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ORIGINAL CONTRIBUTION

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Effect of a PEG lipid (DSPE-PEG2000) and freeze-thawing process on phospholipid vesicle size and lamellarity

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Abstract Liposomes containing distearoylphosphatidylethanolamine with covalently linked polyethylene glycol of molecular weight 2,000 (DSPE-PEG2000) covering a range of 0–30 mol% were prepared by a mechanical dispersion or detergent-removal method. The effects of DSPE-PEG2000 on particle sizes and lamellarity of liposomes were investigated. The average diameters of vesicles prepared from both methods decreased when the concentration of DSPE-PEG2000 was increased. The decrease in vesicle size with increase in DSPE-PEG2000 was ascribed to the steric hindrance of strongly hydrated PEG. The significant decrease in the sizes of DSPE-PEG2000-containing EggPC vesicles prepared by the detergent-removal method could be explained by the postvesiculation size growth in the process of micelle–vesicle transition. For DMPC vesicles prepared by the detergent-removal method, electron micrographs showed that inclusion of DSPE-PEG2000 promoted vesicle formation. Based on the results of investigation of calcein entrapment efficiency, we concluded that the lamellarity of liposomes is reduced as PEG lipid concentration is increased. Fragmentation of multilamellar vesicles into smaller unilamellar vesicles occurred more readily when the liposome suspension was subjected to repetitive freeze-thawing. After five cycles of freezing and thawing, vesicles containing more than 0.5 mol% DSPE-PEG2000 were fragmented into unilamellar vesicles with diameters smaller than 300 nm.

Introduction

The use of conventional liposomes as a carrier system for drug delivery has been limited because of their short circulation time in the blood. The rapid clearance of conventional liposomes from the blood is mediated primarily through the scavenging function of phagocytic cells located in the liver and spleen, known as the reticuloendothelial system (RES) [1, 2, 3, 4]. In order to avoid capture by the RES, liposomes, to which polymers such as polyethylene glycol (PEG) are grafted, have been developed. The increased circulation time of liposomes containing PEG lipids has been attributed to their steric repulsive barrier by strong hydration against other macromolecules and cells [5, 6, 7, 8, 9]. Their applications to a drug delivery system have been widely studied [8, 9, 10, 11, 12, 13, 14, 15, 16].

Incorporation of PEG covalently bonded to the polar head group of phospholipids, such as distearoylphosphatidylethanolamine–PEG (DSPE-PEG), has been shown to markedly increase their blood circulation time [8, 10, 11, 17, 18]. However, the incorporation of PEG lipid in a bilayer may alter the physicochemical properties of liposomes. While the effects of DSPE-PEG on the structures and physical properties of liposomes with various compositions have been investigated by a variety

of techniques, including X-ray diffraction [7], nuclear magnetic resonance, differential scanning calorimetry [19, 20, 21, 22], and membrane permeability [23], there have been few systematic studies on the effects of PEG lipid on particle sizes and size distribution of liposomes [24, 25, 26]. This is despite the fact that these are important factors affecting the physicochemical properties, encapsulation efficiency, tissue distribution, and in vivo circulation lifetimes of liposomes.

It is well known that the physical properties of liposomes generally depend on the method of preparation as well as the lipid composition. Particle sizes and lamellarity also depend on the method of preparation. Freeze-thawing cycles are often applied to multilamellar vesicle (MLV) suspensions in order to facilitate hydration in the lamellar phase. Repetition of freeze-thawing cycles results in an appreciable increase in trapping efficiencies [27, 28, 29, 30, 31].

We compared the particle sizes of egg-yolk phosphatidylcholine (EggPC) and dimyristoylphosphatidylcholine (DMPC) vesicles containing various concentrations of DSPE-PEG2000, and found that the incorporation of increasing amounts of PEG lipid in liposomes causes reduction of liposome sizes and that the magnitude of this reduction is dependent on the method of preparation and the concentration of DSPE-PEG2000.

We also examined the effects of the number of freeze-thawing cycles on particle sizes and calcein entrapment efficiencies of liposomes, in which PEG lipid had been incorporated. We demonstrated that repetitive freeze-thawing leads to a decrease in particle size of PEG lipid liposomes. Furthermore, we inferred from the calcein entrapment efficiency that freeze-thawing of liposomes containing DSPE-PEG2000 results in the production of unilamellar liposomes more easily than does freeze-thawing of liposomes composed of only EggPC.

Materials and methods

Materials

Phosphatidylcholine from egg yolk (EggPC; purity of PC = 98.8%), dimyristoylphosphatidylcholine (DMPC; purity of PC = 99.8%), and DSPE-PEG2000 (one molecule of distearoylphosphatidylethanolamine is covalently linked with one molecule of polyethylene glycol with molecular weight 2,000) as a PEG lipid were purchased from Nihon Yushi (Tokyo, Japan) and were used without further purification. *n*-Octyl-β-D-glucopyranoside, calcein, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other commercially available reagents used were of analytical grade.

Preparation of liposomes by a mechanical dispersion method

Known amounts of EggPC and DSPE-PEG2000 (PEG lipid) were dissolved in chloroform/methanol (2:1, v/v). The solvent was evaporated to dryness with a nitrogen gas stream to yield a thin lipid film and the dried lipid sample was put under vacuum for more than 6 h to remove residual traces of organic solvent. An appropriate volume of 20 mM Tris buffer (150 mM NaCl, 20 mM Tris, pH 7.4) was added to the dry film and then vigorously shaken with a vortex mixer (vortexing) for over 10 min to produce multilamellar vesicles (MLV).

The MLV suspensions were frozen and thawed for a specified number of cycles (1–35 cycles). A single freeze-thawing cycle consisted of freezing for 3 min at liquid nitrogen temperature (−196 °C) and thawing for 3 min in a water bath at 40 °C.

Preparation of liposomes by a detergent-removal method

Liposomes were prepared according to the method previously reported [32, 33] with slight modification. The dried lipid film, prepared as described above, was solubilized in 1 mL of an aqueous solution of 125 mM *n*-octyl-β-D-glucopyranoside in 20 mM Tris buffer to give a final concentration of 10 mM phospholipid and vigorously shaken to produce a mixed micelle solution. The solution (1 mL) was dialyzed at 25 °C against 1 L of surfactant-free aqueous buffer for 6 h, then dialyzed again for 24 h using 1 L of a new surfactant-free buffer solution.

Size measurements

The average diameter and size distribution of vesicles were measured using a laser particle-size analyzing system (Photal LPA-3000/3100, Otsuka Electronics, Osaka, Japan) at 25 °C.

Calcein entrapment efficiency measurement

The calcein entrapment efficiency of the vesicles was determined according to Oku et al. [34] with slight modification. The vesicles were prepared in a 10 μM calcein solution and the fluorescence of untrapped calcein was quenched by the addition of an appropriate amount of 100 mM $CoCl_2$. The fluorescence intensity of the sample was recorded continuously subsequent to this dilution (zero time) on an RF-5000 fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) (excitation $\lambda = 490$ nm, emission $\lambda = 520$ nm) equipped with temperature control accessories and a magnetic stirrer. The entrapment efficiency was calculated by the following equation:

$$\text{Entrapment efficiency (\%)} = \left(f_{\text{in}} \cdot r_1 - f_{\text{total.q}} \cdot r_2\right) / \left(f_{\text{total}} - f_{\text{total.q}} \cdot r_2\right) \times 100$$

where f_{in} is the fluorescence intensity of the internal calcein; f_{total} and $f_{\text{total.q}}$ are the total fluorescence intensity of calcein before and after vesicle destruction, respectively; and r_1 and r_2 are correction factors for the volume increase upon adding a $CoCl_2$ solution and the Triton X-100 solution, respectively. They were 2620/2600 and 2625/2600 in our experiments.

The relative entrapment efficiency was calculated by the following equation:

$$\text{Relative calcein entrapment efficiency} = \frac{\left\{\frac{\text{calcein entrap}_{\text{V}}\ \text{(exp)}}{\text{calcein entrap}_{\text{V}}\ \text{(cal)}}\right\}}{\left\{\frac{\text{calcein entrap}_{\text{E}}\ \text{(exp)}}{\text{calcein entrap}_{\text{E}}\ \text{(cal)}}\right\}}$$

where calcein entrap$_{\text{V}}$ (exp) and calcein entrap$_{\text{E}}$ (exp) are the calcein entrapment efficiencies of liposomes prepared by the vortex and extrusion methods, respectively, which are obtained by experiment. Calcein entrap$_{\text{V}}$ (cal) and calcein entrap$_{\text{E}}$ (cal) are the calcein entrapment efficiencies of liposomes calculated from the

particle size of liposomes prepared by the vortex and extrusion methods, respectively, where the vesicles are assumed to be unilamellar.

Negative-staining electron microscopy

Liposomal suspension was applied to carbon-coated grids, washed with stain solution (2% phosphotungstate solution), and blotted with a filter paper. The specimens were examined at 20,000× magnification with an electron microscope (JEM200 CX, JEOL, Tokyo, Japan).

Freeze-fracture electron microscopy

The specimens for freeze-fracture microscopy were prepared according to the procedure previously reported [35] with slight modification as follows. Liposomal 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, Tokyo, Japan). After fracturing the sample, an electric discharge was applied to deposit Pt/C, then C on the surface of the fractured samples at angles of 45 and 90°. The replicas were removed from their holders by submersion in a solution of commercial bleach and distilled water. The cleaned replicas were mounted on 300-mesh Ni grids, dried, and examined at 5,000–10,000× magnification with an electron microscope (JEM200 CX, JEOL, Tokyo, Japan).

Results

Effect of DSPE-PEG2000 concentration on the particle size of EggPC liposomes prepared by a mechanical dispersion method

Figure 1 shows the diameters of EggPC liposomes containing 0–40 mol% (in the membrane phase) of PEG lipid prepared by mechanical dispersion. Inclusion of increasing amounts of PEG lipid resulted in a decrease in particle sizes. The average diameter of EggPC MLV decreased from 10 μm at a PEG lipid concentration of 0% to 300 nm at a PEG lipid concentration of 30%. The mean diameter of the MLV decreased significantly when 0.1 mol% DSPE-PEG2000 was added.

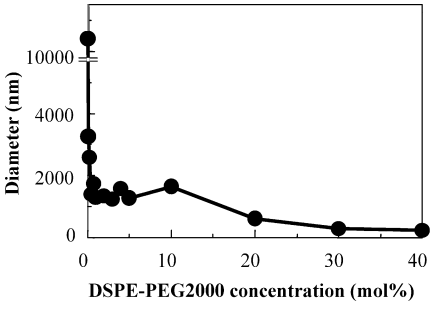

Fig. 1 Diameter of EggPC/DSPE-PEG2000 MLV prepared by the mechanical dispersion method as a function of DSPE-PEG2000 concentration

Effect of DSPE-PEG2000 concentration on diameter of liposomes prepared by a detergent-removal method

The average diameter of EggPC/PEG lipid liposomes prepared by a detergent-removal method depends strongly on PEG lipid concentration (Fig. 2). The mean diameters of EggPC vesicles without PEG lipid and EggPC vesicles with 30 mol% PEG lipid were 205 and 48 nm, respectively.

The average diameter of DMPC liposomes decreased with increase in PEG lipid concentration (Fig. 3), as was the case for EggPC liposomes prepared by mechanical dispersion or by detergent removal. The magnitude of the decrease in the mean diameter of DMPC/PEG lipid liposomes was greater than that of EggPC/PEG lipid liposomes. The average diameter decreased from 10 μm at a DSPE-PEG concentration of 0% to 30 nm when 30 mol% of PEG lipid was incorporated.

Figure 4 shows freeze-fracture electron micrographs of DMPC/PEG lipid liposomes with PEG lipid

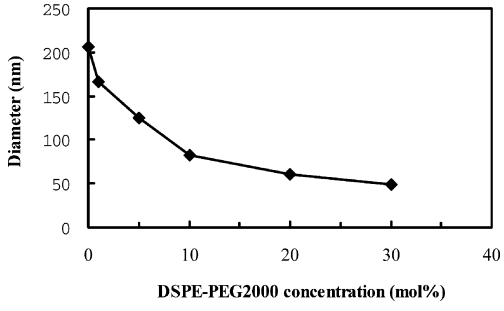

Fig. 2 Dependence of diameters of EggPC/DSPE-PEG2000 liposomes prepared by the detergent-removal method on DSPE-PEG2000 concentration

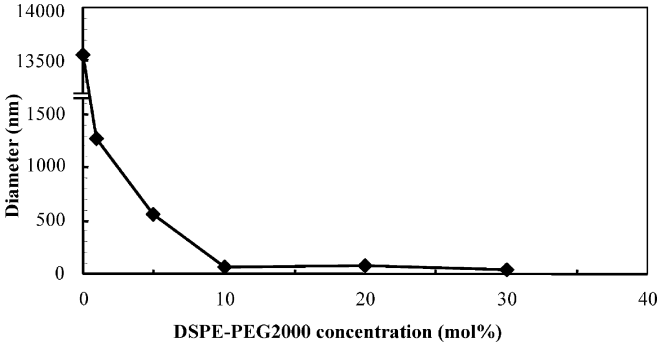

Fig. 3 Relationship between the diameters of DMPC/DSPE-PEG2000 liposomes prepared by the detergent-removal method and DSPE-PEG2000 concentration

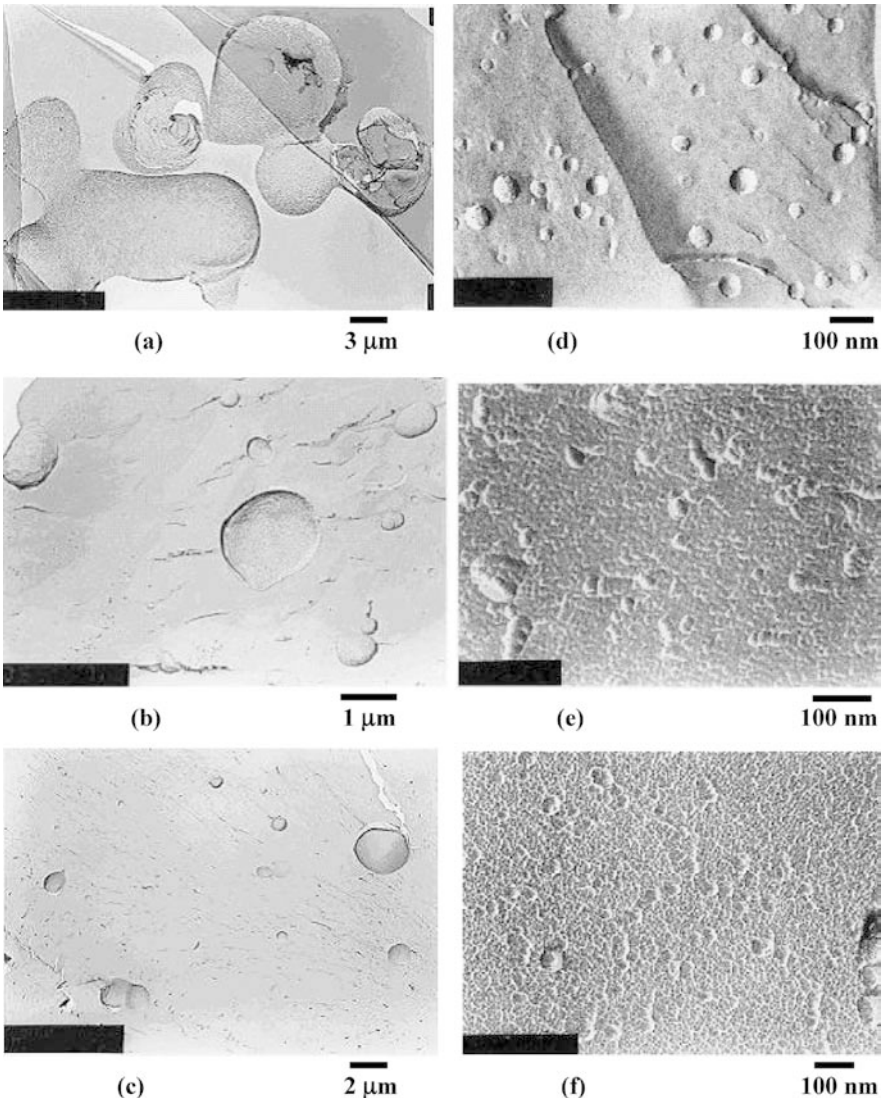

Fig. 4a–f Freeze-fracture electron micrographs of DMPC/DSPE-PEG2000 vesicles prepared by the detergent-removal method at DSPE-PEG2000 concentrations of 0 (**a**), 1 (**b**), 5 (**c**), 10 (**d**), 20 (**e**), and 30 (**f**) mol%

concentrations of 0–30 mol% prepared by the detergent-removal method. A significant structural change in DMPC liposomes was observed when the concentration of PEG lipid was increased. In the absence of PEG lipid, DMPC aggregated into large clusters of distorted liposomes or multilayered liposomes. Inclusion of a small amount of PEG lipid made the aggregates more spherical and unilamellar. At a concentration of more than 20 mol% of PEG lipid, the liposomes became distorted again.

Effect of number of freeze-thawing cycles on the size of liposomes

After freezing and thawing under our experimental conditions, the average diameter of EggPC/PEG lipid vesicles prepared by the mechanical dispersion method was reduced as the number of freeze-thaw cycles was increased (Fig. 5). An increase in the number of freeze-thawing cycles from one to ten resulted in a significant decrease in the diameter of liposomes. Further increases in the number of cycles caused a gradual decrease in vesicle diameter. The magnitude of the reduction in diameters of vesicles with large initial diameters was greater than that of vesicles with small initial diameters. After 20 freeze-thawing cycles, the average size of the liposomes was around 200 nm regardless of initial size. Figure 6 shows negative-staining electron micrographs of EggPC/PEG lipid liposomes obtained after 35 cycles of freeze-thawing. Most of the liposomes were spherical with diameters of less than 300 nm.

Figure 7 shows the average diameters of EggPC/PEG lipid liposomes after five cycles of freeze-thawing as a function of DSPE-PEG2000 concentration. The particle size of PEG lipid-free liposomes was more than

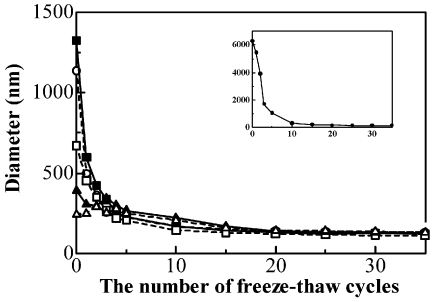

Fig. 5 Influence of the numbers of freeze-thaw cycles on the diameters of EggPC/DSPE-PEG2000 MLV prepared by the mechanical dispersion method. DSPE-PEG2000 concentration:○, 1 mol%; ■, 5 mol%; □, 10 mol%; ▲, 20mol%; △, 30mol%. The *inset curve* shows the diameters of EggPC MLV in the absence of DSPE-PEG2000 as a function of the number of freeze-thaw cycles

1,000 nm after five cycles of freeze- thawing. The size of liposomes containing only 1% PEG lipid was drastically reduced to about 250 nm. The average diameters at all concentrations of PEG lipid (1 to 30 mol%) were almost the same.

Relative entrapment efficiency of DSPE-PEG2000-containing liposomes

Figure 8 shows the relative entrapment efficiencies of calcein for EggPC/PEG lipid vesicles prepared by mechanical dispersion with (five cycles) and without

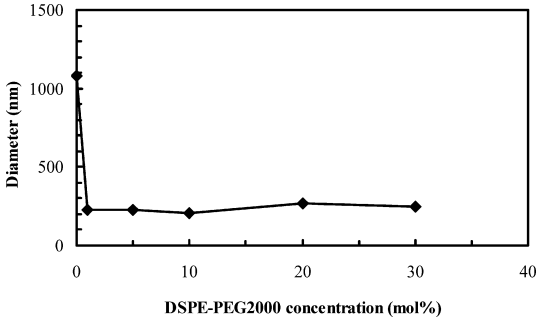

Fig. 7 Diameters of EggPC/DSPE-PEG2000 liposomes prepared by the mechanical dispersion method after five cycles of freeze-thawing as a function of DSPE-PEG2000 concentration

freeze-thawing as a function of PEG lipid mol%. For samples after vortexing, the relative entrapment efficiency of calcein increased with increase in PEG lipid concentration up to 30 mol%, at which the relative entrapment efficiency was close to unity. At a concentration of 40 mol%, the calcein entrapment efficiency slightly decreased. After five cycles of freeze-thawing, the calcein entrapment efficiency of liposomes containing PEG lipid increased significantly. Addition of 0.5 mol% PEG lipid brought the ratio up to 1. Further increase in the concentration of PEG lipid resulted in no significant change in calcein entrapment efficiency. However, at a high concentration of DSPE-PEG2000 (40 mol%), the calcein entrapment efficiency decreased to that of liposomes before freeze-thawing.

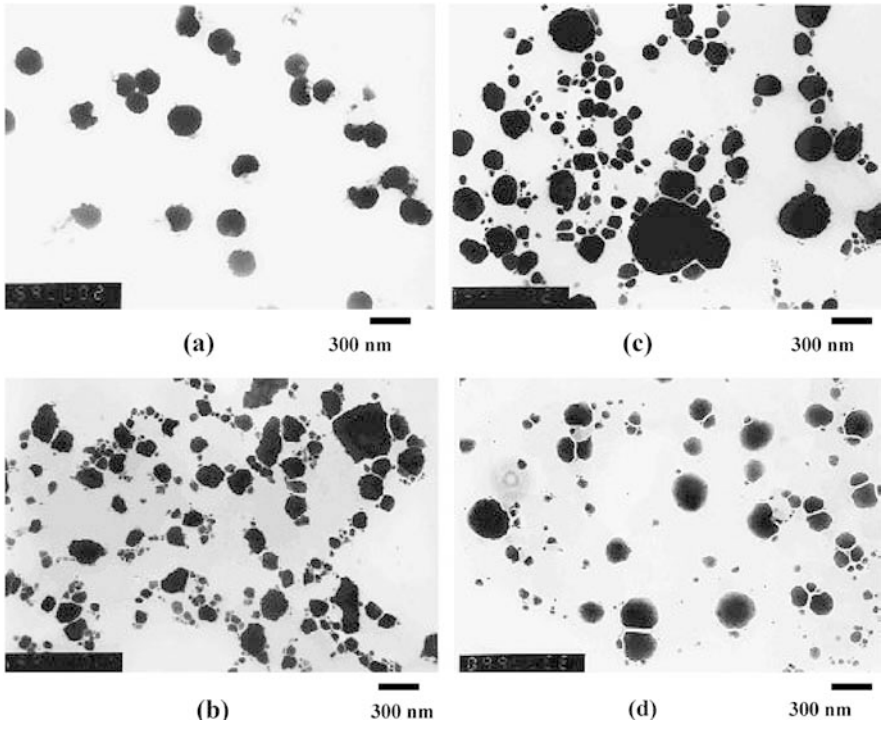

Fig. 6a–d Negative-staining electron micrographs of Egg-PC/DSPE-PEG2000 vesicles prepared by the mechanical dispersion method after 35 cycles of freeze-thawing at DSPE-PEG2000 concentrations of 5 (**a**), 10 (**b**), 20 (**c**), and 30 (**d**) mol%

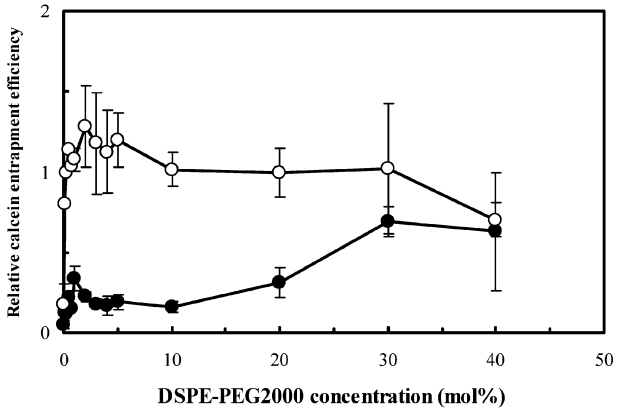

Fig. 8 DSPE-PEG2000 concentration dependences of relative calcein entrapment efficiencies of EggPC/DSPE-PEG2000 liposomes prepared by vortexing without freeze-thawing (●) and after five cycles of freeze-thawing (○)

Discussion

Effect of DSPE-PEG2000 concentration on particle sizes

The mean diameter of EggPC/PEG lipid liposomes prepared by the mechanical dispersion and detergent-removal methods decreased as a function of PEG lipid concentration, that is, incorporation of PEG lipid into the membrane phase made the membrane curvature large. For liposomes prepared by the mechanical dispersion method (Fig. 1), the presence of a very low concentration (below 1 mol%) of PEG lipid resulted in a significant decrease in particle size. With increase in the concentration of PEG lipid, the vesicle diameter gradually decreased to 250 nm at a concentration of 40 mol%. On the other hand, in the case of liposomes prepared by the detergent-removal method (Fig. 2), the vesicle diameter continuously decreased with increase in PEG lipid mol%, from 200 nm at 0 mol% to 50 nm at 40 mol%.

The effect of PEG lipid concentration on the diameter of liposomes using DMPC instead of EggPC as a phospholipid is shown in Fig. 3. The liposomes were prepared by the detergent-removal method. The particle size of DMPC/PEG lipid liposomes also showed a tendency to decrease with increase in PEG lipid concentration, as was the case for EggPC/PEG lipid liposomes. However, as shown in Fig. 4a, liposomes cannot form properly without PEG lipid as can be seen by the distorted shapes shown in the freeze-fracture electron micrograph. However, after a small amount of PEG lipid had been added, spherical liposomes were observed (Fig. 4b) and further increase in PEG lipid concentration resulted in a decrease in particle size (Fig. 4c). We also observed that in the absence of PEG lipid, liposomes aggregated into large white clusters in a dialysis tube (data not shown). These clusters disappeared when a small amount of PEG lipid was added. These findings indicate that inclusion of a small amount of PEG lipid enables DMPC liposomes to easily form in the detergent-removal method. At a concentration of PEG lipid of more than 20 mol%, the shapes of particles seem to deviate from spherical and the entrapment efficiency becomes very low (data not shown), suggesting destruction of liposomes.

The relative calcein entrapment efficiency of liposomes after vortexing increased with increase in PEG lipid concentration up to 30 mol% (Fig. 8), indicating that the number of lamellae of liposomes decreases with increase in PEG lipid concentration. This might be due to steric repulsion caused by hydration of large ethylene oxide head groups. Belsito et al. [26] found by spectrophotometric measurements that addition of PEG lipids causes disaggregation of liposomes and a reduction in the number of lamellae. At a concentration of 40%, the relative calcein entrapment efficiency decreased. As was demonstrated in our previous study [23], EggPC liposomes containing 40% PEG lipid exhibit coexistence of large vesicles, small vesicles, and micelles. A reduction in entrapment efficiency might be due to partial micelle formation.

Inclusion of PEG lipid affects not only particle size but also vesicle lamellarity. An increase in the concentration of PEG lipid enhances the lateral repulsive properties of the surface of lipid bilayers 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. Therefore, curving of the bilayer to reduce the intensity of lateral repulsion is the most plausible explanation for the decrease in particle size with increase in PEG lipid concentration. PEG lipid also increases interlamellar repulsion, bringing about a decrease in lamellarity.

The particle sizes of liposomes prepared by both the mechanical dispersion and detergent-removal methods decreased with increase in PEG lipid concentration. While liposomes prepared by the former method became about 300 nm in diameter with 30 mol% of PEG lipid, the particle size of liposomes prepared by the latter method was much smaller, about 50 nm at the same mol% of PEG lipid. This discrepancy could have been caused by the difference in liposome preparation methods. We have shown that postvesiculation size growth is a crucial factor to regulate vesicle size in the process of vesicle formation by detergent removal from phospholipid/detergent mixed micelles; that is, initially formed vesicles are small, but the size increases slowly thereafter by fusion [36, 37, 38, 39]. In the present study, strong hydration around the head group in PEG lipid could have prevented contact among initially formed vesicles and subsequent fusion, resulting in limited size growth in the process of detergent removal.

Effect of number of freeze-thawing cycles on particle sizes

Repeated freezing and thawing of MLV containing PEG lipid caused a decrease in particle size (Fig. 5) and an increase in trapping efficiency (Fig. 8). However, the average of diameters of all EggPC/PEG lipid liposomes after five cycles of freeze-thawing are almost the same (200–300 nm) regardless of PEG lipid concentration (Fig. 7). The results of the determination of relative calcein entrapment efficiency (Fig. 8) showed that inclusion of PEG lipid results in an increase in the relative calcein entrapment efficiency ratio. These findings indicate that freeze-thawing of liposomes containing DSPE/PEG lipid results in the production of unilamellar liposomes more easily than does freeze-thawing of liposomes composed of EggPC alone (without PEG lipid). A dramatic increase in calcein entrapment efficiency was observed after PEG lipid had been incorporated into liposomes (Fig. 8), suggesting that PEG lipid has a marked effect on reduction in lamellarity. At a PEG lipid concentration of 0.5%, the calcein entrapment efficiency of freeze-thawed liposomes corresponds to that of extruded liposomes, as indicated by the relative calcein entrapment efficiency of about 1 (Fig. 8). A comparison of the relative calcein entrapment efficiencies of liposomes after vortexing and after freeze-thawing indicates that a unilamellar formation of freeze-thawed liposomes is achieved more easily than that of vortexed liposomes. This indicates that, in the presence of PEG lipids, the freeze-thaw process results in complete fragmentation of the original multilamellar vesicles into much smaller unilamellar vesicles. The results of particle size and calcein entrapment efficiency measurements indicate that, after having been dispersed in a buffer and subjected to five cycles of freeze-thawing, the average size and lamellarity of phospholipid vesicles containing PEG lipid are comparable to those of large unilamellar vesicles (LUV) obtained by the extrusion method.

It is well known that repetitive freeze-thawing enhances trapping efficiency due to the breakage of MLV and the formation of a more homogeneous vesicle population that appears to be LUV [27, 28]. Repeated freezing and thawing of MLV results in physical disruption of liposomal phospholipid bilayers, probably due to ice crystals formed during the freezing process. Bilayers may break off or partially fragment. Damaged bilayers will reassemble due to the "hydrophobic effect" and form new liposomes possibly of a different size. Since PEG lipid possesses a large hydrophilic head group, the presence of a strongly hydrated polyethylene group extending from the liposome surface may have sterically inhibited contact between vesicles, resulting in the formation of smaller unilamellar liposomes.

In summary, the present study demonstrated that PEG lipid had remarkable effects on particle sizes and lamellarity of liposomes. The average diameter and number of lamellae were reduced as the content of PEG lipid was increased. Reductions in particle size and number of lamellae were more significantly observed when liposomes were subjected to freeze-thawing.

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