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Effect of PEG lipid on fusion and fission of phospholipid vesicles in the process of freeze-thawing

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Abstract

The effects of freeze-thawing on the sizes and size distribution of egg yolk phosphatidylcholine (EggPC) liposomes in the presence of 0–40 mol% distearoylphosphatidylethanolamine–polyethyleneglycol 2000 (DSPE-PEG2000) prepared by detergent removal method were studied by quasielastic light scattering (QELS), gel exclusion chromatography, and freeze-fracture electron microscope. Especially, gel exclusion chromatography successfully provided the size distribution of polydisperse vesicle suspension. The mean diameters of the liposomes, which had originally large size, decreased with increase in the number of freeze-thawing cycles. On the contrary, the mean diameters of the liposomes, which had originally small size, increased with increase in the numbers of freeze-thawing cycles. After freeze-thawing over 10 times, the liposomal mean diameters fell into a range from 80 to 150 nm in spite of their original size. Gel exclusion chromatography showed that in the process of freeze-thawing of the liposomes containing DSPE-PEG 2000, two opponent events, one is fission and the other is fusion, occurred at the same time.

Keywords: Liposome; DSPE-PEG; Gel exclusion chromatography

1. Introduction

The application of conventional liposomes in a drug delivery is drastically limited by their rapid detection and removal from the blood by the recticuloendothelial system (RES) [1–5]. The rate of clearance from the blood varies widely depending on a variety of parameters such as particle size, surface charge, lipid composition, and membrane fluidity [3,6–12]. The half-lives of liposomes decrease with increasing diameters, negative surface charge densities and fluidity. In order to overcome above disadvantage, many attempts have been made. One venture is to use liposomes as a material for chemoembolization therapy against cancer [13]. The other is to use liposomes as a material of vaccine [14]. These attempts are topical use of liposomes instead of conventional drug carrier in blood.

It is well known that the incorporation of a lipid derivatized with a hydrophilic polymer, polyethylene glycol (PEG), into liposome bilayers results in a prolongation of blood circulation times of liposomes [15–18]. Furthermore, it was shown that clearance of PEG-lipid-grafted liposomes is less sensitive to liposome diameter than that of conventional liposome [10,18,19]. The application of these PEG-lipids liposomes for drug delivery system was envisioned as described in many reviews and papers [15–17,20–31].

The methods of preparation of liposomes with a surface coating with PEG are similar to those of conventional liposomes. PEG-lipid derivatives were added to the lipid mixture, then liposomes have been prepared with the known techniques such as hydration method, extrusion method, sonication method and detergent removal method [32]. It has been widely demonstrated that a number of liposome preparation methods have a freeze-thawing step as an important part. The effects of freeze-thawing on phospholipids suspensions under a variety of condition have been investigated. Repetitive freeze-thawing cycles are often applied to multilamellar vesicles (MLV) in order to homogenize their lipid composition [33]. Freeze-thawing

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procedure also enhances trapping efficiency [34–36] and possibility leading to the formation of unilamellar liposome more readily [35,37].

Additionally, the freezing-thawing procedure leads to the change in the sizes of liposomes. Freeze-thawing was demonstrated to cause fusion of ultrasonicated small unilamellar vesicles (SUV) [38,39]. However, it has been demonstrated that repeated freeze-thawing does not have the same effect for all lipid mixtures. Contrary to the fusion of SUV, the freeze-thawing procedure lead to fragmentation of MLV into smaller vesicles in the presence of electrolytes in the case of vesicle suspension prepared from dioleylphosphatidylcholine (DOPC) [39,40]. Whilst the effects of freeze-thawing on the physiochemical properties of conventional liposomes has been extensively studied as described above, little has been published about the effect of freeze-thawing on the liposome incorporated with PEG-lipids.

The influences of polymer-grafted lipids on the physical properties of lipid bilayers have been studied as described in many literatures [41–46]. In a previous study, we have reported that increasing in the content of polyethylene glycol-phosphatidylethanolamine (PEG–PE) resulted in decreasing in the mean diameters of the vesicles prepared by extrusion method [41]. In the present study, we investigated the effect of freeze-thawing on the sizes and size distribution of liposomes containing various concentrations (0–40 mol%) of distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG2000) prepared by detergent-removal method. We here demonstrated the alteration of size and size distribution of EggPC/DSPE-PEG2000 liposomes upon the process of freeze-thawing. To evaluate the size and the size distribution of the vesicles before and after freeze-thawing, dynamic light scattering is an extremely rapid and simple method to analyze. However, disadvantage to this technique is that it only yields the average size of the liposome. If the liposome preparations are heterogeneous systems exhibiting bimodal or more complex size distributions, the misleading results can be obtained [47]. To provide the more information about the particle size and size distribution of liposome, gel exclusion chromatography and freeze-fracture electron microscopy is also performed. To examine how the preparation procedure and the initial mean diameter affects the alteration of EggPC/DSPE-PEG2000 liposomes upon the freeze-thawing process, EggPC liposomes, varying in initial size, prepared by two other methods, which are extrusion method and sonication method, were also investigated.

It has been found that in the presence of DSPE-PEG2000, the freeze-thawing process lead to the formation of large liposomes which have diameters 200–600 nm. Formation of these large vesicles depends on the number of freeze-thawing cycles and the concentration of DSPE-PEG2000 incorporated in the vesicles. The number of large vesicles increased as a function of both freeze-thawing cycles and the concentration of DSPE-PEG2000.

2. Materials and methods

2.1. Materials

Egg yolk lecithin (EggPC; purity of PC = 98.8%), DSPE-PEG2000 were purchased from Nihon Yushi (Tokyo, Japan) and were used without further purification. *n*-Octyl-β-D-glucopyranoside, calcein and Tris–hydroxymethylaminomethane (Tris) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other commercially available reagents used were of analytical grade.

2.2. Preparation of liposomes by the detergent-removal method

EggPC liposomes containing 0–40 mol% of DSPE-PEG2000 were prepared in 20 mM Tris buffer (150 mM NaCl, 20 mM Tris, pH 7.4) by detergent-removal method previously reported [48]. Briefly, lipid mixtures were dissolved in chloroform–methanol (2:1, v/v), the solvent was evaporated under a stream of nitrogen gas and resulting lipid films were stored under vacuum for at least 6 h to remove a trace of residual solvent. The dried lipid film was hydrated in 1 ml of an aqueous solution of *n*-octyl-β-D-glucopyranoside in 20 mM Tris buffer (150 mM NaCl, 20 mM Tris, pH 7.4) to give a final phospholipids concentration of 10 mM and vigorously vortexed to produce mixed micelle solution. One ml of mixed micelle solution was dialyzed against 1 l of surfactant-free aqueous buffer for 6 h, then dialyzed again for 24 h using a new 1 l of surfactant-free buffer. In the case of measurement of the amount of micelle in EggPC/DSPE-PEG2000 suspension, a buffer solution (300 mM Sucrose, 20 mM Tris, pH 7.4) instead of the buffer solution (150 mM NaCl, 20 mM Tris, pH 7.4) was used to prepare vesicles.

2.3. Preparation of liposomes by extrusion method

Lipid films of EggPC (10 μmol) and DSPE-PEG (0–40 mol%) mixtures were prepared by the same procedure as previously described. A thin lipid film was hydrated with 1 ml of 150 mM NaCl, 20 mM Tris buffer. The resulting multilamellar vesicles were frozen (liquid nitrogen, −196 °C) and thawed (water-bath, 40 °C) five times, then extruded 10 times through double-stacked polycarbonate membranes (Nucleopore®, Costar Co., USA) with decreasing pore sizes (600, 200, 100 and 50 nm) with an extruder (Lipex Biomembranes Inc.).

2.4. Preparation of liposomes by sonication method

A thin lipid film of EggPC was hydrated in 150 mM NaCl, 20 mM Tris buffer (pH 7.4). The small unilamellar vesicles (SUV) suspension was prepared by sonication using a probe-type sonicator (US-600TS, Nissei, Japan). The multilamellar vesicle suspension was ultrasonicated three times for 20 min, with a rest time of 10 min, at an ice-bath temperature. Titanium fragments and multilamellar aggregates were removed by centrifugation at 100 000*g* for 60 min at 4 °C, then diluted to 10 mM by the buffer.

2.5. Freezing and thawing procedure

Subsequent freezing and thawing cycles were accomplished by freezing each preparation of liposomes at −196 °C by immersing in liquid nitrogen for 3 min and thawing for 3 min at 40 °C in a water bath. The freezing and thawing process was repeated for a specified number of cycles.

2.6. Liposome size determination

The mean diameters of vesicles were determined by quasielastic light scattering (QELS) at a scattering angle of 90° using an LPA-3000/3100 laser particle analyzer (Otsuka Electronics, Osaka, Japan). The measurements of the average diameters of the liposomes were made on liposomes suspension before freeze-thaw and after subjection to 1, 2, 3, 4, 5, 10, 20, 30 and 35 freeze-thaw cycles.

2.7. Separation of micelles from vesicles

To determine the amount of micelle in EggPC/DSPE-PEG2000 suspension, ultracentrifugation separation technique was used to separate micelles from the vesicles. The liposomes suspension was centrifuged at 50 000*g* for 2 h to obtain both the supernatant and the centrifugal pellet. Both samples were freeze-dried and adjusted to an appropriate concentration by addition of purified water. Due to the difference of specific gravity inside and outside of the liposomes, the small vesicles can be separated from micelles. The amount of phospholipids and DSPE-PEG2000 both in the supernatant and the pellet were determined. The results are demonstrated as the relative proportions of phospholipid concentration in the lamellar (pellet) and micelle (supernatant) to the total phospholipid concentration.

2.8. Determination of phospholipid concentration

Phospholipid concentrations (phosphatidylcholine + PEG lipid) in supernatant and centrifugal pellet were determined as phosphorus based on the method of Ames [49], and calculated in percent of total phospholipids. The total recovery of phospholipids was more than 90%. The relative phospholipids concentration is expressed as

$$\text{Relative phospholipids concentration} = \frac{P_{\text{conc in supernatant or pellet}}}{(P_{\text{conc in supernatant}} + P_{\text{conc in pellet}})}$$

2.9. Gel exclusion chromatography

The size distribution of liposomes before freeze-thawing and after 5 and 35 freeze-thawing cycles were determined by gel exclusion chromatography. The vesicle suspensions were prepared in 20 μM of calcein (a low molecular weight fluorescent probe) solution. Liposome suspension was applied to a Sephacryl®S-1000 Superfine (Pharmacia Biotech, Sweden) column (48 × 1 cm) equilibrated with a buffer solution (20 mM Tris, 150 mM NaCl, pH 7.4), whose gel was presaturated with the phospholipids by passing sonicated EggPC liposomes until the total recovery of phospholipids was more than 80% of loading amount. The eluent from the column was collected. Phospholipid content and fluorescence intensity of each fraction was measured. Phospholipid concentrations were determined by phosphorus assay by the method of Ames [49]. Fluorescence intensities (Ex: 490 nm, Em: 520 nm) were determined by a RF-5000 spectrofluorophotometer (Shimadzu, Japan).

2.10. Freeze-fracture electron microscopy

Liposome suspensions 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 C on the surfaces of the fractured samples at an angle of 45 and 90°, respectively. Then the replicas were moved from holder by submersion in solution of commercial bleaches and distilled water. The cleaned replicas were mounted on 300-mesh Ni grids, dried, and examined with a transmission electron microscope (JEM200CX, JEOL, Tokyo, Japan).

3. Results

3.1. Effect of DSPE-PEG2000 on the particle sizes of EggPC vesicles prepared by detergent removal method

The evolution of the mean diameter measured by QELS of EggPC/DSPE-PEG2000 vesicles prepared by detergent removal method versus the concentration of DSPE-PEG2000 is illustrated in Fig. 1(a). The mean diameter decreased with increasing DSPE-PEG2000 content and finally decreased to 40 nm at 40 mol% of DSPE-PEG2000.

We have also characterized the size of EggPC/DSPE-PEG2000 suspension by gel exclusion chromatography. Fig. 1(b) shows the elution profiles of phospholipids concentration of EggPC vesicles prepared by detergent removal method obtained as a function of DSPE-PEG2000

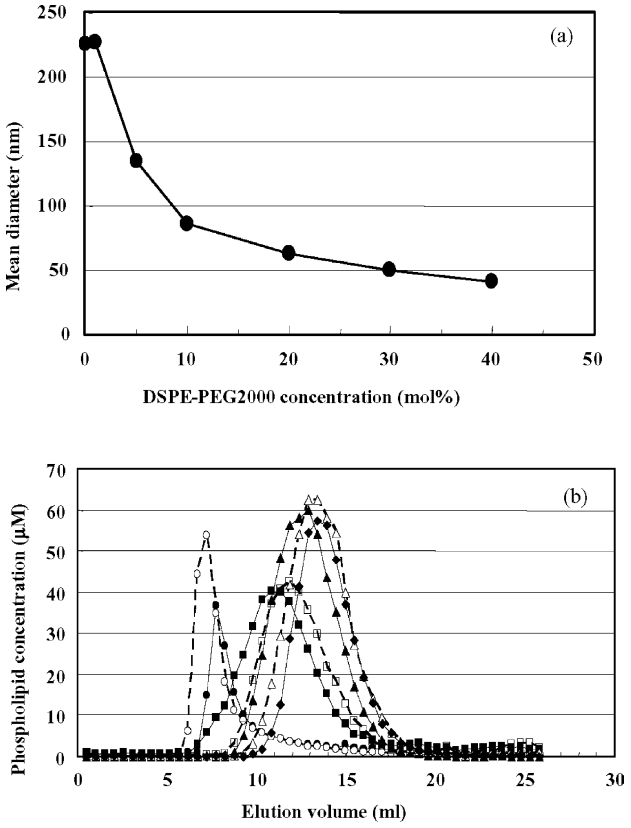

Fig. 1. (a) The evolution of the mean diameter measured by QELS of EggPC vesicles prepared by detergent removal method versus the concentration of DSPE-PEG2000. (b) Elution profiles of EggPC with various concentration of DSPE-PEG2000. (●) 0%, (○) 1%, (■) 5%, (□) 10%, (▲) 20%, (△) 30%, (♦) 40%.

concentration. The maxima of the chromatograms shift to smaller particles region when increasing DSPE-PEG2000 content. This significant shift of their gel chromatography peaks toward the elution region of small particles agrees with the mean diameters measured by QELS (Fig. 1(a)) showing that the progressive decrease in size with increasing DSPE-PEG2000 concentration.

Fig. 2(a)–(g) depict the chromatograms (fluorescence intensity and phospholipids concentration versus elution volume (V_e) curve) for EggPC containing 0–40 mol%. Fluorescence detection curve has revealed two elution peaks. The first peak position coincided with that of the chromatogram of phospholipid concentration, this peak corresponding to the calcein contained in vesicles. The second peak was attributed to free calcein molecules. A notable feature of Fig. 2 was that progressive addition of DSPE-PEG2000 markedly decreased the intensity of the first peak of calcein. The value of fluorescence intensity decreased rapidly from 200–300 for dispersion of EggPC alone and EggPC/1 mol% DSPE-PEG2000 to 10–40 at 5–30 mol% DSPE-PEG2000, and finally decreased to nearly zero when the concentration of DSPE-PEG2000 is 40 mol%. The decrease in fluorescence intensity may be explained by the decrease in diameter of vesicle, contributing to decrease in encapsulated volume, or the formation of micelle structure.

In order to investigate that the decrease in mean diameter of EggPC/DSPE-PEG2000 suspensions and the fluorescence intensity are due to micelle formation or not, we have determined micelle formation by ultracentrifugation experiment. Micelle formation induced by DSPE-PEG2000 could be quantified by determined phospholipid concentration in supernatant of the liposome dispersion separated by ultracentrifuge.

Fig. 3 shows the relative concentration of phospholipids that determined in pellet and supernatant to total phospholipids of EggPC vesicles as a function of DSPE-PEG2000 concentration. When the concentration of DSPE-PEG2000 was less than 5 mol%, almost all of the phospholipids existed in pellet. Increasing DSPE-PEG2000 concentration more than 5 mol% resulted in increasing of phospholipids concentration in supernatant. This increase in phospholipids concentration in supernatant corresponds to an increase in the micelle formation of the phospholipids/DSPE-PEG2000 mixtures. In the case of EggPC/40 mol% DSPE-PEG2000, approximately 75% of total phospholipid was determined in supernatant.

3.2. Influence of freeze-thawing on EggPC/DSPE-PEG2000 vesicles

Fig. 4 shows variation in the mean diameters of the vesicles containing various concentrations of DSPE-PEG2000 with the number of freeze-thaw cycles. When freeze-thaw cycles were applied to these preparations, the mean diameters of EggPC liposomes containing 0–5 mol% of DSPE-PEG2000 significantly decreased following repeated freeze-thawing. For EggPC and EggPC/1 mol% DSPE-PEG2000 vesicle dispersions, the mean diameters decreased from ~250 to ~140 nm after 10 times freeze-thawing. Subsequent increased in the number of freeze-thaw cycles bring no significant change in the vesicle mean diameters. In contrast to these marked reduction in the mean diameters, the mean sizes of EggPC liposomes containing DSPE-PEG2000 more than 10 mol% revealed an increase in the mean diameter with each freeze-thaw cycle. The mean diameter of EggPC/40 mol% DSPE-PEG2000 increased from ~50 to ~150 nm after 15 times freeze-thawing. At 35 freeze-thaw cycles, the mean diameters of all EggPC/DSPE-PEG2000 vesicles were in the range from 100 to 150 nm.

To determine the size distribution of the preparation, we have performed gel exclusion chromatography of EggPC vesicles containing 0–40 mol% of DSPE-PEG2000. Fig. 5 shows gel exclusion chromatograms of EggPC/DSPE-PEG2000 vesicles before and after 5 and 35 freeze-thawing cycles. As shown in Fig. 5(a), in the absence of DSPE-PEG2000 before freeze-thawing, a sharp elution peak of phospholipids was observed at elution volume (V_e) = 7.2 ml. The elution peaks became broader and the maxima of chromatograms shift to V_e = 9.8 and 11.9 ml after 5 and 35

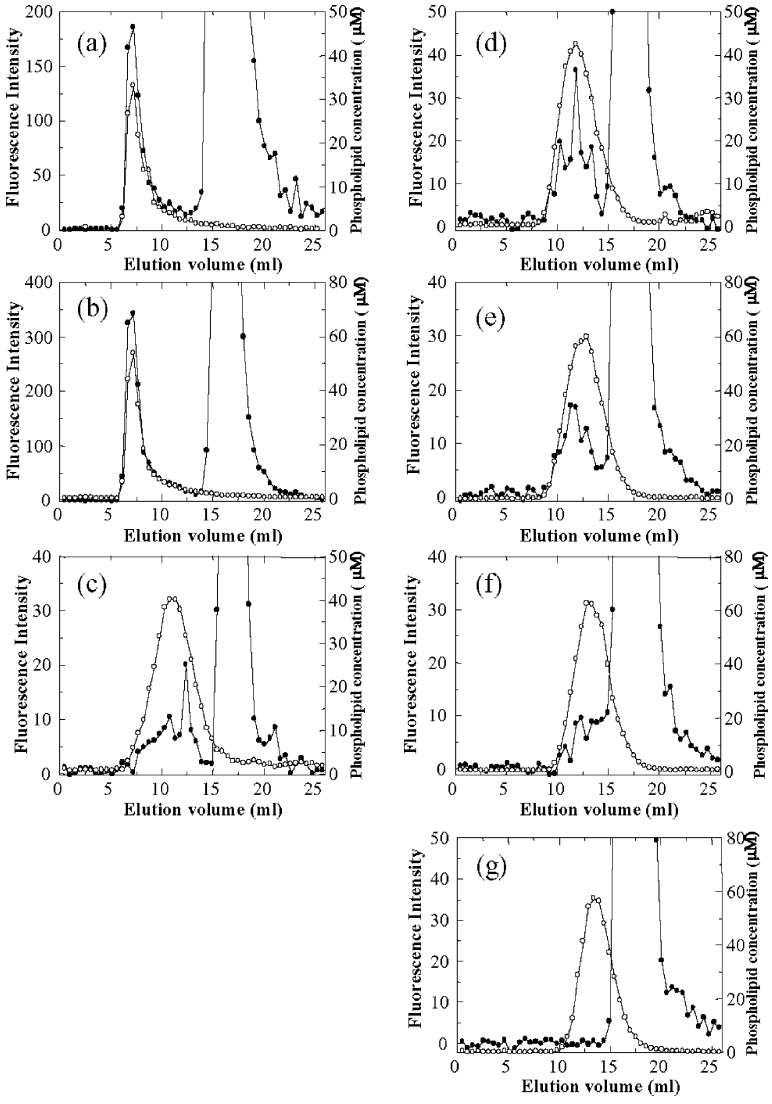

Fig. 2. Gel exclusion profiles of EggPC vesicles at DSPE-PEG concentration of 0% (a), 1% (b), 5% (c), 10% (d), 20% (e), 30% (f), and 40% (g). —●— florescence intensity —○— phospholipid concentration.

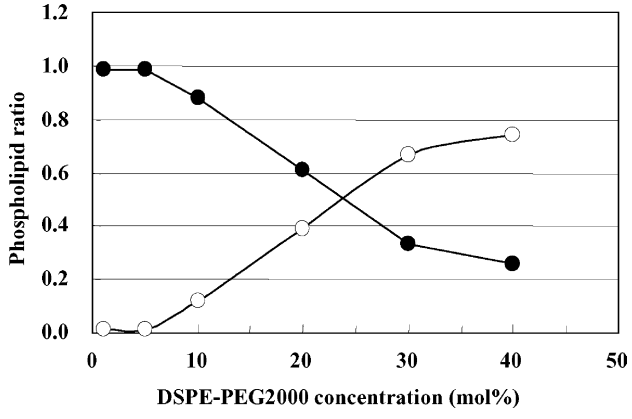

Fig. 3. Relative phospholipid concentration determined in pellet and supernatant to total phospholipids of EggPC vesicles as a function of DSPE-PEG2000 concentration. —●—, pellet; —○— supernatant.

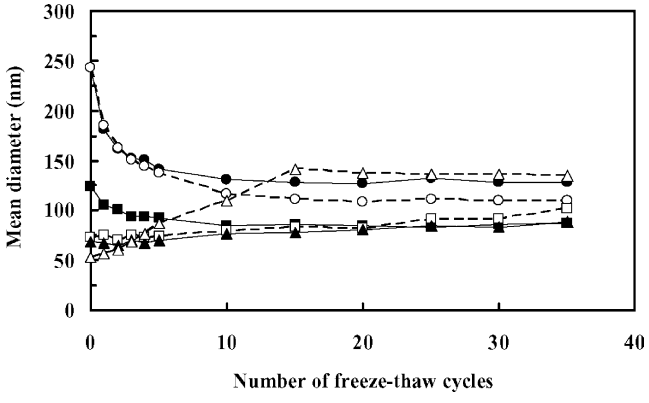

Fig. 4. Influence of the number of freeze-thaw cycles on the mean diameter of EggPC/DSPE-PEG2000 liposomes prepared by detergent removal method. DSPE-PEG2000 concentration: (●) 0, (○) 1, (■) 5, (□) 10, (▲) 20, (△) 30%.

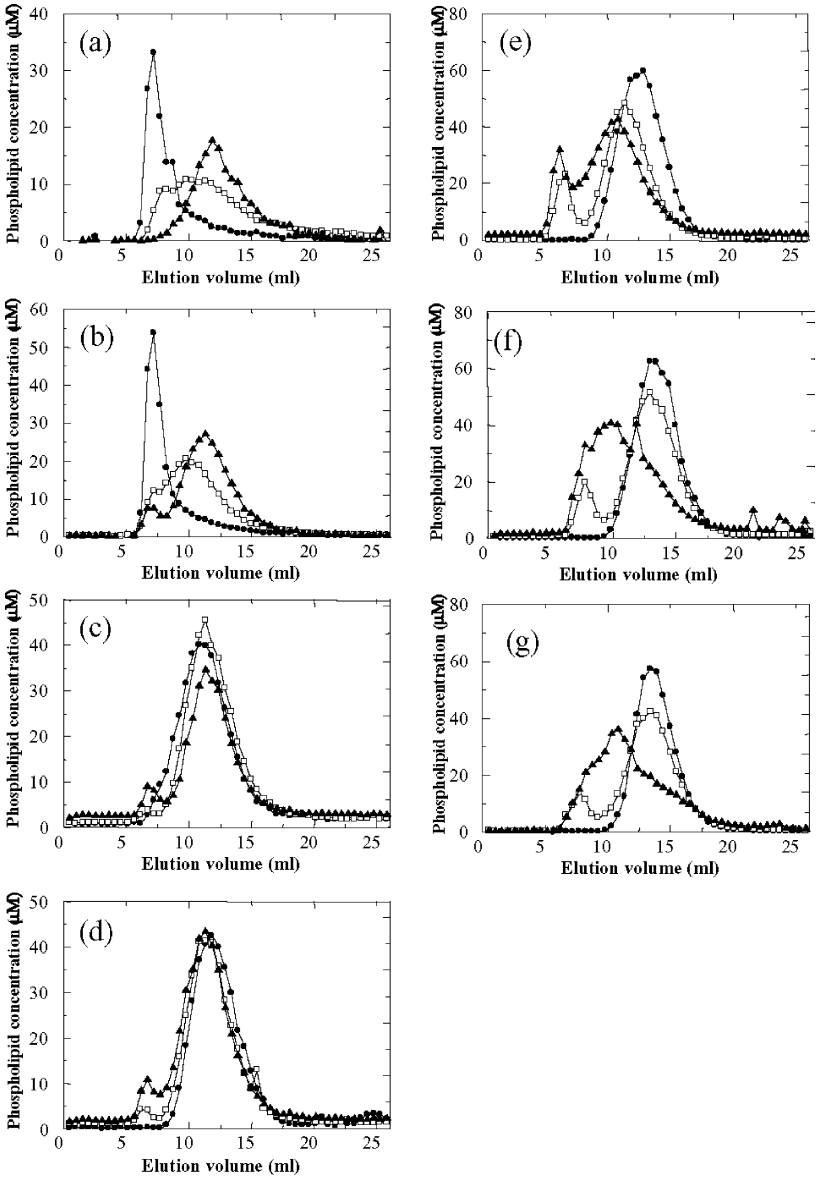

Fig. 5. Elution profiles of phospholipids concentration of EggPC/DSPE-PEG2000 vesicles. DSPE-PEG2000 concentration; (a) 0 mol%; (b) 1 mol%; (c) 5 mol%; (d) 10 mol%; (e) 20 mol%, (f) 30 mol%; (g) 40 mol%. Phospholipid concentrations were determined before freeze-thaw (●), after 5 times freeze-thawing (□), and after 35 times freeze-thawing (▲).

cycles freeze-thawing, respectively. This tendency was in accordance with the result obtained from QELS that demonstrates the decrease in particle size (Fig. 4) and increase in size distribution of the vesicles with an increase in DSPE-PEG2000 concentration (data not shown). When 1–5 mol% of DSPE-PEG2000 was incorporated, the maximum of chromatogram also shifted to the elution region of smaller particle as the number of freeze-thawing cycles was increased (Fig. 5(b) and (c)). As can be seen in Fig. 5(a)–(c), the shifts of elution peaks upon freeze-thawing decreased when increasing the concentrations of DSPE-PEG2000. The maxima of chromatogram of EggPC/5 mol% DSPE-PEG2000 barely change from $V_e = 10.8$ ml before freeze-thawing to $V_e = 11.3$ ml after 35 cycles freeze-thaw. On the other hand, as can be seen from Fig. 5(d)–(g), the sizes of EggPC vesicles containing 10–40 mol% DSPE-PEG2000, of which the mean diameters prior to freeze-thawing are smaller than 100 nm, increase following freeze-thaw cycles. This size enlargement of the vesicles containing higher DSPE-PEG2000 concentrations, which possess smaller mean diameters before freeze-thaw, are greater than the vesicles containing lower DSPE-PEG2000 concentrations. Significant increase in the size of vesicle after freeze-thawing is observed when the concentration of DSPE-PEG2000 more than 30 mol%. After 35 freeze-thawing cycles, the maxima of chromatograms shift from $V_e = 12.9$ and 13.4 ml to $V_e = 9.8$ and 10.8 ml for DSPE-PEG2000 concentration of 30 and 40 mol%, respectively.

When DSPE-PEG2000 is incorporated into the vesicles, upon the freeze-thawing process not only the shifts of the

maxima of the chromatogram, but also the additional peaks locating at $V_e = 6.2–7.7$ ml, which correspond to the presence of large vesicles, are observed (Fig. 5(b)–(g)). The amplitudes of these peaks increase in proportion to the number of freeze-thaw cycles and the concentrations of DSPE-PEG2000. The progressive addition of DSPE-PEG2000 to the vesicles significantly increases the amplitudes of these new peaks. To determine whether the large phospholipids particles, which were eluted at $V_e =$ 6.2–7.7 ml were vesicles or other aggregated particles, EggPC/DSPE-PEG vesicles containing calcein were fractionated. Fig. 6(a)–(d) show the chromatograms of EggPC vesicles containing DSPE-PEG at the concentration of 0, 1, 20 and 40 mol%, respectively. EggPC without DSPE-PEG were analyzed as a reference (Fig. 6(a.1)–(a.3)). As shown in Fig. 6(b.2)–(b.3), (c.2)–(c.3), and (d.2)–(d.3), at the position of the additional peaks of phospholipid ($V_e = 6.2–7.7$ ml) the fluorescence intensities were also observed, suggesting that the large phospholipid particles which were formed during freeze-thawing are vesicles. In addition to the experiment of gel exclusion chromatography, we have used freeze-fracture electron microscopy to investigate the aggregate structure of EggPC/DSPE-PEG vesicles before and after freeze-thawing. Figs. 7 and 8 show the sequence of structural transitions of EggPC vesicles containing 0–40 mol% DSPE-PEG after 0, 5, and 35 cycles freeze-thawing. In agreement with the results from QELS and gel exclusion chromatography, the vesicles size decreased as the concentration of DSPE-PEG2000 increased. For EggPC without DSPE-PEG, the vesicle sizes were significantly

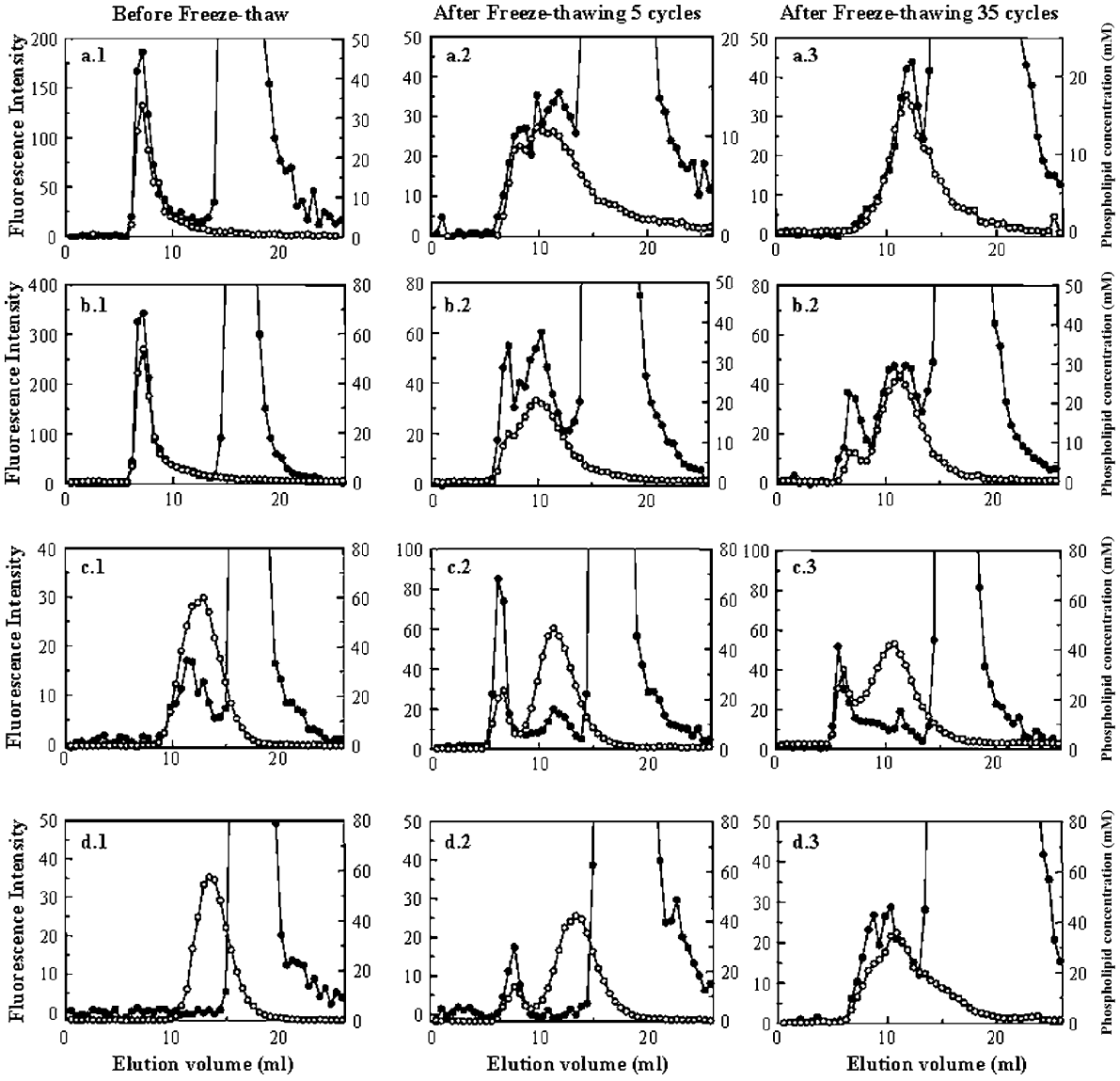

Fig. 6. Gel exclusion profiles of EggPC/DSPE-PEG2000 vesicles. DSPE-PEG2000 concentration; (a) 0 mol%; (b) 1 mol%; (c) 20 mol%; (d) 40 mol%. (1) Before freeze-thawing, (2) after freeze-thawing 5 cycles, (3) after freeze-thawing 35 cycles. —●— florescence intensity —○— phospholipid concentration.

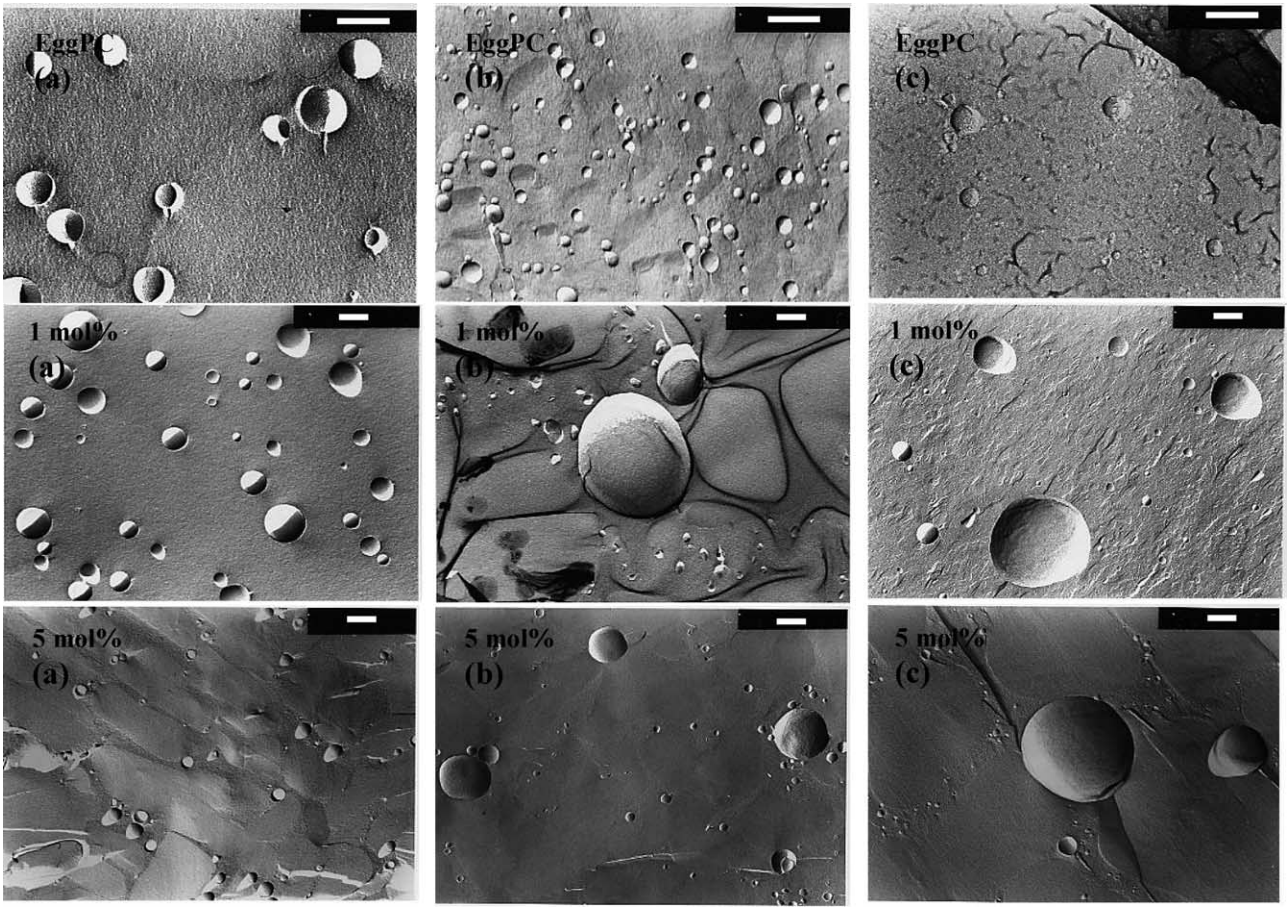

Fig. 7. Freeze-fracture electron micrographs of EggPC vesicles containing 0–5 mol% DSPE-PEG2000 prepared by detergent-removal method before (a) and after 5 (b) and 35 (c) cycles of freeze-thawing, Bar = 200 nm.

smaller than the original vesicles after 35 times freeze-thawing. In the case of EggPC containing DSPE-PEG, after freeze-thawing large spherical structures with 200–600 nm in diameter were observed, regardless DSPE-PEG concentration. The fraction of these large vesicles appeared as increase with increasing DSPE-PEG concentration and the number of freeze-thaw. These progressive increase in the number of large vesicles agrees with the amplification of the additional peak ($V_e = 6.2$–7.7 ml) observed in the gel exclusion chromatography.

To ascertain whether the formation of the large vesicles of EggPC/DSPE-PEG after freeze-thawing is due to the incorporation of DSPE-PEG, to the size of vesicle prior to freeze-thaw, or to the preparation method, we have also studied the size and size distribution change upon freeze-thawing of EggPC vesicles with various original size, prepared by extrusion and sonication method. Fig. 9 illustrates the mean diameter changes with increase in the number of freeze-thaw cycles of EggPC liposomes without DSPE-PEG prepared by extrusion and sonication method, which are measured by QELS method. Similar to the results of EggPC/DSPE-PEG prepared by detergent-removal method, the mean diameter of EggPC vesicles prepared by extrusion, which had original size of 184.5 or 133.9 nm, decreased as the number of freeze-thawing cycles. On the contrary the mean diameters increase following freeze-thawing for small vesicles prepared by extrusion (74.5 nm in diameter) or vesicles (60 nm in diameter) prepared by sonication method. Gel exclusion chromatographs of EggPC without DSPE-PEG2000 prepared by extrusion and sonication methods are similar to EggPC vesicles prepared by detergent removal method. Elution profiles of EggPC vesicles prepared by both extrusion and sonication methods revealed no additional peak after repetitive freeze-thaw cycles in the absence of DSPE-PEG (data not shown).

The size alteration upon freeze-thawing of EggPC/20 mol% DSPE-PEG 2000 prepared by extrusion method was also investigated. The mean diameter decreased as a function of number of freeze-thaw cycles. The elution profiles of this preparation are similar to the EggPC/DSPE-PEG vesicles prepared by detergent removal method. After 35 freeze-thaw cycles, the additional peak at $V_e = 6.2$–7.7 ml were observed and freeze-fracture electron micrograph revealed large vesicles with a diameter of 100 nm (data not shown).

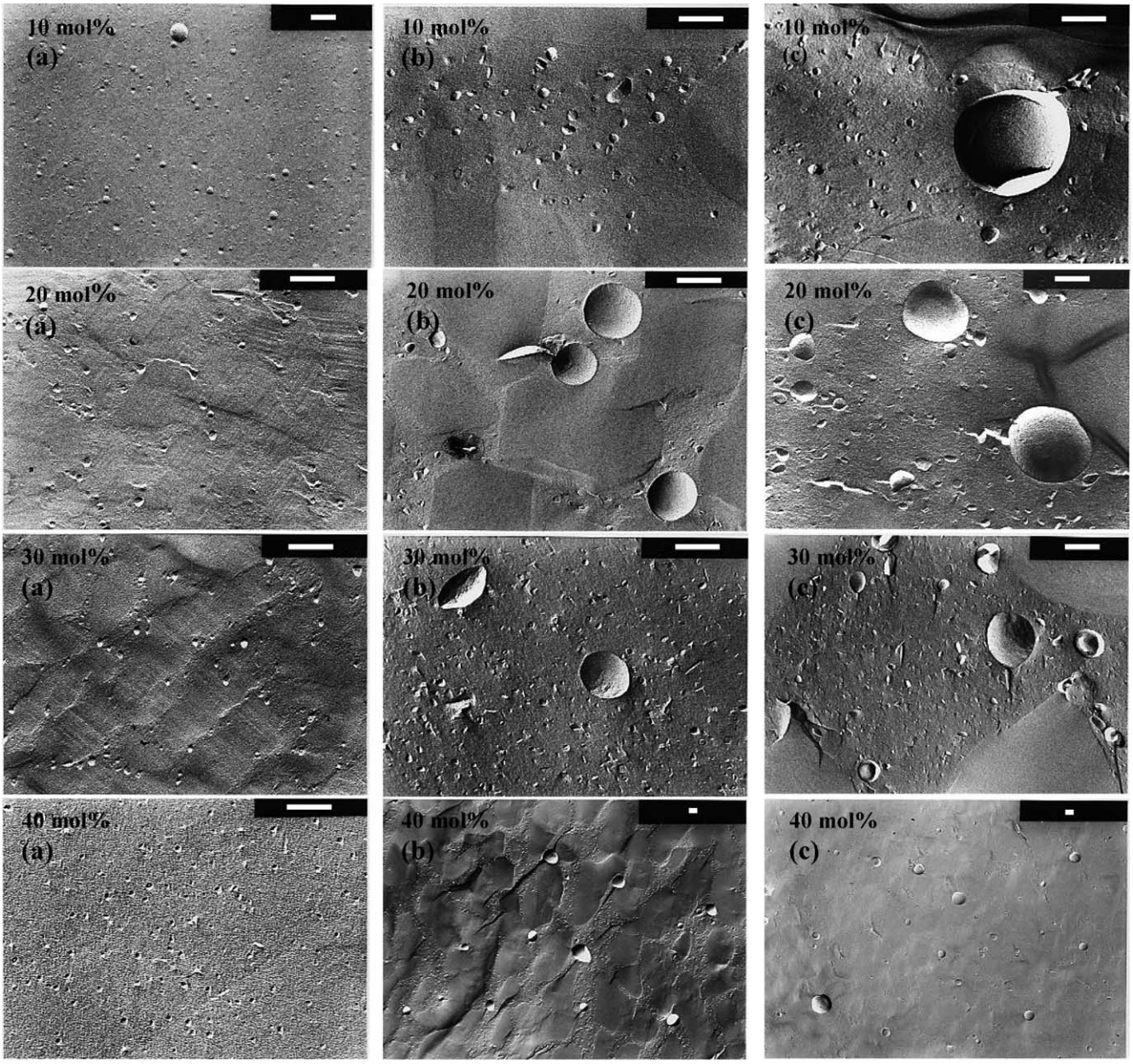

Fig. 8. Freeze-fracture electron micrographs of EggPC vesicles containing 10–40 mol% DSPE-PEG2000 prepared by detergent-removal method before (a) and after 5 (b) and 35 (c) cycles of freeze-thawing, bar = 200 nm.

4. Discussions

4.1. Effect of DSPE-PEG2000 on the size of EggPC vesicles

The results obtained from QELS (Fig. 1(a)), gel exclusion chromatography (Fig. 1(b)) and freeze-fracture electron microscopy Figs. 7 and 8) demonstrated that the size of EggPC/DSPE-PEG2000 liposomes prepared by detergent removal method decreased as a function of DSPE-PEG concentration. When the concentration of DSPE-PEG is more than 10 mol%, not only decrease in diameter of vesicles, but also micelle formation are observed, as illustrated by drastically decreasing in fluorescence intensity in gel exclusion chromatography (Fig. 2) and appearance of phospholipids in supernatant in ultracentrifugation experiment (Fig. 3). An incorporation of amphiphiles with big polar head into vesicles generally makes the vesicle size smaller by lateral steric repulsion [50,51]. As was described in our previous study [43], an increase in the concentration of PEG-lipid enhances the lateral repulsive properties of the surface of lipid bilayer by extensive hydration around the head group. In order to reduce the degree of repulsion, the vesicle sizes decrease so as to increase the curvature of the grafting surface.

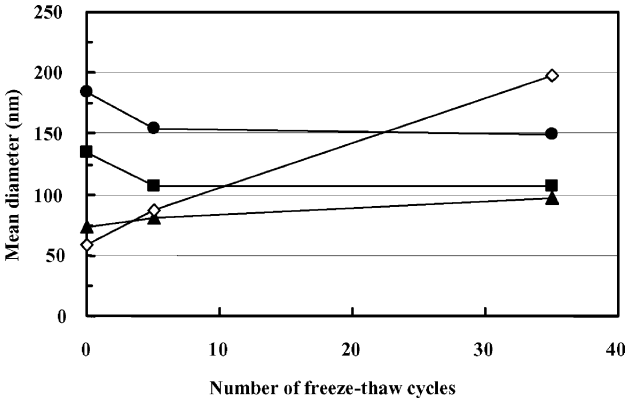

Fig. 9. The mean diameter of EggPC liposome as a function of the number of freeze-thaw cycles determined by QELS. (●) EggPC liposomes prepared by extrusion method (d=184.5 nm); (■) EggPC liposomes prepared by extrusion method (d=133.9 nm); (▲) EggPC liposomes prepared by extrusion method (d=73.5 nm); (◇) EggPC liposomes prepared by sonication method (d=58.7 nm).

The micelle formation induced by PEG-lipid has been demonstrated in a number of studies [4,41,42,44–46]. From our present experiments the micelle structure were formed at ~10 mol% of DSPE-PEG2000. This value was comparable with Edward et al. [46], and a little lower than that of Hristova et al. [45]. This difference may be due to difference in preparation method and kind of PEG-lipids.

4.2. Influence of freeze-thawing on EggPC/DSPE-PEG2000 vesicles

The effects of freeze-thawing on particle sizes of vesicles in the absence of PEG lipid have been documented by many researchers [39,40,52–54]. On the other hand, in the presence of DSPE-PEG, gel chromatography of EggPC vesicles containing 1–5% DSPE-PEG2000 (Fig. 5(b) and (c)), as well as EggPC vesicles without DSPE-PEG2000 (Fig. 5(a)), showed that most of the vesicles fragmented to the smaller vesicles when they are subjected to successive cycles of freeze-thawing as illustrated by the shift of the 'main' peak to the smaller particles size position with freeze-thawing cycles. On the other hand, the average diameter of EggPC vesicles containing 10–40 mol% DSPE-PEG2000 which have mean diameter prior to freeze-thaw less than 100 nm is increased as the number of freeze-thawing is increased. Increase in mean diameter of smaller vesicles is greater than the larger ones (Fig. 4). Furthermore, gel chromatography of EggPC liposomes containing DSPE-PEG (Fig. 5(b)–(g)) demonstrated that not only a large fraction of vesicles have smaller diameters (in the case of EggPC containing 1–5 mol% DSPE-PEG2000) or larger diameters (in the case of EggPC containing 10–40 mol% DSPE-PEG2000) than the vesicles prior to freeze-thaw, but the formation of large vesicle with a diameter of hundreds nm which was eluted at V_e=6.2–7.7 ml are also observed upon the repetitive freeze-thawing. The freeze-fracture electron micrographs after 5 and 35 freeze-thaw cycles of EggPC containing DSPE-PEG2000 (Figs. 7 and 8) confirm the presence of the large vesicles with diameter of 200–600 nm.

As demonstrated by gel exclusion results obtained with various concentration of DSPE-PEG2000 (Fig. 5), it appears that the more freeze-thaw cycles the greater the number of large vesicle. Moreover after freeze-thawing, the vesicles with higher concentration of DSPE-PEG2000 fuse to large vesicle more than the dispersion with lower concentration and the amount of large vesicles increase in proportion to the number of freeze-thaw cycles. The formation of these large vesicles is independent of the method of preparation. Vesicles made of EggPC and DSPE-PEG2000 prepared by extrusion method gave rise to formation of large vesicle same as those prepared by detergent removal method. Thus, the formation of large liposome with a diameter of 200–600 mm is absolutely dependent upon the concentration of DSPE-PEG2000 and the number of freeze-thaw cycles.

About conventional liposomes, it has been discussed that much of the potential damage to liposome is directly related to the behavior of water in the dispersion by ice formation during freeze-thawing [55,56]. Furthermore, small vesicles with large curvature are known to be thermodynamically unstable. When these vesicles are subjected to freeze-thaw, the process of fusion is greatly accelerated [32,57].

On the other hand, an acceleration effect of PEG lipid on size evolution during freeze-thawing is not entirely clear. The presence of strongly hydrated PEG groups extending from the liposome surface must take part in this event. One possibility is unstabilizing of membrane by steric hindrance of large PEG group, especially against inner leaflet of the vesicles during freeze-thawing. Another possibility is fusion induction effect of polyethylene glycol (PEG). It has been known that fusion took place only above a threshold concentration of PEG [58,59]. Freezing process involves the formation of ice crystals and the concomitant concentration of solutes in the unfrozen fraction. Therefore, it is possible that concentration of PEG-grafted liposomes should be brought about during freeze-thawing process. The presence of PEG-lipid mixed micelles is also possibly involved in the size evolution during freeze-thawing. Although freeze-thawing of DSPE-PEG liposome suspension without micelle (1–5 mol%) (Fig. 3) also resulted in formation of large vesicles (Fig. 5(a) and (b)), the amounts of large vesicles increase significantly (Fig. 5(e)–(g)) after freeze-thawing of high DSPE-PEG containing liposome suspension which micelles are preferentially to form (Fig. 3). In order to clarify the mechanism of fusion-enhancing effect by PEG-lipid during freeze-thawing process, further experiments should be necessary.

5. Conclusion

In this paper gel exclusion chromatography, QELS

measurement, and freeze-fracture electron microscopy have been used to determine the sizes and size distribution of EggPC liposomes containing DSPE-PEG2000. The results from gel exclusion chromatography, QELS and the direct image obtained from freeze-fracture electron microscopy reveal the new information on the effect of freeze-thaw on size evolution and size distribution of vesicles composing of EggPC and DSPE-PEG2000. Mean diameters, which had originally large size, decreased with increasing in the number of freeze-thawing cycles. On the contrary, mean diameters of the liposomes, which had originally small size, increased with increase in the number of freeze-thawing cycles. After freeze-thawing over 10 times, the liposomal mean diameters fell into a range of 80–150 nm in spite of original size. Gel exclusion chromatography showed that in the process of freeze-thawing, two opponent events one is fission and the other is fusion, occurred at the same time. PEG-lipid accelerate fusion event.

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