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Effect of preformed egg phosphatidylcholine vesicles on spontaneous vesiculation of oleate micelles

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Abstract An addition of oleate micellar solution to two original sizes (180 nm and 50 nm) of preformed vesicles was studied using gel exclusion chromatography, dynamic light scattering and freeze fracture electron microscopy. The effect of molar ratios of phospholipid and oleate on size distribution of newly formed vesicles was investigated by varying molar concentrations of these two components. After adding an equiamount of oleate to 180 nm-preformed vesicles or 50 nm-preformed vesicles, a relatively monodisperse population of newly formed vesicles was detected. For the high amount of oleate addition to two original sizes of preformed vesicles, the results were quite different. New large vesicles and a number of new small vesicles were observed in samples of mixed EggPC/oleate suspension in the presence of preformed vesicles with 180 nm of size, whereas, only some new large vesicles were detected in samples of mixed EggPC/oleate suspension in the presence of preformed vesicles with 50 nm of size. We assumed that the number of new small vesicles, with size close to preformed vesicles, increased in the latter case. The transformation of mixed EggPC/oleate micelles to mixed vesicles was investigated. The results showed that transformation of mixed EggPC/oleate micelles to vesicles was remarkably faster than transformation of mere oleate micelles to vesicles. The above findings suggested that new mixed EggPC/oleate vesicles with small size were presumably formed by partial solubilization.

Keywords Micelle · Vesicle · Solubilization · Sodium oleate · Egg yolk phosphatidylcholine

Introduction

The studies on the size distribution and the vesicle morphology, after incorporation of surfactants on vesicles, have been continuously reported [1–4]. These reports mainly used surfactants that usually form micelle, whereas, fatty acids (a kind of surfactants) can form vesicles at a particular pH. With its unique property, size and size distribution of fatty acid vesicles at a variety of pHs have been studied [5–10]. Size distribution of the newly formed vesicles by the incorporation of fatty acids on preformed vesicles have been studied by Luisi's group [11–17]. Final size distribution in the presence of preformed vesicles was more monodispersed than in the absence of preformed ones, as if the preformed vesicles were the template of newly formed vesicles. This phenomenon was called "matrix effect" [11, 12]. Luisi et al. [13–17] have proposed the mechanism that monomers were uptaken by preformed vesicles following fission.

Our previous study focused on the size distribution of mixed EggPC/oleate vesicles after the addition of oleate

to two different sizes of preformed vesicles [18]. When the oleate micellar solution was added to 230 nm-extruded vesicles, new vesicles with larger and smaller sizes than those of preformed vesicles were observed. In contrast, when the oleate solution was added to 50 nm-preformed vesicles, the new vesicles appeared to be larger than the preformed ones. As the model, supposed by Luisi et al., was not enough to explain our results, we started the following experiment.

In the present works, size distribution of mixed EggPC/oleate vesicles was investigated in detail by varying molar concentrations of these two components. The distributions of EggPC and oleate in the newly formed vesicles were also examined. Though there are many reports that fatty acids can rapidly and quantitatively bind to the phospholipid vesicles [19–22], the distribution of phospholipid and oleate in the newly formed vesicles after the addition of oleate to preformed vesicles still remains unclear.

We found that the size regulation of newly formed vesicles was strongly dependent on the amount of oleate added to preformed vesicles. Addition of small amount of oleate led to a narrower size distribution than adding a large amount of oleate. The size distribution after adding a high amount of oleate to preformed vesicles became heterogeneous with the mixture of newly large and small vesicles. Furthermore, the presence of new small vesicles with diameter of 45–60 nm was found at any amount of oleate addition to preformed vesicles. In order to clarify this phenomenon, we studied the relationship of EggPC/oleate mixtures and vesicular sizes. The transformation of mixed EggPC/oleate micelle to mixed vesicles was also investigated. Based on these experiments, the mechanism of new small vesicle formation was discussed.

Materials and methods

Materials

Egg yolk phosphatidylcholine (EggPC; purity of PC = 98.8 %) was purchased from Nihon Yushi (Tokyo, Japan). Sodium oleate (cis-9-octadecenoic acid sodium salt, 99%) was obtained from Sigma (St. Louis, MO, USA). Boric acid (99.5%) and other reagents were purchased from Nacalai Tesque (Kyoto, Japan). All these materials were used without further purification.

Preparation of vesicle suspensions

A desired amount of EggPC was dissolved in chloroform/methanol (2:1, v/v) and dried into a thin film with the nitrogen gas stream. After complete removal of residual traces of organic solvent, the lipid films were dispersed in a buffer containing 150 mM NaCl and 0.1 M borate (pH 8.5) by vortexing, forming multilamellar vesicles (MLV) suspension. The MLV were sized down by repeated extrusions through polycarbonate filters (Nucleopore, Costar Co., USA) using THE EXTRUDER (Lipex Biomembranes) after five times freeze-thaw cycles. Repeated extrusions (ten times) were made first by using two stacked filters with pores of 400-nm size and then by two stacked 200-nm filters. The resulting vesicular size was roughly 180 nm by quasi-elastic light scattering.

Small unilamellar vesicles (SUV) were prepared by five cycles of sonication of the lipid suspension cooled in an ice-water bath for 10 min with a rest time for 10 min. The sonicated dispersion was centrifuged at 50,000 rpm for 1 h at 4 °C to remove titanium and lipid debris.

Preparation of oleate vesicles and mixed EggPC/oleate vesicles in borate buffer

A 200-μl aliquot of an aqueous sodium oleate solution was added into a 800-μl borate buffer solution (pH 8.5) or a 800-μl of the preformed EggPC vesicle suspension at various concentrations followed by 1 min stirring. The mixed EggPC/oleate vesicular suspensions were run through gel filtration chromatography, 1 day after oleate addition. The total final concentration of mixed EggPC/oleate vesicles was kept at 30 mM. In the case of 180-nm preformed vesicles, the final molar ratio of phospholipid:oleate was 2:1, 1:1, 1:2, 1:5 and 1:9. The variation of molar ratios of phospholipid (in 50-nm preformed vesicles):oleate was 1:1 and 1:9. Oleate vesicles and the mixed EggPC/oleate vesicles in the molar ratio of 1:1 were used for studies on time progress of vesicle formation at 5 min and 1 h after mixing by freeze-fracture electron microscopy. The morphology of oleate vesicles was also performed after 15 min mixing. Furthermore, size and morphology of the mixed EggPC/oleate vesicles of molar ratio 1:1 and 1:9 were studied with freeze-fracture electron microscopy.

Optical density measurement

Optical density measurement was performed with a UV-160A spectrophotometer (Shimadzu) at 400 nm, using a quartz cell with a path length of 1 cm. A solution of 200 μl of 20-mM oleate micelle solution was added either to 800 μl of borate buffer (pH 8.5), or to 800 μl of 2.5 mM, or 800 μl of 0.05 mM preformed EggPC vesicles. The final concentration of mixture was approximately 4 mM and the final molar ratio of EggPC/oleate was 1:1 and 1:100. The size of preformed vesicles was 180 nm.

Gel filtration chromatography

Fractionation of the EggPC/oleate vesicles by gel filtration chromatography through Sephacryl S-1000 (Pharmacia Biotech, Sweden) column (1×48 cm) was performed as described before [23]. Each of mixed EggPC/oleate suspension was previously passed through the gel column several times before applying samples in order to avoid possible absorption. Two hundred microliters of mixed vesicle suspensions with concentration of 30 mM was loaded on the column. Extruded EggPC vesicles (180 nm) with concentration of 20 mM and extruded oleate vesicles (180 nm) with concentration of 15 mM were also applied on the gel column as control. The void volume and total volume of the column were 8.60 ml and 18.67 ml, respectively. The phospholipid concentration in each gel filtration fraction was determined based on the lipid phosphorus according to Ames [24]. The vesicle sizes in the fractions containing phospholipid were measured by a laser particle-size analyzing system (Photal LPA-3000/3100, Otsuka Electronics, Osaka, Japan) and quantification of oleic/oleate by HPLC.

Determination of oleic/oleate concentration

The amount of oleic/oleate was determined by high-performance liquid chromatography (HPLC) on a LC-6A Liquid chromatography with SPD-6A UV spectrophotometric detector (Shimadzu, Japan). The UV absorbance spectra of oleic acid were recorded with a chromatopac model C-R6A (Shimadzu, Japan). The HPLC condition was slightly modified from the method of Schmitt and Lehr [25]. The separations were carried out on a Nucleosil 100 C_{18} analytical column (150×3.2 mm, I.D., particle size 5 μM) protected with a Waters C_{18} guard column. A 20-μl of each sample, which contained 4-undecyloxybenzoic acid as an internal standard (0.6 μg/100 μl) was injected onto the HPLC system. The slightly unclear fractions were diluted with methanol before injection. The mobile phase was acetonitrile–water–phosphoric acid (85%) (80:20:0.1, v/v/v) and was routinely filtered before use. The chromatographic system employed a flow-rate of 0.4 ml/min and a spectrophotometric detection at 200 nm.

Electron microscopy

The microstructures of oleate vesicles and EggPC/oleate vesicles were examined by freeze-fracture method as described previously [26]. The samples were mixed with 30 wt% glycerol to avoid crystallization during freeze process [27]. Then, the samples were cryofixed by dipping into a suspension at −96 °C of liquid nitrogen and fractured them at −120 °C with a freeze replica apparatus (FR-7000B, Hitachi, Tokyo, Japan). The preparations were subsequently shadowed using Pt/C at an angle of 45° and then carbon-backed. The cleaned replicas were mounted on 300-mesh Ni grids and examined at 5,000–50,000× magnification with a JEOL JEM-200 CX electron microscope. Each sample was examined for a large number of areas and the micrographs shown were selected to give as representative picture of each one. The final concentration of each freeze fracture sample was kept constant at 15 mM.

Results

Time course in the process of oleate or mixed EggPC/oleate vesicles formation

Figure 1 represents time course of optical density change, when oleate solution is added to borate buffer (pH 8.5) and to the suspensions of preformed vesicles. The optical density change during the first 10 min is faster in the presence of even small amount of preformed vesicles (EggPC:oleate = 1:100). The change of optical density is remarkably faster with the higher amount of preformed ones (EggPC:oleate = 1:1).

Freeze-fracture electron micrographs of oleate vesicles and mixed EggPC/oleate vesicles in the ratio of 1:1 were shown in Fig. 2. Figure A, B and C show structural change of oleate vesicles at 5 min, 15 min and 1 h after addition of oleate micellar solution to borate buffer (pH 8.5). At 5 min, there were some preliminary structures with rough surface in the size range of 40–120 nm (Fig. 2a).

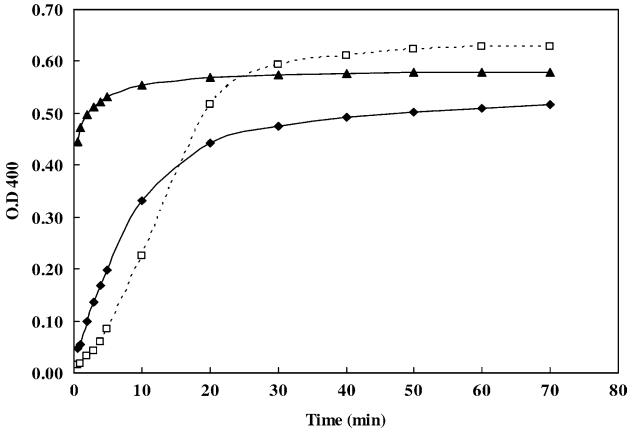

Fig. 1 Time courses of optical density of oleate vesicles (*open square*) EggPC/oleate (1:1) vesicles (*filled triangle*) and EggPC/oleate (1:100) vesicles (*filled diamond*) in borate buffer (pH 8.5)

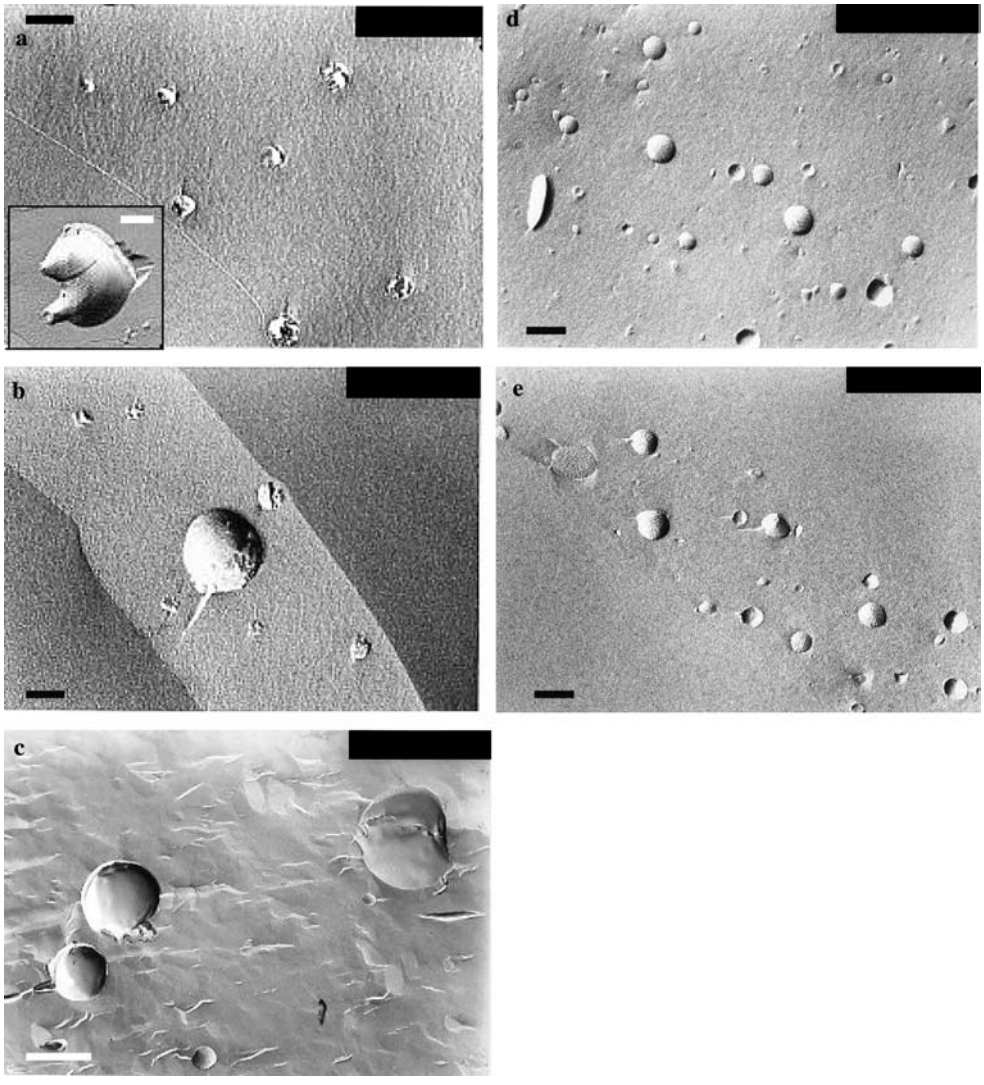

Fig. 2 Microscopic study of time course in the process of vesicle formation. **a–c** Display oleate vesicles at different time after adding oleate to borate buffer (pH 8.5) at 5 min (**a**), 15 min (**b**) and 1 h (**c**). **d, e** Display mixed phospholipid/oleate vesicles (EggPC:oleate = 1:1) after adding oleate to 180-nm preformed vesicles at 5 min (**d**) and 1 h (**e**). The *inset* picture in Fig. 2a shows a structure of oleate vesicle with one side convex at 5 min. *Black bars* in **a, b,d** and **e** correspond to 200 nm; *white bar* in **c** and *inset* picture correspond to 1 μm

The one side convex structure with smooth surface was also found as shown in inset picture. Freeze fracture micrograph of oleate vesicles after 15 min mixing (Fig. 2b) reveals somewhat round shape structure with a diameter of 400 nm in coexistence with some morphologies similar to that found in Fig. 2a. One hour later after mixing, the preliminary structure is disappeared and a broad size distribution of oleate vesicles is obtained as shown in Fig. 2c. The oleate vesicular size varied from 200 nm up to 1.5 μm.

A system of EggPC:oleate in molar ratio of 1:1 at 5 min and 1 h after mixing is displayed in Fig. 2d, e. The appearance and size of mixed EggPC/oleate vesicles seemed to remain unaltered upon time change. Mixed vesicles appeared round shape with the size range of 50–165 nm.

The distribution of EggPC and oleate in newly formed vesicles

Phospholipid and oleate concentration of mixed EggPC/oleate vesicles at different molar ratios are plotted against elution volume in Fig. 3. The pattern of preformed EggPC vesicles (vesicular size = 180 nm) and oleate vesicles (vesicular size = 188 nm) are also presented in Fig. 3a. Gel filtration chromatograms of both preformed vesicles reveal only a single peak at low elution volume. In contrast, all profiles of mixed EggPC/oleate vesicles at different molar ratios display new peak at higher elution volume (Fig. 3b–f) in addition to the peak at low elution volume like Fig. 3a. The elution patterns of phospholipid as shown in Fig. 3b–f are correlated to oleic/oleate patterns. The oleate and EggPC concentrations at elution volume of 8.00–

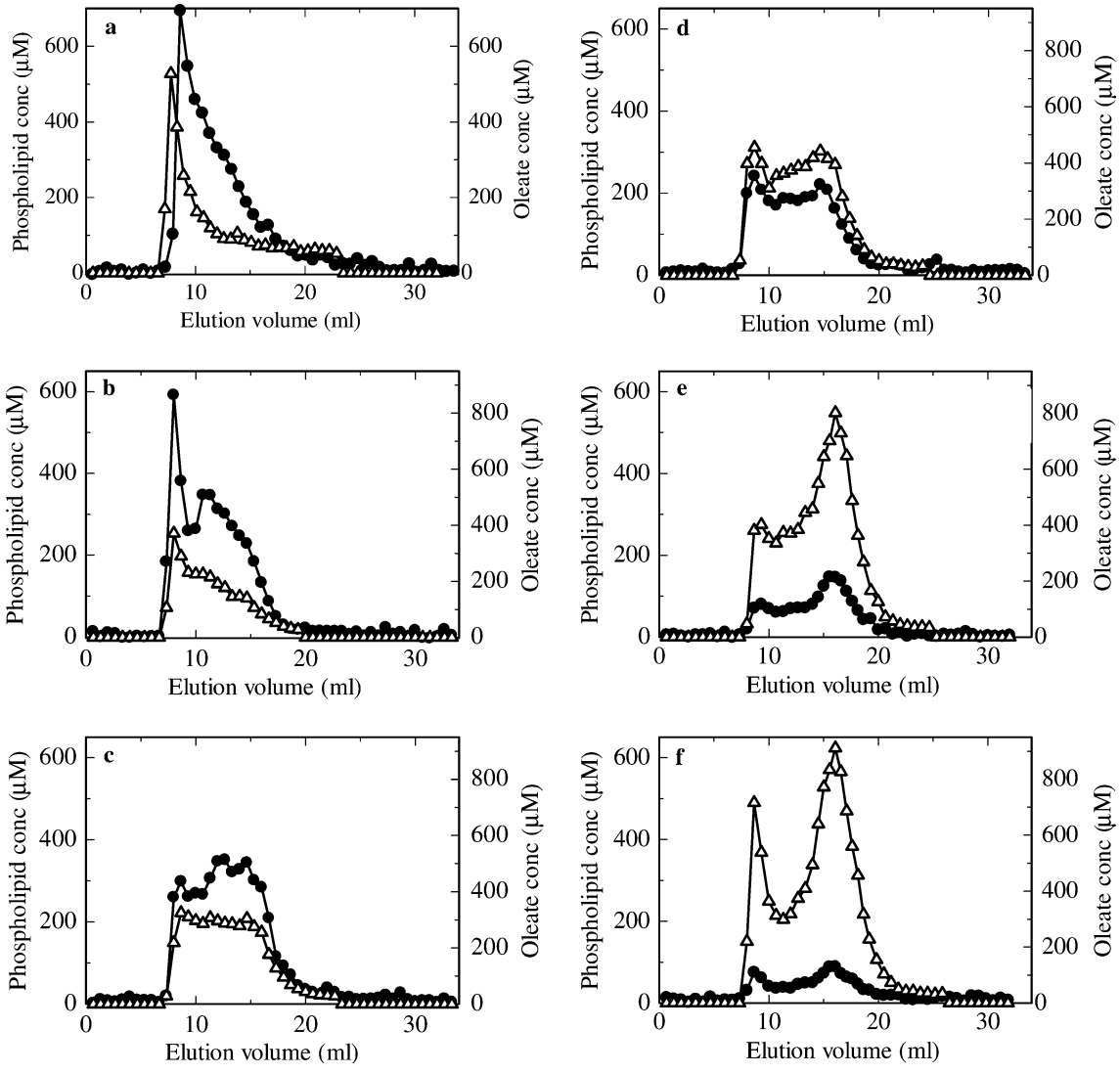

Fig. 3 Elution profiles of phospholipid and oleate concentration of 180-nm preformed EggPC vesicles and mixed vesicles at various molar ratios in borate buffer (pH 8.5). **a** 180-nm preformed EggPC vesicles and 188 nm oleate vesicles; **b** EggPC/oleate (2:1) vesicles; **c** EggPC/oleate (1:1) vesicles; **d** EggPC/oleate (1:2) vesicles; **e** EggPC/oleate (1:5) vesicles; **f** EggPC/oleate (1:9) vesicles. (*filled circle*) phospholipid concentration; (*open triangle*) oleate concentration

8.67 ml were assigned to large vesicles, whereas, the concentrations at elution volume of 11.33, 14.67 and 16.07 ml were representative to the small vesicles. The ratios of oleate/EggPC concentration at top of two peaks representative to large and small vesicles against each formulation of mixed EggPC/oleate vesicles are displayed in Fig. 4. As total concentration of each mixed EggPC/oleate formulation was constant at 30 mM, EggPC and oleate in molar ratio of 2:1, 1:1, 1:2, 1:5 and 1:9 correspond to the concentration of EggPC(mM):oleate(mM) at 20:10, 15:15, 10:20, 5:25 and 3:27, respectively. Thus, the ratio of molar fraction of oleate/EggPC of each formulation was 0.5, 1.0, 2.0, 5.0 and 9.0. These values were correlated well to the final ratios of oleate/EggPC concentration at top of two peaks shown approximately as 0.6, 0.9, 2.0, 5.4 and 10. However, at the mixed EggPC/oleate vesicles at 1:2, 1:5 and 1:9, the ratio of oleate/EggPC concentration of small vesicles was slightly larger than that of the large ones.

Figure 3b–f also shows the profiles change of phospholipid and oleate at various molar ratios of mixed EggPC/oleate vesicles. After addition of oleate micellar solution to 180-nm preformed vesicles, the new peaks were observed as described previously. When the molar ratio of EggPC is higher than the molar ratio of

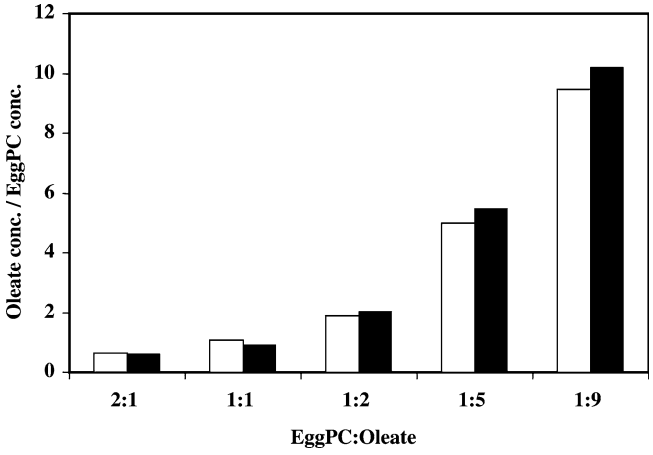

Fig. 4 The relationship between oleate concentration (μM) /EggPC concentration (μM) in both large and small vesicles and molar ratios of EggPC:oleate of each formulation. Each concentration was calculated from the top of the peak in the elution profile of gel filtration. (*open square*) large vesicles; (*filled square*) small vesicles

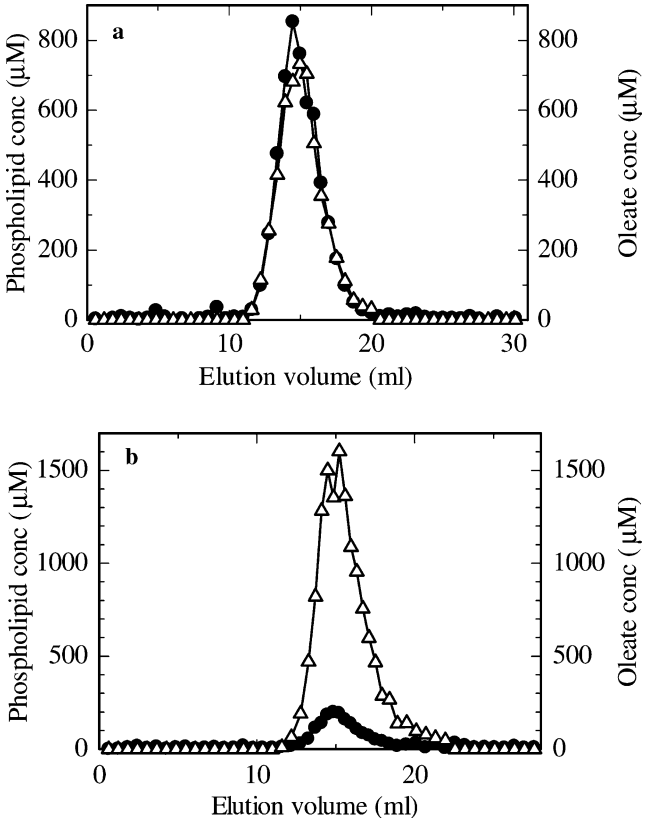

Fig. 5 Elution profiles of phospholipid and oleate concentration of mixed EggPC/oleate vesicles (1:1) (**a**) and mixed EggPC/oleate vesicles (1:9) (**b**). The size of preformed vesicles was 50 nm. (*filled circle*) phospholipid concentration; (*open triangle*) oleate concentration

oleate, new peak position is still adjacent to the first peak of large preformed vesicles (EggPC/oleate at molar ratio = 2:1, Fig. 3b). The area of this new peak is larger and shift to higher elution volume in the case of EggPC/oleate at molar ratio of 1:1 (Fig. 3c). A continuous increase in molar ratio of oleate led to further shift of this second peak to higher elution volume corresponding increase of peak area. This incident is remarkably observed in the EggPC/oleate at molar ratio 1:5 and 1:9 (Fig. 3e, f). At EggPC/oleate dispersion (mole ratio 1:9), there were two peaks which were partly resolved. The peak containing main area at higher elution volume indicated that most of the lipid was small vesicles.

The phospholipid and oleate patterns of mixed SUV/oleate at molar ratio of 1:1 (Fig. 5a) are not much different from mixed SUV/oleate at molar ratio of 1:9 (Fig. 5b). The final molar ratios of oleate/EggPC calculated from top of each peak appeared to be 0.86 and 8, representative to the EggPC/oleate vesicles at molar ratio 1:1 and 1:9, respectively.

Effect of equimolar and higher molar concentration of oleate on newly formed vesicles

Figure 6 shows some representative electron micrographs of 180 nm EggPC vesicles before and after oleate addition. Micrograph obtained from 180-nm preformed vesicles is dominated by spherical shape with a size in the range of 100–150 nm (Fig. 6a). Micrograph of mixed EggPC/oleate vesicles in the molar ratio of 1:1 (Fig. 6b) reveals mixed vesicles with similar size to that found for preformed ones and some small vesicles are also found. At high concentration of oleate (EggPC/oleate = 1:9), small vesicles are observed together with large vesicles (Fig. 6c). In addition to size distribution, this sample revealed morphological alterations (see inset) and also vesicles with rough surface (arrow).

The micrographs of 50-nm preformed vesicles and mixed EggPC (50-nm preformed vesicles)/oleate at the ratio of 1:1 and 1:9, respectively, were demonstrated in Fig. 7a–c. Sample containing equimolar amounts of EggPC and oleate, corresponding to Fig. 7b, does not reveal any changeability of vesicular size or structure when compared to 50-nm preformed vesicles (Fig. 7a). When the molar fraction of oleate is increased (EggPC/oleate = 1:9), large mixed vesicles with diameter of about 120–200 nm can be visualized in coexistence with small vesicles having diameters ranging from 40 nm to 60 nm (Fig. 7c). A larger mixed vesicle of about 300-nm diameter appeared in the other micrograph (data not shown). Mixed vesicles with rough surface marked by arrow were also found in this mixed suspension.

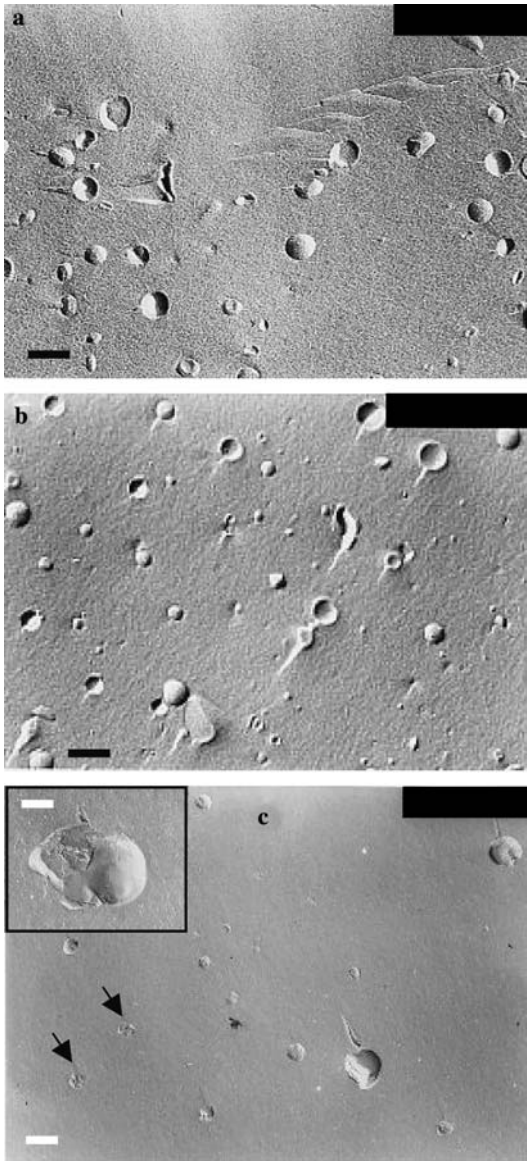

Fig. 6 Freeze fracture electron micrographs of 180-nm preformed vesicles (**a**), mixed EggPC/oleate (1:1) vesicles (**b**) and mixed EggPC/oleate (1:9) vesicles (**c**). The *inset* picture in Fig. 6c shows a morphological change of mixed EggPC/oleate (1:9) vesicles. These mixed vesicles were prepared by oleate addition to 180-nm preformed vesicles. The lengths of *black bars* in **a, b** correspond to 200 nm; the lengths of *white bars* in **c** and *inset* picture correspond to 400 nm

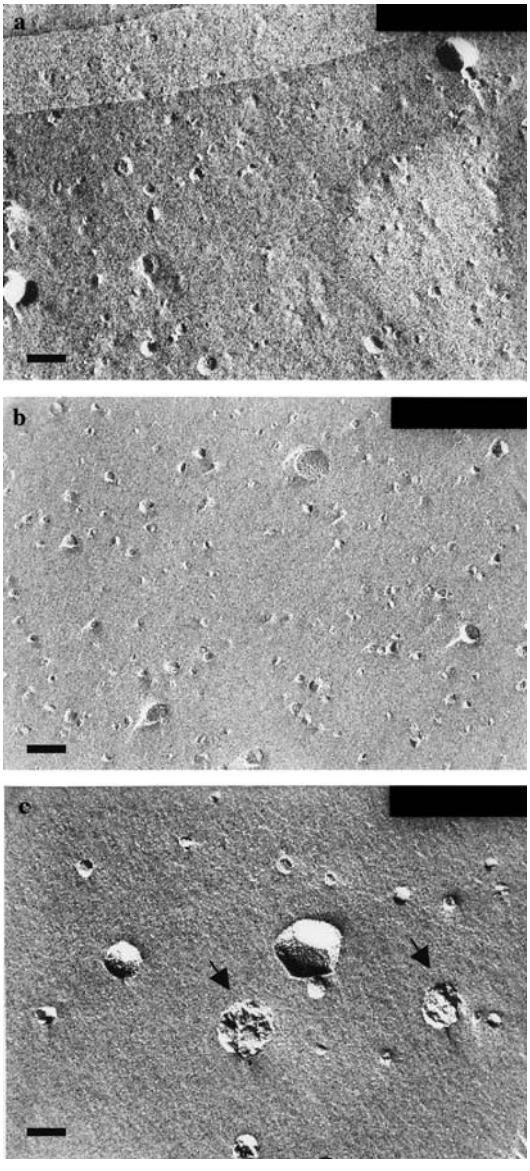

Fig. 7 Freeze fracture electron micrographs of 50-nm preformed vesicles (**a**), mixed EggPC/oleate (1:1) vesicles (**b**) and mixed EggPC/oleate (1:9) vesicles (**c**). These mixed vesicles were prepared by oleate addition to 50-nm preformed vesicles. The lengths of bars in **a, b** and **c** correspond to 100 nm

Discussion

Time course in the process of oleate and mixed EggPC/oleate vesicles formation

Optical density change upon oleate addition to borate buffer (pH 8.5) or to preformed vesicles in Fig. 1 indicates that the process of mixed EggPC/oleate vesicle formation is markedly fast in the presence of preformed vesicles as reported before [11–18]. Even though the oleate solution was added to small amount of preformed vesicles (EggPC:oleate at molar ratio = 1:100), it could still accelerate the mixed vesicle formation during the first 10 min. The micrographs in Fig. 2a, b demonstrate the morphological change of oleate vesicles, which is not complete in short time (< 15 min). On the other hand, in the presence of preformed vesicles (EggPC/oleate = 1:1) the uncompleted structure of mixed vesicles was not found even at the first 5 min (Fig. 2d). This indicated

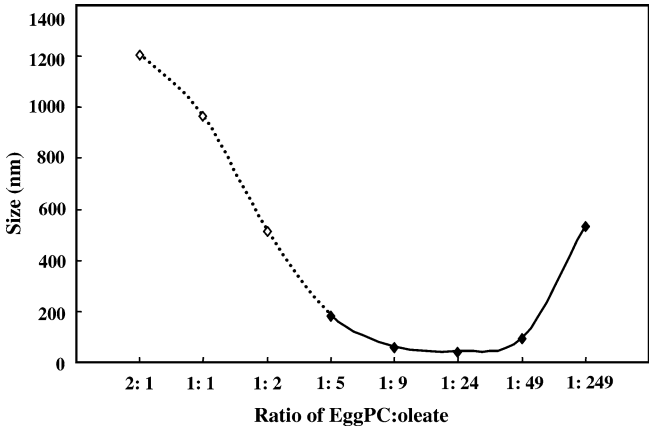

Fig. 8 The relationship between various molar ratios of EggPC/oleate and average size of vesicles after addition of mixed EggPC/oleate micelle suspensions in borate buffer (pH 8.5). (*open diamond*) is marked to the addition of turbid suspensions in borate buffer, whereas (*filled diamond*) is marked to the addition of clear solutions in borate buffer

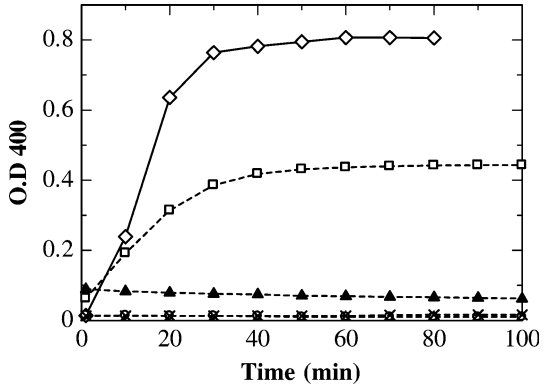

Fig. 9 Time courses of optical density of oleate vesicles and mixed EggPC/oleate vesicles at various ratios in the borate buffer (pH 8.5). A solution of 200 µl of oleate micelle solution or a variety of mixed EggPC/oleate suspensions/solutions was added to 800 µl of borate buffer. Oleate vesicles (*open diamond*), molar concentration of EggPC/oleate: 1:9 (*filled triangle*), 1:24 (*cross*) 1:49 (*open circle*) 1:249 (*open square*)

that mixed EggPC/oleate vesicles were absolutely formed within 5 min and morphology of these mixed vesicles was not changed after 1 h. Consequently, these micrographs could support the acceleration of mixed vesicle formation with the presence of preformed vesicles.

The distribution of EggPC and oleate in newly formed vesicles

As is presented in Fig. 3b–f, the phospholipid distribution patterns are coordinate to the oleate distribution patterns. The final ratios of oleate/EggPC in large and small vesicles, shown in Fig. 4, appear that the ratios are nearly corresponded with the initial concentrations of each agent. However, the distribution of oleate/EggPC among large and small vesicles is slightly different (Fig. 4). With a increase in added oleate content, the oleate/EggPC proportion in small vesicles seemed to be higher than the oleate/EggPC proportion in large ones. This result could be hypothesized that these new small vesicles might contain a little higher amount of oleate than EggPC.

Effect of molar ratio on newly formed EggPC/oleate vesicles

As shown in Fig. 3b–f compared to pure EggPC and oleate vesicles in Fig. 3a, the oleate addition to preformed vesicles of size 180 nm give rise to the additional peak at higher elution volume. This is correlated well with our earlier study that the oleate addition to 230-nm preformed vesicles indicated a mixture of large and small vesicles [18]. The addition of oleate to 180-nm preformed EggPC vesicles at molar ratio of EggPC/oleate from 2:1, 1:1 and 1:2 (Fig. 3b–d) yielded sizes of new small vesicles in the range of 135–150 nm (vesicular size of the second peaks) and large vesicles in the range of 190–195 nm (vesicular size of the first peak).

At the higher amount of oleate addition (EggPC/oleate = 1:5, 1:9), the size of new small vesicles appeared to be smaller and the population likewise increased (Fig. 3e, f). It is apparent that the higher the molar ratio of sodium oleate, the higher the formation of new small vesicles. The vesicular size of new small vesicles at second peak was around 120–130 nm. But the size distribution of these new peaks measured by dynamic light scattering was not sharp, these fractions also contained small vesicles with size less than 100 nm. As have been known, size measurement by dynamic light scattering is susceptible to larger particles more than smaller ones [28]. The position of these second new peaks was close to those of pure 50 nm EggPC vesicles (data not shown). The aggregates corresponding to 16.07 ml of elution volume may contain some micelles in the light of the report [29], but it could not be verified in the present experiments.

Freeze-fracture electron microscopy was attempted to observe the appearance of mixed EggPC/oleate vesicles. When oleate solution is added to equimolar amount of 180-nm preformed vesicles, it displays new small vesicles (Fig. 6b) as similar as previously described [14, 15, 17]. Although these small ones are also found in pure EggPC vesicles (Fig. 6a), the number of them seem to increase after oleate addition (Figs. 2d, 6b). This presented micrograph was therefore in good agreement with gel filtration pattern (Fig. 3c).

The micrograph of EggPC/oleate at 1:9 confirms the high degree of heterogeneity in the particle size distribution (Fig. 6c). This micrograph and the elution profiles, in Fig. 3f, revealed new large vesicles corresponding with a number of new small vesicles as similar as the results of Lonchin et al. [12] and Berclaz et al. [13].

After the addition of oleate solution to 50-nm preformed vesicles, some new large vesicles are found in the micrograph of mixed EggPC/oleate vesicles at ratio of 1:9 (Fig. 7c). It was apparent that the growth of mixed vesicles became substantial when fatty acid concentration was much higher than phospholipids concentration. This result is also correlated well to our prior study [18] and similar behavior observed by Cryo-TEM has previously reported [9]. Although some large vesicles were visualized, gel filtration peak was at the same position. It was presumably due to a few number of these large vesicles. On the other hand, the number of new small vesicles with a diameter around 45–60 nm increased. It implied that the main population of new vesicles after higher amount of oleate addition to SUV was small vesicles having size similar to preformed vesicular size.

To obtain a better insight into the vesicle sizes of mixed EggPC/oleate, the following experiment was carried out by preparing mixed lipid films of EggPC/oleate at various ratios. These mixed lipid films were solubilized with pure water prior adjusted pH to 12.5 with 1 N NaOH. After dissolved with water, the mixed films of EggPC/oleate at molar ratio of 1:5, 1:9, 1:24, 1:49 and 1:249 were clear solution, whereas, the others (2:1, 1:1 and 1:2) were turbid. Then, 200 μl of these suspensions were added into 800 μl of borate buffer (pH 8.5). Vesicle size and turbidity against time were further measured. The relationship of average sizes of mixed vesicles and molar ratio of EggPC/oleate is illustrated in Fig. 8. This preliminary experiment indicated a tendency that the higher the molar fraction of oleate the smaller the size of mixed vesicles. However, at much higher molar ratio of oleate than EggPC (1:49 and 1:249), vesicular size became larger. Therefore, the smallest mixed vesicles that can be formed were in the range of 45–60 nm (EggPC/oleate at 1:9 and 1:24). This study suggested that mixed EggPC/oleate micelles can spontaneously form mixed vesicles with smaller size dependent on increasing oleate molar ratio when the molar ratio is not too much. This tendency was likewise observed for mixed EggPC/oleate vesicles prepared by dialysis method (data not shown). It has been well known that a mixture of phospholipid and single chain surfactant forms smaller size vesicles than phospholipid alone, and incorporation of single chain surfactant to preformed vesicles brings about fission to make small vesicles due to geometrical shape of the surfactant molecule [3, 30, 31]. Similar results are reported about fatty acid to a lipid bilayer [32, 33].

The time progress of turbidity after addition of mixed micellar solutions to borate buffer is shown in Fig. 9. It was apparent that the transformation of mixed EggPC/oleate micelle into mixed vesicles was very fast. Therefore, incorporation of phospholipid in oleate micelles could accelerate vesicle formation more than mere oleate micelles. However, the rate of mixed vesicle formation became slower with increase in oleate molar fraction. These findings indicated that the proper ratio of mixed EggPC/oleate micelles could easily form vesicles. Luisi et al. has proposed a mechanism that oleate molecules were uptaken by phosphatidylcholine vesicles followed by fission [13–17]. However, the results led us to postulate another mechanism that these new small vesicles may be formed from the partial solubilization of phosphatidylcholine vesicles by added oleate micelles followed by rapid vesiculation of lipid-containing oleate micelles. In the absence of lipid, the transition from micelles to vesicles requires lag time over 1 h, while the lipids-containing oleate micelles can abruptly change to small vesicles, although it is unknown whether oleic acid and oleate in mixed micelles make the pair or not, which has been pointed out in oleic acid/oleate vesicle formation [29].

The results obtained in this study could describe a more detailed characteristic after oleate addition to preformed vesicles. Herein, the regulation of size distribution of newly formed vesicles was dependent on the amount of oleate added to preformed vesicles. The size distribution of mixed vesicles was nearly monodisperse at eqimolar ratio of EggPC/oleate. Increase of added oleate content led to a broad size distribution of newly formed vesicles. Based on the experimental results of components in new small vesicles and easy transformation from mixed EggPC/oleate micelles to mixed small vesicles having size in the range of 45–60 nm, partial solubilization was proposed as a possible mechanism of this phenomenon.

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