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ORIGINAL CONTRIBUTION

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Study on size growth in the vesicle formation upon detergent removal from phospholipid/detergent mixed micelles

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Abstract When 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) was removed from the mixed CHAPS/EggPC micelles, large vesicles were prepared by dialysis or by slow step-by-step dilution, but small vesicles were prepared by fast one-step dilution. When sodium cholate was removed from the sodium cholate/EggPC micelles, small vesicles formed either by dialysis or by dilution; however, in the presence of 5 mM Ca^{2+} large vesicles were produced by dialysis, while small vesicles were prepared by dilution. The size growth was related to a detergent-induced fusion of the vesicles containing a large amount of detergent. Using spectrophotometry, quasielastic light scattering and freeze–fracture electron microscopy the fusion events were investigated both through the process of vesicle solubilization by adding detergent and through the process of vesicle formation by diluting a mixed micelle. The results suggest that a rapid CHAPS-induced fusion of the vesicles led to the large resultant vesicles and that no fusion of vesicles containing sodium cholate is responsible for the formation of small vesicles. Furthermore, the ultimate vesicle size related to rapid or slow detergent removal is dependent on the kinetic aspects of the fusion.

Key words Vesicle formation · Detergent removal · Size growth · Detergent-induced fusion · Different dilutions

Introduction

The mechanism of the micelle–vesicle transition has been studied in close relationship to the functional reconstitution of membrane proteins [1–3] and to some physiological activities [4]. The size of the vesicles formed upon detergent removal from mixed detergent/phospholipid micelles is dependent on the kind of detergents and the removal method. For example, larger vesicles were prepared by dialysis than by depleting the detergent with hydrophobic porous beads or gel filtration and the size of vesicles prepared by removing octyl glucoside by dialysis was large, while that obtained by removing sodium cholate, sodium dodecyl sulfate (SDS) or poly(oxyethylene alkyl ether) was small. Early work by Almog et al. [5] described that the overall process of vesicle formation upon the depletion of sodium cholate involved a rapid prevesiculation equilibration of the micelle, a relatively rapid membrane closure to form small vesicles and a slow postvesiculation size growth of the initially formed small vesicles, which was induced by lipid exchange. On the other hand, the investigation of small vesicles containing a large amount of detergent in the process of small unilamellar vesicle (SUV) solubilization by a detergent, octyl glucoside, has provided convincing evidence to indicate that a detergent-induced fusion of those small vesicles increases their size, giving rise to large vesicles [6]. Furthermore, Rotenberg and Lichtenberg [7] diluted infinitely the mixed micelles of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/EggPC, leading to a small vesicle, which was in contrast to the large vesicles formed by

dialysis, and pointed out that if the detergent-induced size growth processes were faster than detergent removal, size growth would occur.

The previous studies in our laboratory on the solubilization of vesicles by detergent have shown that small vesicles containing a large amount of detergent can fuse to increase their size time-dependently, resulting in the formation of large vesicles if octyl glucoside is used as a detergent, but they are not fusable if sodium cholate is used. It was suggested that the inhibition of fusion could be due to an electrostatic repulsion on the surface of the vesicles [6]. In this perspective, in the present study we used sodium cholate in the presence of Ca^{2+} or CHAPS as a detergent to monitor the behavior of the vesicles containing detergent and to induce size growth. The size growth was focused on the fusion of the vesicles containing a large amount of detergent. The fusion events were observed through the process of the vesicle solubilization by detergent. Furthermore, the effect of the fusion on the formation of large vesicles was investigated by many methods related to slow or fast detergent removal and the kinetic behavior of SUV* (small vesicles containing a large amount of detergent) fusion is discussed regarding the ultimate vesicle size.

Materials and methods

EggPC (egg yolk lecithin) was obtained from Nihon Yushi, Co., Japan. Sodium cholate was from Nacalai Tesque (Kyoto, Japan) and CHAPS was purchased from Wako Chemicals (Osaka, Japan). All samples were used without further purification.

EggPC was dried overnight under vacuum after the removal of $CHCl_3/CH_3OH$ and was suspended at a concentration of 25 mM in the buffer system of 150 mM NaCl and 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.0). The SUV suspension was prepared by sonication using a probe-type sonicator at 4 °C, then diluted to 10 mM by the buffer as a stock SUV suspension.

The vesicle solubilization was performed by adding detergent solutions with a series of concentrations to SUV suspensions prepared by sonication with a syringe while stirring the suspensions. The phospholipid concentration was fixed at 5 mM. The solubilization process was observed by turbidity and size measurements of those suspensions. The turbidity, as an indicator of the structural change of aggregates, was measured at 350 nm using a UV spectrophotometer (UV-160, Shimadzu, Japan) and the size was measured using a quasielastic light scattering apparatus (LPA-3000, Otsuka electronics, Japan). To ensure the same experimental conditions, the same mixed micelles formed by the SUV solubilization were used in the preparation of vesicles from the detergent removal either by dialysis or by dilution. The dialysis of the mixed micelles was carried out 2 times in 1 l of the buffer for 1 ml of the micelle suspension. The tenfold dilution of the mixed micelles into the resultant vesicles was in two ways: slow step-by-step dilution and fast one-step dilution. In the former the micelles were diluted ten times, each time at intervals of one-tenth of the total phospholipid concentration, with an average equilibration time of 20 min and in the latter the micelles were diluted to the resultant vesicles only in one step. The initial mixed micelles contained 5 mM EggPC. The process of vesicle formation by dilution was also demonstrated in terms of the changes in turbidity and size. The phospholipid concentration was determined as phosphorous according to Ames [8].

Negative stained vesicle preparations for electron microscopic observation were made by mixing a vesicle suspension with phosphotungstate $[H_3(PO_4W_{12}O_{36})\cdot 14H2O]$ solution (2%) and incubating the mixture for about 1 min. The freeze-fractured preparations of vesicles or micelles were made by flash-freezing of a suspension at −160 °C of a liquid Freon and fracturing it under vacuum (6×10^{-6} torr). Those preparations were shadowed using platinum at a 45° angle and then carbon-backed, all at −120 °C under high vacuum. The cleaned, shadowed replicas on carbon-coated films on copper grids were examined at 80 kV, usually at 30,000× magnification with an electron microscope (JEM-200CX, Jeol, Japan).

Results

To investigate the influence of different kinds of detergents on the vesicles, the sizes of vesicles formed by removing sodium cholate or CHAPS were examined. The results obtained are shown in Table 1 (phospholipid concentration of about 5 mM). Under our experimental conditions, the size of the vesicles formed upon the removal of sodium cholate was about 50 nm, in the presence of Ca^{2+} it was about 150 nm and with CHAPS it was about 380 nm. In Fig. 1A the large vesicles formed by removing sodium cholate in the presence of 5 mM Ca^{2+} are multilamellar ones. CHAPS is a zwitterionic derivative of a trihydroxy bile salt and has the same steroid structure in the hydrophobic moiety as sodium cholate, but no net charge in the hydrophilic one. From the viewpoint of molecular structure it is suggested that the anionic headgroup of sodium cholate affected the size of the resultant vesicles. Recently the size growth of vesicles was suggested to be a determining

Table 1 The size change with the different detergent removal (nm)

Ways of detergent removal	Phospholipid:CHAPS		Phospholipid: Chol-Na	Phospholipid:Chol-Na/Ca	
	5 mM:7 mM	5 mM:4 mM	5 mM:10 mM	5 mM:7 mM	5 mM:6 mM
Dialysis	46 → 380[a]		40 → 50	50 → 150	
Step-by-step dilution	46 → 335	300 → 345	40 → 45	50 → 60	117 → 90
One-step dilution	46 → 40	300 → 270	40 → 38	50 → 46	117 → 60

[a] The size of initial mixed micelles → the size of resultant vesicles

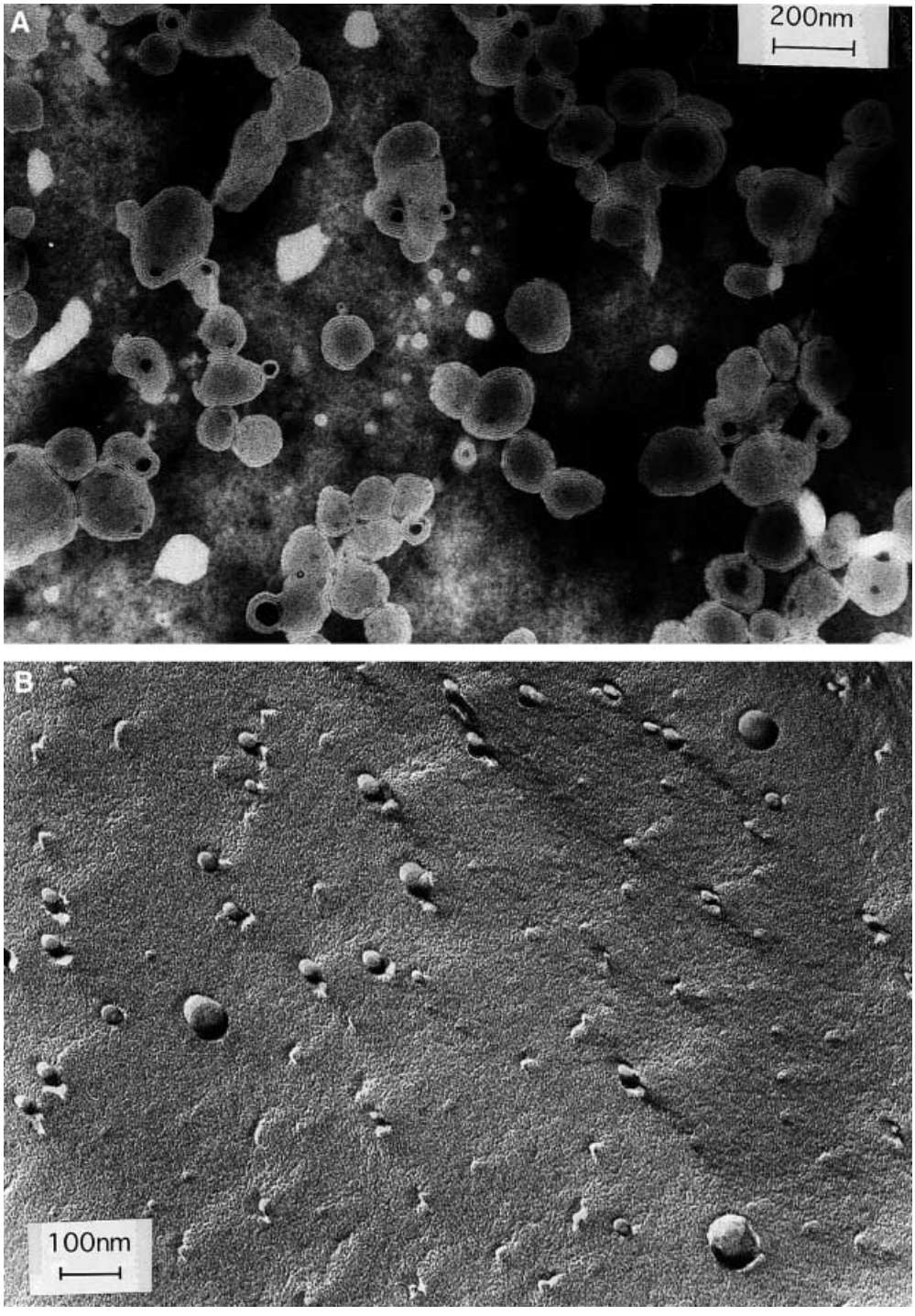
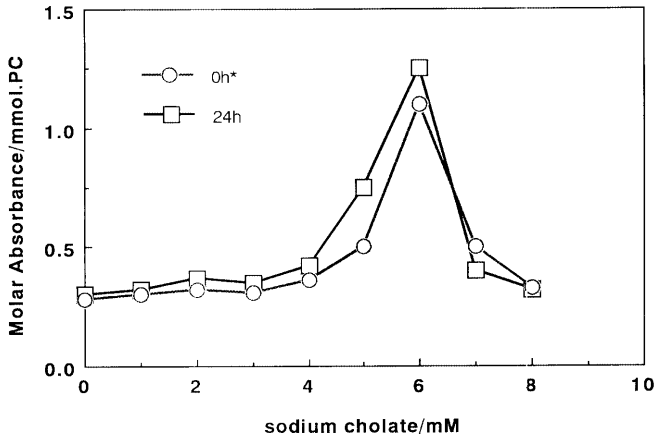


Fig. 1 **A** Negative stained electron micrograph of the vesicles formed upon the removal of sodium cholate in the presence of 5 mM Ca^{2+} by dialysis and **B** the freeze–fracture electron micrograph at the critical concentration of 4 mM sodium cholate in the presence of 5 mM Ca^{2+}

factor for the formation of large vesicles [6, 7, 9]. Therefore, the size-growth events would be related to the behaviors, such as fusion, of the vesicles modified by detergent.

For the size growth of the vesicles containing detergents, the process of the vesicle solubilization by detergent was investigated. In many studies, the variation of turbidity and apparent size with detergent concentrations illustrated the evolution of the aggregate structures [10, 11]. The changes in the turbidity and apparent size in the SUV solubilization by sodium cholate or CHAPS are shown in Figs. 2 and 3. In general, there were several breakpoints in the curves for the turbidity and apparent size. At concentrations of



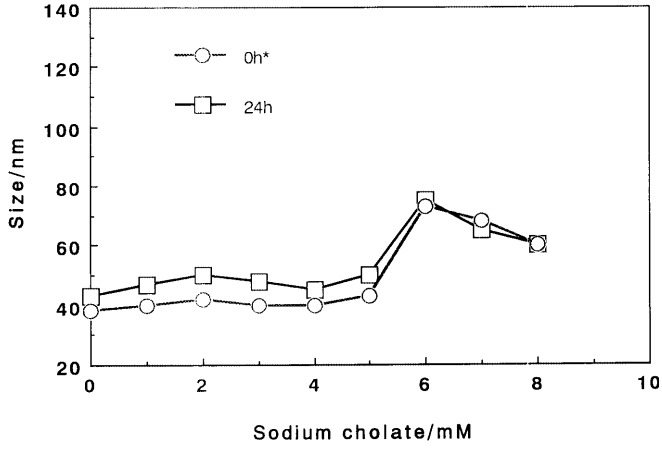
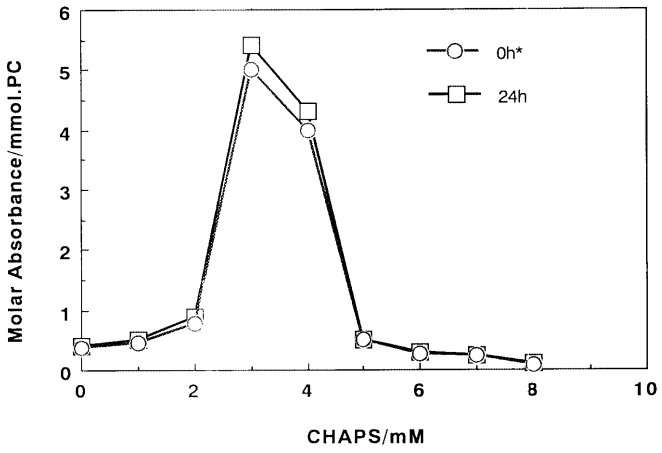


Fig. 2 The dependence of turbidity and apparent size on the concentrations of sodium cholate in the suspension of EggPC and sodium cholate. (*) The turbidity and apparent size were measured within 10 min after the addition of sodium cholate



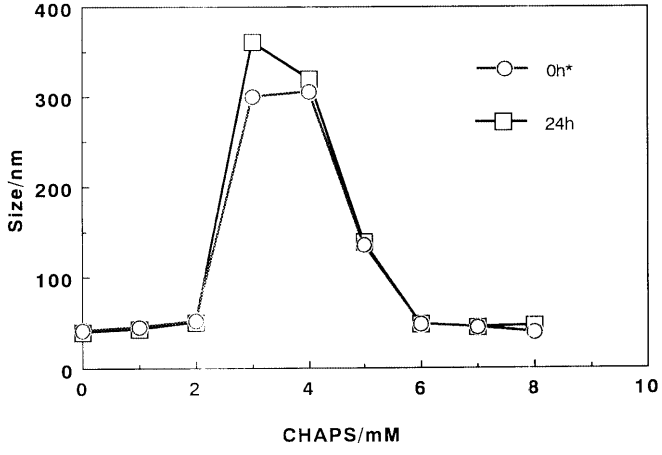


Fig. 3 The dependence of turbidity and apparent size on 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) concentrations in the suspension of EggPC and CHAPS. (*) The turbidity and apparent size were measured within 10 min after the addition of sodium cholate

4 mM sodium cholate and 3 mM CHAPS, the freeze–fracture electron microscopic images showed the vesicle morphology, as shown in Figs. 4A and 5A. Beyond this concentration a slight increase in the detergent content of the suspensions caused a drastic change in the turbidity and apparent size. At a concentration of 6 mM sodium cholate or 4 mM CHAPS, no vesicle morphology was found, as shown in Figs. 4B and 5B. At this point, the vesicles were transformed into an intermediate structure. Apparently, up to the critical detergent concentration for the vesicles, 4 mM sodium cholate and 3 mM CHAPS, the addition of the detergents to SUV suspensions resulted in the distribution of detergent into the vesicle membrane. In the case of sodium cholate, no significant change in turbidity and apparent size was found and the small vesicles remained. On the other hand, when CHAPS was added to the SUV suspension, there was a fast increase in turbidity and apparent size at the critical concentration of 3 mM, as shown in Fig. 3. Furthermore, in Fig. 5A aggregation (a) and fusion (b) of the small vesicles were observed, indicating that a rapid detergent-induced fusion of vesicles took place. Thus, SUV containing a large amount of CHAPS is easily fusible. It should be noted that the SUV with a large amount of CHAPS and with a large amount of sodium cholate are very different in the detergent-induced fusion. The small vesicles containing a great amount of detergent are defined as SUV*. They are similar to SUV prepared by sonication in morphology, but are more fusible or are potentially fusible. SUV* appeared before the vesicle destruction as a common phenomenon in spite of the variety of detergents, even in the solubilization process of large unilamellar vesicles [12]. Otherwise, at the critical concentration of 4 mM shown in Fig. 6, a slow size growth of SUV* containing sodium cholate was induced in the presence of 5 mM Ca^{2+}, from 40 nm to about 110 nm, which was

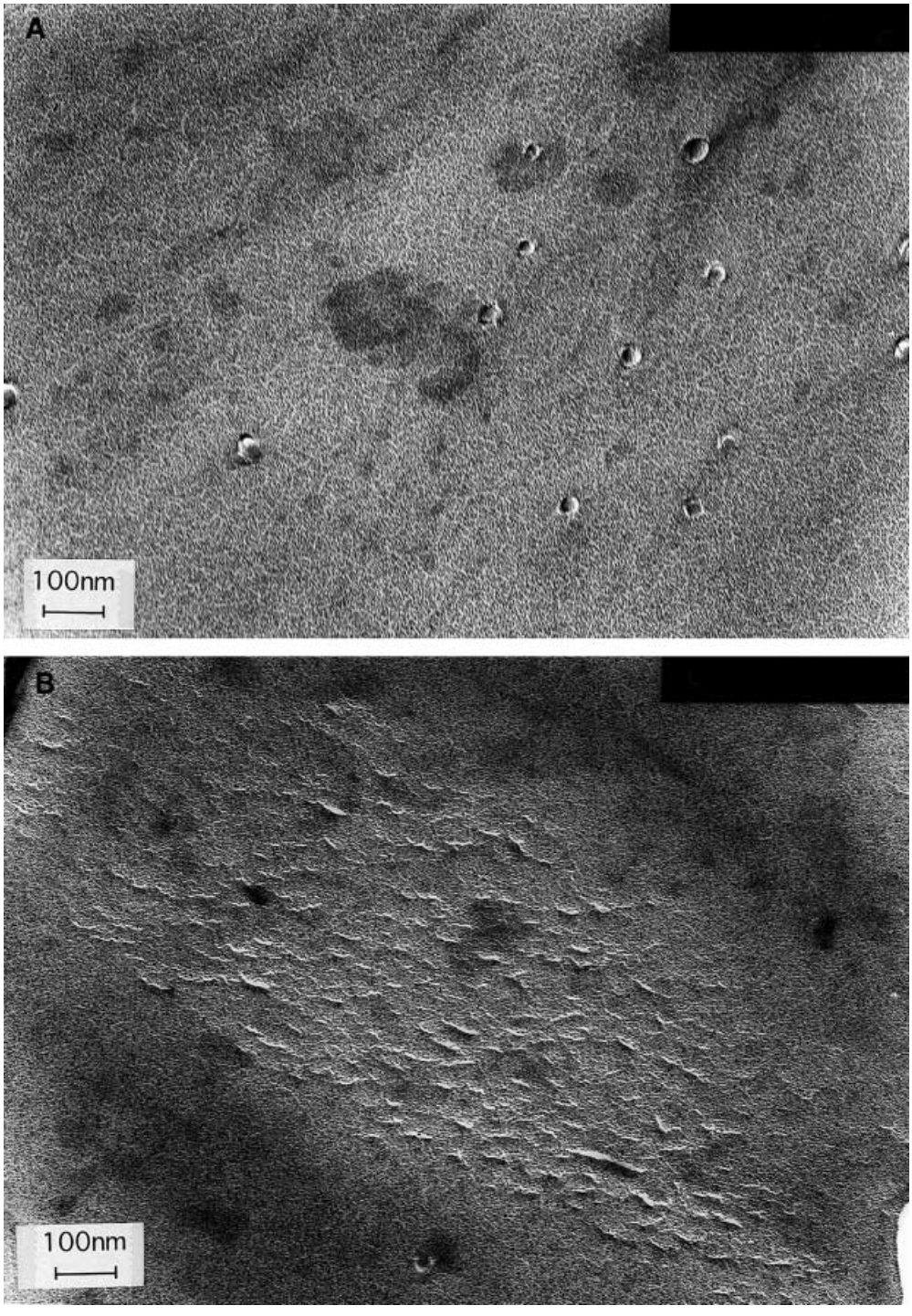


Fig. 4 Freeze–fracture electron micrographs at **A** the critical concentration of 4 mM sodium cholate and **B** the drastic point of 6 mM 24 h after the addition of sodium cholate

measured 24 h after adding sodium cholate. In Fig. 1B the freeze–fracture electron microscopic appearance showed the presence of some large vesicles. From the previous observations the size-growth ability of the vesicles containing a large amount of detergent is dependent on the kind of detergent and the experimental conditions.

It has been known that in the process of detergent removal the mixed micelles are transformed into initially formed vesicles, followed by the conversion of them to the resultant vesicles [7]. Apparently, the size growth happens during the detergent removal. The detergent removal is also performed by diluting the mixed micelles of detergent and phospholipid, which causes the depletion

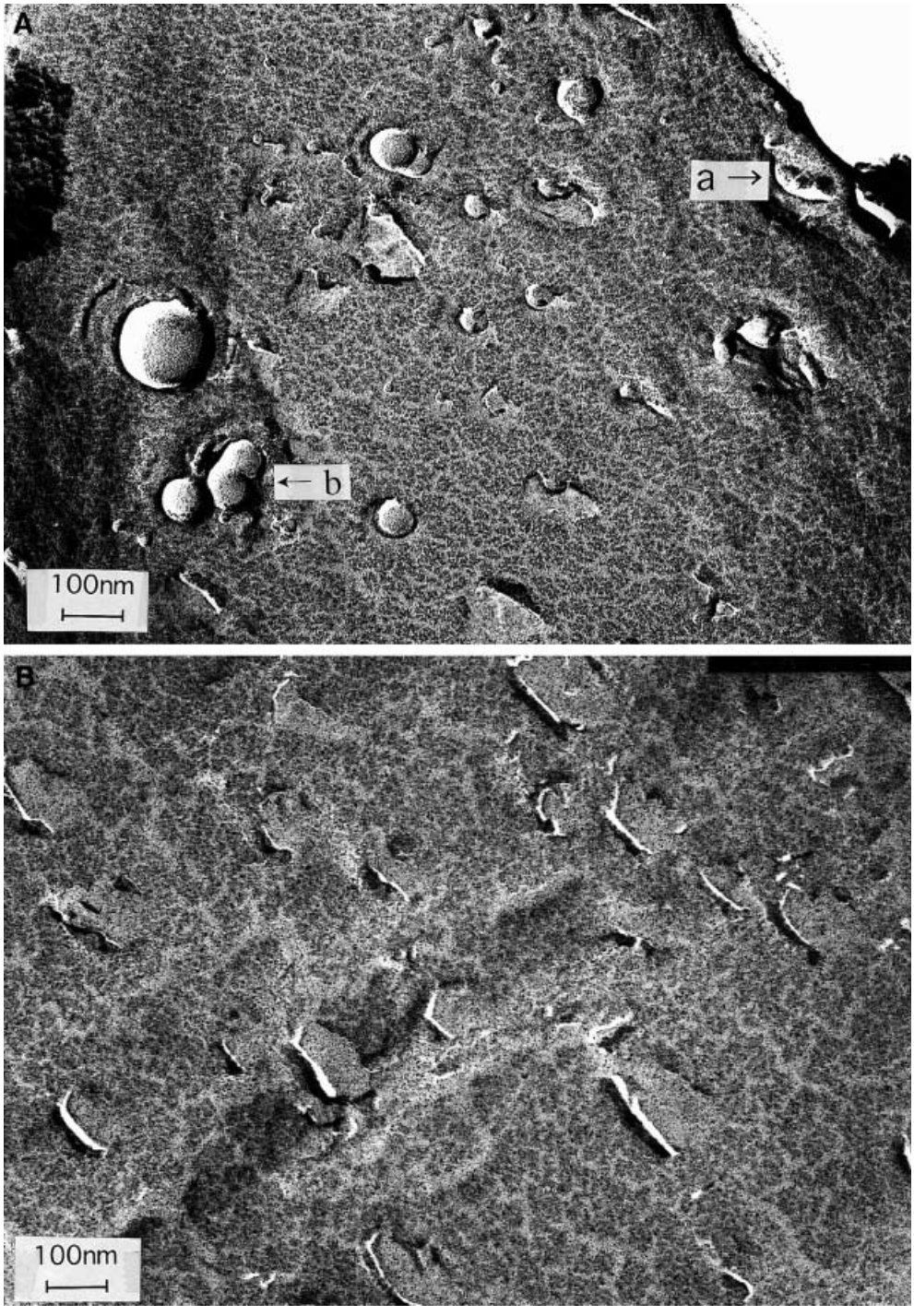
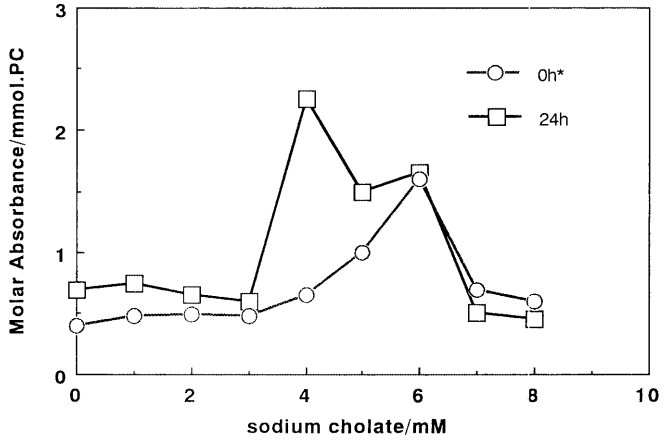


Fig. 5 Freeze–fracture electron micrographs for **A** the mixed vesicles at the critical concentration of 3 mM CHAPS and **B** micelles at the drastic point of 4 mM 0 h after the addition of CHAPS

of the detergent from mixed micelles, leading to the transition to vesicles [5, 13–15]. To investigate the influence of rapid or slow detergent removal from the initially formed vesicles on the size growth, the dilution of the mixed micelles was performed in two ways, as described in the Materials and methods. The results are shown in Table 1. Large vesicles formed when the mixed CHAPS/EggPC micelles were diluted step by step, but small vesicles were formed by fast one-step dilution. In the presence of 5 mM Ca^{2+}, small vesicles were produced from the mixed sodium cholate/EggPC micelles either by step-by-step dilution or by one-step dilution. In order to demonstrate the fusion of the initially formed vesicles, the intermediates, such as at the points of 4 mM CHAPS



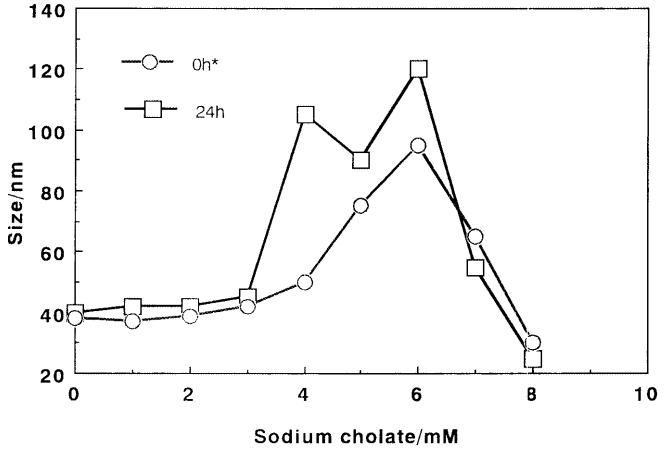


Fig. 6 The dependence of turbidity and apparent size on the concentrations of sodium cholate in the suspension of EggPC and sodium cholate in the presence of 5 mM Ca^{2+}. (*) The turbidity and apparent size were measured within 10 min after the addition of sodium cholate

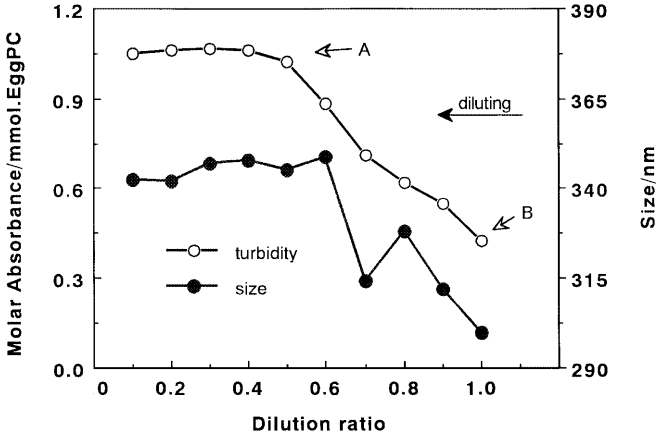


Fig. 7 Transition of mixed CHAPS/EggPC micelles to vesicles by slow definite dilution. Dilution ratio, c/c_o; c is the EggPC concentration after dilution and c_o is the EggPC concentration (5 mM) before dilution

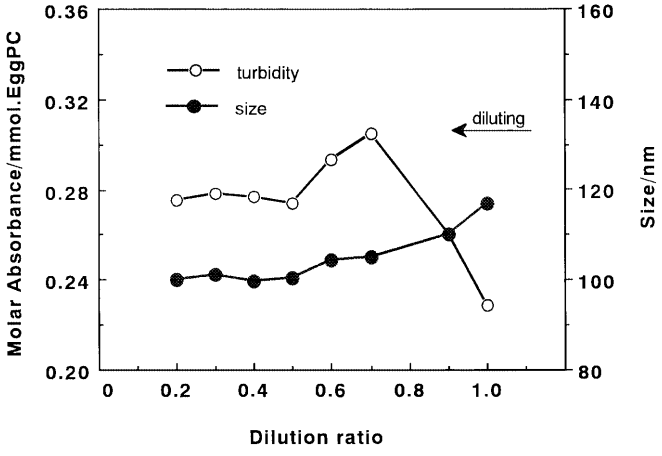


Fig. 8 Transition of mixed sodium cholate/EggPC micelles to vesicles by slow definite dilution in the presence of 5 mM Ca^{2+}

in Fig. 3 and 6 mM sodium cholate in Fig. 6, were diluted step by step so that the initially formed small vesicles could grow for 20 min. When the intermediate of CHAPS/EggPC was diluted step by step, the turbidity and apparent size varied with each dilution, showing that the aggregate structures were changing (Fig. 7). At the breakpoint A the formation of large vesicles was suggested based on no change in molar turbidity and apparent size when these suspensions were diluted further. On the other hand, for the process of vesicle formation by diluting the intermediates of sodium cholate and EggPC in the presence of Ca^{2+} shown in Fig. 8, the size growth of the initially formed vesicles was not observed, evidenced by the resultant vesicles being smaller than those prepared by dialysis, as shown in Table 1. In the case of CHAPS, the fast one-step dilution resulted in small vesicles, about 40 nm, which is in agreement with the results reported by Rotenberg and Lichtenberg [7].

Discussion

In our studies, the freeze-fractured electron microscopic appearances showed the detergent-induced fusion of SUV* containing CHAPS and correspondingly large vesicles were prepared by the removal of CHAPS from the mixed micelles by dialysis, while the freeze-fractured electron microscopic appearances did not show the detergent-induced fusion of SUV* containing sodium cholate and small vesicles formed. Additionally, in the presence of Ca^{2+} size growth of SUV* containing sodium cholate was induced and large vesicles were produced by dialysis. Size growth is important either to elucidate the mechanism of the micelle–vesicle transition or to control the preparation of the functional reconstitution of membrane proteins. For some systems of

detergents and phospholipids, composed of sodium cholate and phospholipids or of octyl glucoside and phospholipids, size growth has been investigated [5, 6]. In these earlier investigations, as in the present study, the growth of the initially formed vesicles is responsible for the ultimate size of the vesicles; however, regarding the size growth conflicting results have been reported. Lasic et al. [9] reported that CHAPS/EggPC mixed micelles would fuse more readily than CHAPS/EggPC vesicles and therefore yield large vesicles, whereas, Rotenberg and Lichtenberg [7] suggested that in the fast infinite dilution of the CHAPS/EggPC mixed micelle system the ultimate size of the vesicles is likely to be equal to their initial size, 21.8 nm, which is due to no size growth of the initially formed vesicles. Our results demonstrate that the preparation of large vesicles with the detergent removal method was related to the size growth of the vesicles containing a large amount of detergent. The vesicle formation upon the detergent removal and the vesicle solubilization by detergent have been known to be opposite symmetry as observed in the behaviors of turbidity and apparent size [16–18], in both of which there should be similar aggregate structures at the point of the same molar ratio (Re) of detergent and phospholipid in the membrane. Furthermore, the initially formed vesicles contained a large amount of detergent and should have similar properties to SUV* appearing in the process of the vesicle solubilization by detergent; therefore, the fusion of the initially formed vesicles could be investigated through the SUV*. The size growth of those vesicles is generally induced through detergent-induced fusion or through lipid exchange, in which some of the small vesicles grow by themselves [5]. In the case of CHAPS, we suggested that the detergent-induced fusion of the vesicles is responsible for the size growth. This fusion was demonstrated further through the turbidity increase in the process of diluting the intermediate into the large vesicles step by step as shown in Fig. 7. Since the fast dilution led to small vesicles, the initially formed vesicles would be small [7]. The molar turbidity of the small vesicles is less than that of the intermediate structure, as shown in Fig. 3. So, the turbidity increase is not likely to be caused by the formation of the initially formed vesicles; however, the initially formed small vesicles are strongly fusible just as SUV* in the process of the vesicle solubilization by CHAPS because they both contained a great amount of CHAPS. The turbidity increase resulted from the fusion of the initially formed vesicles increasing their sizes. All those facts suggested the following scheme for the formation process of large vesicles: the mixed micelles were transformed into small vesicles containing a great amount of CHAPS, followed by the fusion of those small vesicles leading to large vesicles.

Irrespective of the precise mechanisms of the size growth, it is interesting that the size of the resultant vesicles is dependent not only on the kinds of detergent but also on fast or slow detergent removal. In our study, when a mixed micelle was diluted step by step, large vesicles formed upon CHAPS removal, while the small vesicles were produced by sodium cholate removal in the presence of Ca^{2+}. It is not clear why the similar slow detergent removal resulted in the different results, but it may be related to a kinetic problem between the size growth and conversion of the initially formed vesicles. Rotenberg and Lichtenberg [7] pointed out that if the detergent-induced size growth process were faster than detergent removal, size growth would occur. Apparently, the formation of large vesicles corresponded with a rapid fusion of SUV* with CHAPS, as shown in Fig. 3, whereas, the formation of small vesicles was in harmony with a slow size growth of the SUV* containing sodium cholate in the presence of Ca^{2+}, as shown in Fig. 6. The latter is similar to case of octyl glucoside, where the fusion of SUV* containing octyl glucoside was slow and the vesicles prepared by dialysis were larger than those by fast detergent removal using hydrophobic porous beads [10]. In addition, we can also imagine that the one-step dilution speeded up the depletion of detergent from the mixed vesicles, causing the rapid conversion of the initially formed vesicles and simultaneously reduced greatly the phospholipid concentration in a short time, slowing down the vesicle fusion. If the fusion of the initially formed vesicles were slower than their conversion, the size growth could not occurand small vesicles would form. In this sense the size growth of the vesicles is related to the rate of detergent removal. The same explanation would also apply to the formation of small vesicles by rapid detergent removal using hydrophobic porous beads or gel filtration [19–21].

In summary, the size growth of the vesicles containing a large amount of detergent determined the ultimate size of the vesicles when the detergent was removed from the mixed micelles. Furthermore, the results from the different dilutions indicate that sometimes the ways to dilute mixed micelles into vesicles could not show the detailed process of the vesicle formation upon detergent removal, at least in kinetics, because the size growth is dependent on detergent concentration and time and, moreover, the mixed micelles are easily over diluted.

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