 mol% DSPE-PEG2000 LUV

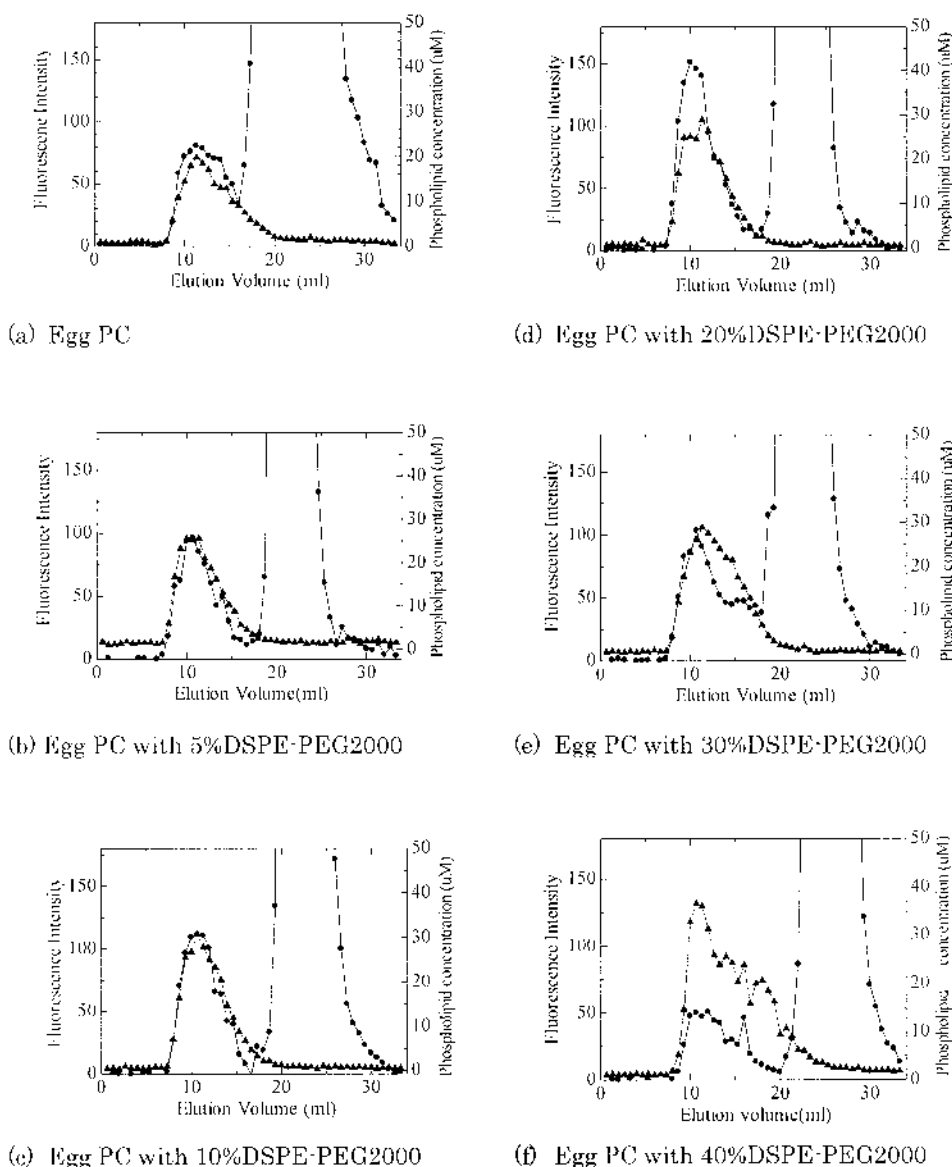


Fig. 4. Gel Exclusion Profiles of EggPC Vesicles at DSPE-PEG2000 Concentration of 0% (a), 5% (b), 10% (c), 20% (d), 30% (e), and 40% (f) —●—, fluorescence intensity; —▲—, phospholipid concentration.

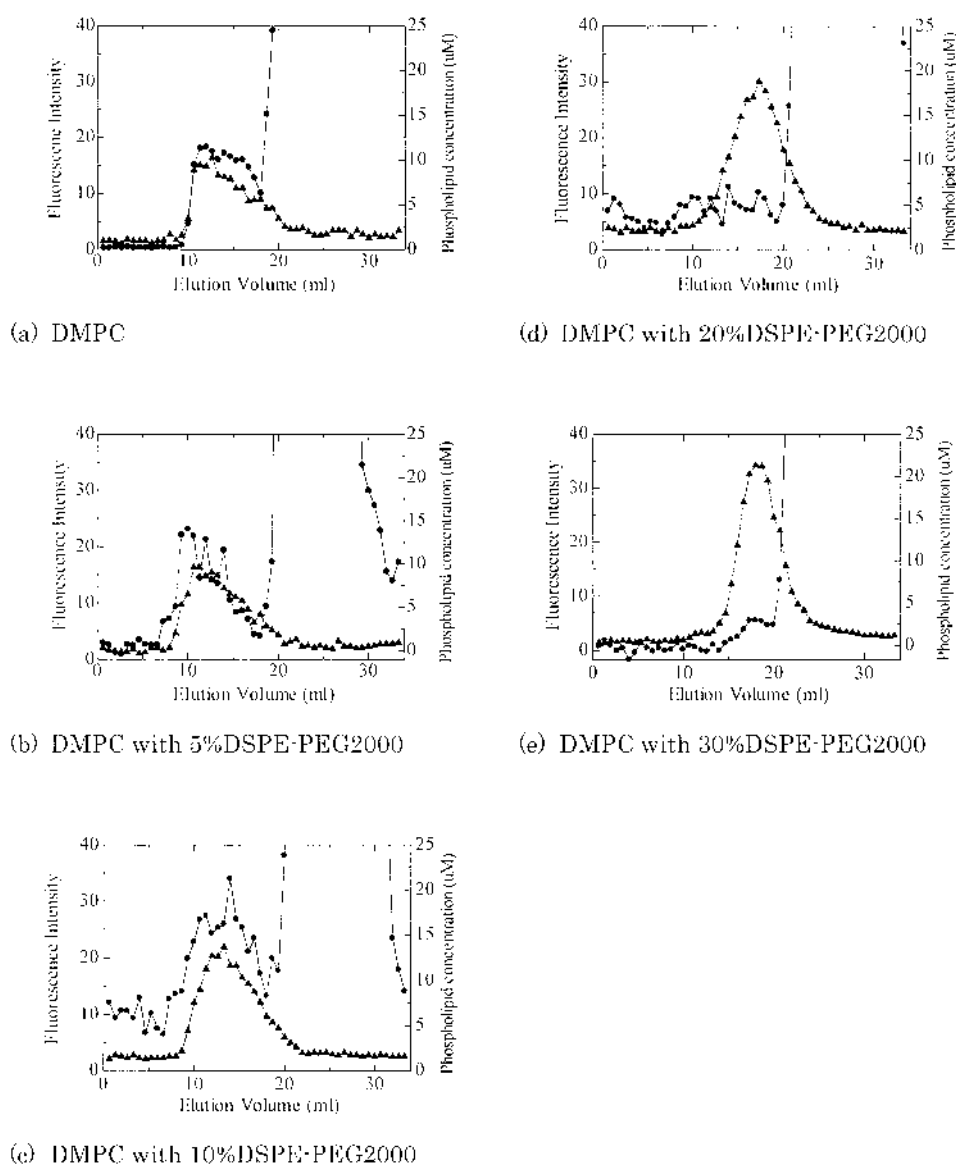


Fig. 5. Gel Exclusion Profiles of DMPC Vesicles at DSPE-PEG2000 Concentration of 0% (a), 5% (b), 10% (c), 20% (d), and 30% (e) —●—, fluorescence intensity; —▲—, phospholipid concentration.

vesicles cause transition from vesicles to micelle. At these concentrations the small vesicles coexist with the micelles. These results are in accordance with freeze-fracture electron micrograph (Fig. 2b) that demonstrates the decrease in amount of vesicles and increase in the formation of irregular structures.

Fig. 6a shows the elution profiles of phospholipid concentration of EggPC vesicles obtained as a function of DSPE-PEG2000 concentration. The maxima of the chromatograms vary in narrow interval, but when the concentration of DSPE-PEG2000 is 30 mol%, the elution profile displays a shoulder ($V_e=13.3$ – 14.6 ml). The sample profile containing 40% DSPE-PEG2000 displays a similar pattern (Fig. 4f). At $V_e=13.3$ – 14.6 ml, chromatogram of phospholipid concentration and fluorescence intensity posses sharp peaks which corresponding to small particle. Nevertheless, at this concentration the elution profile of phospholipid concentration shows the additional peak locating at the region of very small particles ($V_e=16.6$ – 20.0 ml).

The elution profiles of DMPC vesicles are shown in Fig. 6b. The maxima of the chromatograms for high concentration of DSPE-PEG2000 (20–30 mol%) are located in 17.3–18.0 ml. These values are markedly greater than those found for DMPC vesicles at low DSPE-PEG2000 concentration (5–10 mol%) varying in a narrow interval ($V_e=12.6$ – 13.3 ml). This significant shift of their gel chromatography peaks toward the elution region of small particles agrees with the vesicles sizes (Fig. 1) and freeze-fracture electron micrographs (Fig. 2) showing that the progressive decrease in size with increasing PEG-lipid molar ratio.

Calcein Permeation The permeability of vesicles was determined from the release of calcein from the vesicles as expressed by %calcein retention in the vesicles. Figure 7 depicts the calcein permeation of EggPC containing various concentrations of PEG-lipids. As shown in the figures, permeability of calcein increases with increasing DSPE-PEG content. Addition of cholesterol-PEG900 exhibits the different result. As can be seen from Fig. 7c, the permeability of

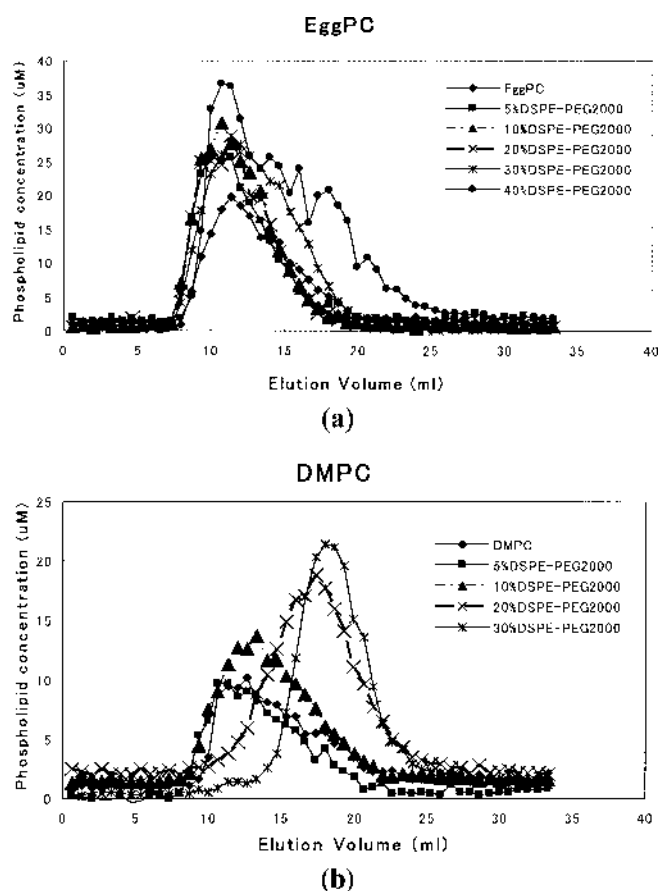


Fig. 6. Elution Profiles of EggPC (a) and DMPC (b) with Various Concentration of DSPE-PEG2000

◆, 0%; ■, 5%; ▲, 10%; ×, 20%; *, 30%; ●, 40%.

the vesicles containing 5–20% cholesterol-PEG is a little lower than cholesterol-PEG-free vesicles, but when increasing the concentration to 30%, permeability of the vesicles increases significantly.

Discussion and Conclusion

Size Evolution The results obtained in this study are applied to the phase behavior and the change in physicochemical properties of PC/PEG-lipid vesicles as a function of polymer concentration. As shown in Fig. 1, the presence of low concentration of PEG-lipid results in a decrease in vesicular sizes. Further increase in the PEG-lipid content leads to a transition from the lamellar membranes to micelles (Fig. 4f, Figs. 5d, e). Vesicles and micelles can be discriminated on the basis of calcein-entrapping ability in addition to their shape and size. DMPC vesicles provide the same sequence of morphological transitions as that observed with EggPC vesicles. Nevertheless, the concentration region of micelles was shifted toward lower PEG-lipid concentrations. Below 20 mol% for EggPC/DSPE-PEG2000 and 10 mol% for DMPC/DSPE-PEG2000 systems, only vesicles are formed, which are shown by the absence of micelles on gel chromatography profiles (Figs. 4a–d, Figs. 5a–c) and by the presence of only vesicle structures in the freeze-fracture electron micrographs (Fig. 2a). In the case of EggPC vesicles, when DSPE-PEG2000 concentration increased up to 30 mol%, the elution profile of phospholipid concentration dis-

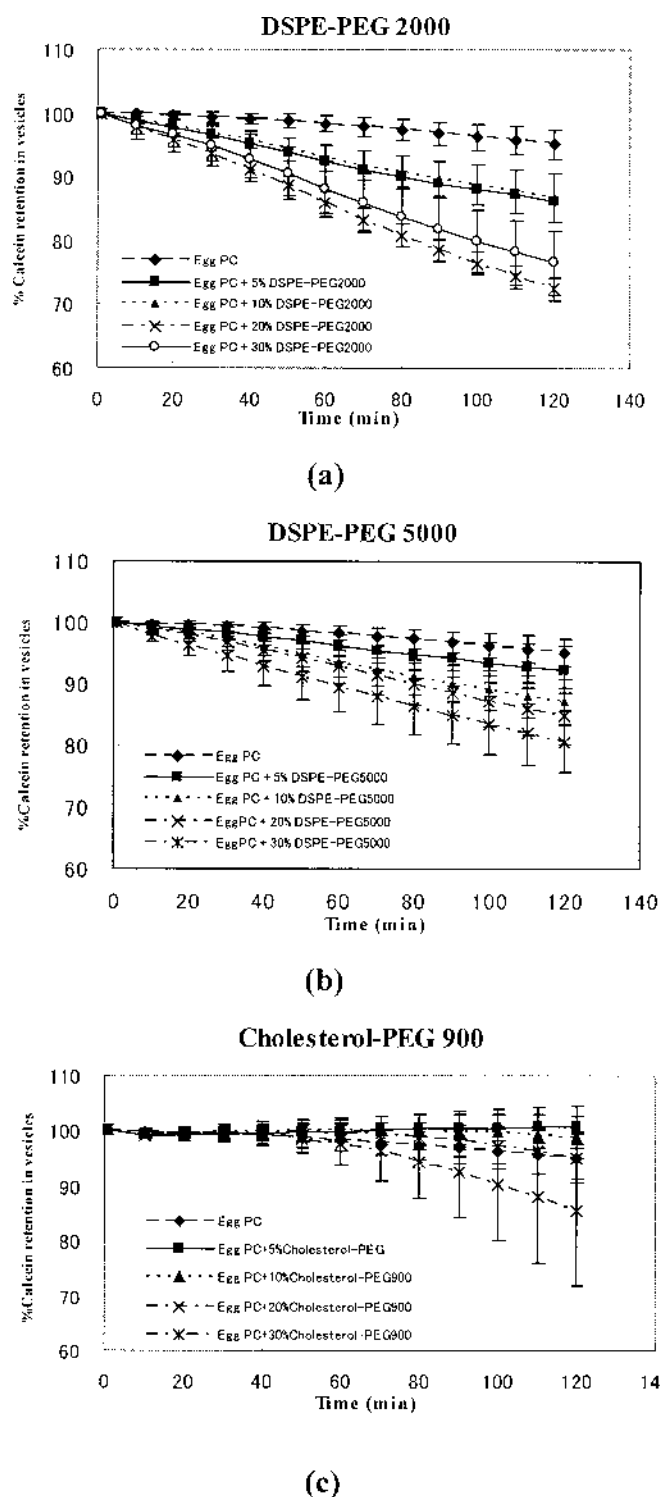


Fig. 7. %Calcein Retention in Vesicles vs. Time Plots of EggPC Vesicles Containing DSPE-PEG2000 (a), DSPE-PEG5000 (b), and Cholesterol-PEG 900 (c)

◆, 0%; ■, 5%; ▲, 10%; ×, 20%; *, 30%. The values are mean \pm S.D. of 3–5 different experiments.

plays a shoulder. The elution pattern of fluorescence intensity (Fig. 4e) shows the peak at the same position of this shoulder region ($V_e = 13.3$ – 14.6 ml). This implies that at 30% DSPE-PEG2000 small vesicles coexist with usual ones. The sample containing 40% DSPE-PEG2000 displays a similar pattern (Fig. 5f). At $V_e = 13.3$ – 14.6 ml, chromatogram of phospho-

lipid concentration and fluorescence intensity possess sharp peaks which correspond to small particles. Nevertheless, at this concentration the elution profile of phospholipid concentration shows the additional peak locating at the region of very small particles ($V_e = 16.6\text{--}20.0\text{ ml}$). This additional peak is clearly a micelle pattern because of its small size and no calcein entrapment. That is sample containing 40% DSPE-PEG2000 exhibits coexistence of large vesicles, small vesicles and micelles. As depicted by very weak fluorescence intensities in gel chromatography profiles (Figs. 5d, e) and freeze-fracture micrographs (Fig. 2b), in the case of DMPC systems containing more than 20% DSPE-PEG2000, the amount of vesicle structures decrease remarkably and that of micelle structures are observed. From our present experiments the micelle structures are observed when DSPE-PEG2000 concentration is greater than 40 mol% for EggPC and 20 mol% for DMPC systems similar to the results of Kenworthy *et al.* and Lasic *et al.*^{24,31)} with a little difference. A possible reason for this difference may result from a consequence of difference in the kind of phospholipid and PEG-lipids.

At the concentration that we used in the present study the PEG chains have the "brush" conformation.^{32,33)} Further increasing the concentration of PEG-lipid improves the repulsive properties of the surfaces of lipid bilayers.^{25,26)} In order to reduce these repulsion, curvature of vesicular surface increase and then sizes of vesicles decrease.^{34,35)} Increase in curvature of the grafting surfaces would reduce the lateral tension in polymer layers, so, higher curvature produce greater relaxation. Therefore, increase in the concentrations of PEG-lipids cause a decrease in sizes of the vesicles. When the concentration of PEG-lipid further increases, solubilization of PEG-lipid-saturated vesicles occurs. A mixture of PEG-lipid-saturated bilayers and phospholipid-saturated mixed micelles exists until enough PEG-lipid is added to convert all the bilayers into mixed micelles.^{36–38)}

EggPC is a phospholipid extracted from egg yolk. Its hydrocarbon chains have different lengths and different degrees of unsaturation, whereas DMPC is a phosphatidylcholine that have a single kind of saturated hydrocarbon chain (C14:0). PEG-lipids possess large hydrophilic head groups. When they are introduced into the bilayers, the destabilizing effect on PEG-lipid-phospholipid mixed vesicles occur. EggPC consist of fatty acid with different chain lengths, therefore when PEG-lipids are incorporated into EggPC bilayers, it can accommodated itself to retain their bilayer structures. Fatty acid chain length is also the important factor that determines the stability of vesicles. Moreover, our experiments were carried out at room temperature, where EggPC is liquid crystalline. In other words, the coexistence of gel and liquid crystalline phases resulting in decreased cohesive forces in bilayer are presented for liposomes made of DMPC, because the phase transition temperature of DMPC is about 24 °C. Because of these results, the concentration of PEG-lipids that can be incorporated in EggPC vesicles is higher than that in DMPC vesicles.

To investigate why the particle sizes of sample are less than 200 nm notwithstanding sample was extruded through 200 nm membrane, we compare the particle sizes of the sample after vortexing and freeze-thawing during their preparation and on final preparation that sample was extruded

through 200 nm polycarbonate filter (data not shown). Both of the MLVs after vortexing and MLVs after freeze-thawing decrease in size as the DSPE-PEG2000 content is increased. For DMPC liposome containing more than 10% DSPE-PEG2000 the diameter of freeze-thawed sample were sufficiently small to be directly pass through polycarbonate membranes with pore size 200 nm. These results denote that the diameter of DMPC liposomes with high PEG-lipids concentration is smaller than 200 nm before they were extruded through 200 nm filter.

Calcein Permeation The permeability of calcein is dependent on the concentration of DSPE-PEG. The permeability increases with concentration of DSPE-PEG. Nicholas *et al.*³⁹⁾ reported that the permeability coefficients for D-glucose increase with mol% DPPE-PEG5000, which was in accordance with our results. Otherwise, permeability decreases slightly with an increase in concentration up to 20% cholesterol-PEG900. The permeability increases beyond this concentration. We suggest that slight decrease of membrane permeability in low concentration of cholesterol-PEG results from the dominated effect of cholesterol. It is well known that inclusion of cholesterol in liposomal membranes reduces the permeability of the membranes to water-soluble molecules.

In conclusion, present study clearly demonstrated that PEG-lipids had remarkable effects on the physicochemical properties of phospholipid membranes, such as, sizes and membrane permeation. The concentration of DSPE-PEG2000 incorporated into vesicles without damaging vesicle structures were <20% for EggPC and <10% for DMPC. Moreover, the effect on membrane barrier efficiency is different with each other between DSPE-PEG and cholesterol-PEG. These physicochemical insights, therefore, PEG-lipids concentration and appropriate PEG derivatives has to be chosen with the greatest care, to optimize the aggregate morphology and properties, as well as the coating thickness responsible for the particle steric repulsion. Our experiments developed here may give useful information for formulation of stable liposomes.

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