Colloid Polym Sci (2003) 282: 69–75
DOI 10.1007/s00396-003-0919-9

ORIGINAL CONTRIBUTION

M. Ueno
N. Hirota
H. Kashiwagi
S. Sagasaki

Process of destruction of large unilamellar vesicles by a nonionic detergent, octylglucoside, and size growth factor in vesicle formation from phospholipid–detergent mixed micelles

Received: 3 December 2002
Accepted: 17 March 2003
Published online: 20 June 2003

M. Ueno (✉) · N. Hirota · H. Kashiwagi
S. Sagasaki
Faculty of Pharmaceutical Sciences,
Toyama Medical and Pharmaceutical
University, 2630 Sugitani,
930-0194, Toyama, Japan
E-mail: mueno@ms.toyama-mpu.ac.jp
Fax: +81-76-4345050

Abstract The process of vesicle solubilization and size growth by detergents, especially by octylglucoside, was examined in detail in order to elucidate the phenomena observed in the vesicle-to-micelle transition and to clarify the size-determining factor of vesicles prepared by removing detergent from phospholipid–detergent mixed micelles. In the vesicle solubilization process, when the detergent concentration in the vesicle membrane reached a critical value, the collapse of large unilamellar vesicles (LUV) into small unilamellar vesicles (SUV) was observed. This newly appeared SUV were named SUV*. The SUV* could be produced by adding an appropriate amount of detergent to the SUV prepared by an ultrasonication method so as to increase the concentration to a little over the critical value, such as, in the case of adding octylglucoside, a molar ratio of 1.0–1.1 to phospholipid in the membrane phase. The SUV* containing octylglucoside were fusible and grow time-dependently, but those containing sodium cholate were not fusible. On the basis of the SUV* data, the following problems were solved: the variety of the size of the vesicles prepared by detergent removal from mixed micelles composed of a phospholipid and different detergents, or by different removal methods; the complex appearance of turbidity or vesicle size observed in vesicle destruction and formation; the conflict between LUV and SUV in the partition behavior of detergent and the size change with addition of detergent.

Keywords Micelles · Vesicles · Detergent · Solubilization · Small unilamellar vesicles

Introduction

The micelle–vesicle transition has been widely studied in close relationship to the functional reconstitution of membrane proteins after they have been purified in detergent solutions [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11]. It has been pointed out that the efficiency of reconstitution of a protein is determined by the micelle–vesicle transition as well as by the properties of the protein itself [12]. The changes in the membrane properties on the addition of detergent, i.e., in the vesicle solubilization, and the mechanism of the micelle–vesicle-transition have been widely studied [13, 14, 15, 16, 17, 18, 19, 20, 21]. In the past 10 years considerable progress has been made on the elucidation of the micelle-to-vesicle transition and the vesicle-to-micelle transition [22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32]. As a minimum scheme, a three-stage model or a four-stage model has been proposed to elucidate the observations made during the micelle–vesicle transition [33, 34, 35]. Although these models are very helpful for understanding the micelle–vesicle transition, the phenomena observed in the micelle–vesicle

transition on an experimental time scale are more complicated and are dependent on the experimental conditions, leading to a consideration of kinetics [14, 31, 33]. In fact, the sizes of the vesicles prepared by the detergent-removal method are dependent on the types of detergents and on the detergent-removal methods; in vesicle formation by removing detergent by dialysis, why are vesicles prepared using octylglucoside as a detergent large (about 200 nm in diameter) and why are vesicles prepared using sodium cholate or sodium dodecyl sulfate small (about 40 nm)? When octylglucoside was used as a detergent, the vesicles prepared by removing detergent by dialysis were large, but the vesicles prepared by rapid detergent removal, such as using hydrophobic porous beads or gel filtration, were small; on the other hand, when sodium cholate was used as a detergent, the resulting vesicle size was not dependent on the detergent-removal methods, being small in both cases. In addition, the models cannot explain the discrepancy of the partition behavior of octylglucoside for large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV); the partition coefficient (which was defined as $k = x / c_f$, where x is the mole fraction of detergent in the membrane phase and c_f is the molar concentration in the water phase) of octylglucoside for SUV remains almost constant over a wide concentration range, being about 33 M^{-1} [36], while the partition coefficient for large vesicles is dependent on the detergent concentration, and is 70–90 M^{-1} in the very low concentration range [5, 16, 38], decreasing with increasing octylglucoside concentration, and it compares interestingly with that for SUV just before vesicle solubilization. Furthermore, it has been reported that the size growth of SUV by incorporation of a detergent cannot be simulated by an equation derived from the effective cross-sectional area of the detergent incorporated into the vesicle membrane by a simple distribution equilibrium between membrane and water phases [36], while the size growth of LUV could be simulated well by a similar equation up to a rather high concentration of detergent (10 mM detergent in water in equilibrium) [32]. In order to clarify these complicated phenomena and to control the vesicle size in the vesicle formation, we conducted a detailed study on the vesicle destruction process, and we found the appearance of detergent-rich small vesicles before LUV solubilization by detergent as a common phenomenon regardless of the types of detergents used. We tentatively named the detergent-rich small vesicles SUV* to distinguish them from conventional SUV prepared by sonication or extruding [39]. The introduction of SUV* in the micelle–vesicle transition can not only explain the previously mentioned discrepancies consistently, but can also suggest how to control the vesicle size in the vesicle preparation by removing the detergent from phospholipid–detegent mixed micelles.

Experimental

Materials

L-α-Phosphatidylcholine from egg yolk was purchased from Nippon Yusi Co. *n*-Octyl-*β*-*d*-glucopilanoside (octylglucoside) was purchased from Wako Chemicals. 5-Doxyl stearate (5-DS) as a spin probe for electron spin resonance (ESR) measurements was obtained from Sigma Co. All other reagents were of reagent grade from Wako Chemicals.

Preparation and size evaluation of vesicles

Detergent-free LUV were prepared by extruding a vesicle suspension five times through two stacked nuclear pore filters with a pore size of 200 nm using THE EXTRUDER (Lipex Biomembranes) after freeze–thawing five times. Detergent-free SUV were prepared by sonication using a probe type sonicator (US-600TS, Nissei Japan) under ice cooling for 40 min followed by the removal of titanium fragments and multilamellar aggregates by centrifugation at 100,000 *g* for 30 min. Mixed vesicles were prepared by the slow addition of octylglucoside solutions of different concentrations into the detergent-free vesicles with stirring. The concentration of the detergent incorporated into the vesicles was determined by a partition equilibrium experiment as previously reported [16]. The characterizations of the mixed vesicles were usually performed at 25 °C 24 h after addition of detergent in order to avoid a significant time effect, except for the time course experiment of size growth. The vesicle size was determined by quasielastic light scattering using a dynamic light scattering apparatus (Otsuka Electronics LPA 3000/3100) at a fixed angle of 90°. The optical density (OD) at 500 nm for LUV or at 350 nm for SUV was measured using a spectrophotometer (Shimadzu UV-160A).

ESR experiment

A spin probe, 5-DS, was incorporated into the membrane phase at a molar ratio of 1/100 to phospholipid. ESR spectra were recorded with an ESR spectrometer (JEOL JM-3x, Japan) at 25 °C. The order parameter, S , used for characterization of the membrane fluidity of the particles (vesicles or micelles), was calculated according to the following equation:

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{ZZ} - 1/2(A_{XX} + A_{YY})}, \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the parallel and perpendicular hyperfine splitting parameters, respectively, and A_{XX} , A_{YY} , and A_{ZZ} denote the hyperfine tensor constants.

Electron microscopy

Freeze–fracture experiments were carried out according to the previous report as follows. About 10 μl sample suspension was placed on a copper plate and quenched in liquid difron 13 and then transferred to liquid nitrogen. The specimens were fractured in an EIKOFD-2A freeze–fracture apparatus under high vacuum (10^{-5} Pa) and below −120 °C. Each fractured specimen was replicated by shadowing with platinum/carbon at an angle of about 45°, followed by carbon shadowing to improve the mechanical stability of the replica for subsequent electron microscopy. Electron micrographs were taken with a JEOL JEM–200 CX electron microscope.

Results

Vesicle size and OD changes with incorporation of octylglucoside

The OD at 500 nm and the relative particle size (diameter of vesicles containing detergent at mole fraction x divided by the diameter of the initial detergent free vesicles, D_x/D_0) are plotted against octylglucoside concentration in Fig. 1a. The size of the initial detergent-free vesicles was about 200 nm and the phospholipid concentration was kept at 4 mM. As the detergent concentration was increased, the OD and the apparent particle size initially increased, then decreased, subsequently increased and decreased again. These observations suggest that at least four distinctive states are included in the process of destruction of LUV by detergent. With more than 30 mM detergent, the OD is almost 0 and the particle size becomes very small, suggesting the formation of mixed micelles.

Membrane state and order parameter

The order parameters of LUV and SUV are shown in Fig. 2. The order parameters are not significantly different between LUV and SUV. Up to 20 mM detergent, the order parameters slightly decrease with an increase in the detergent concentration, but the decrease is small and simple, suggesting a lamellar state is still maintained, even though the OD and the size abruptly changed at about 10 mM detergent concentration. At a concentration over 25 mM, the order parameter abruptly decreased, indicating the beginning of the destruction of the lamellar structure, i.e., vesicle solubilization. The destruction of the lamellar structure is completed at a concentration of 30 mM, where the OD becomes almost 0 and the particle size is very small as shown in Fig. 1.

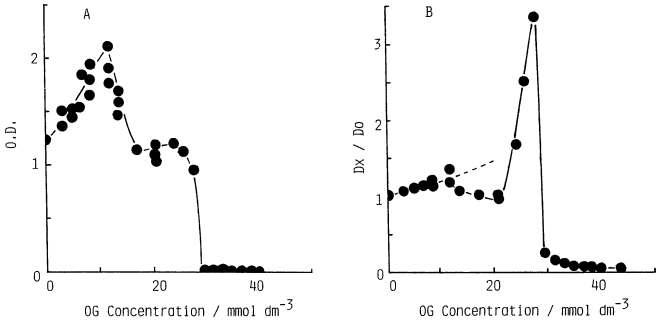


Fig 1 **a** Optical density (*OD*) at 500 nm and **b** relative particle diameter, D_x / D_0, versus total octylglucoside concentration. The phosphatidylcholine concentration was kept at 4 mM. The broken line in **b** represents the theoretical line of Eq. (2)

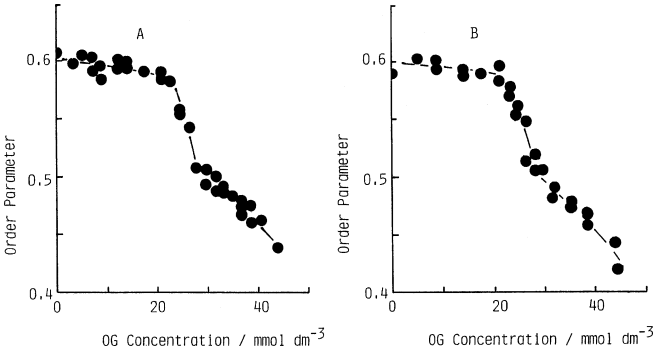


Fig. 2 Order parameter of **A** large unilamellar vesicles (LUV) and **B** small unilamellar vesicles. 5-Doxyl stearate (*5-DS*) was used as a spin probe in the electron spin resonance experiment. The phosphatidylcholine concentration was kept at 4 mM

At a detergent concentration over 20 mM, two phases were separated after 48-h standing. The lower one was viscous and clear and the other (upper one) was turbid, similar to that previously observed by Almog et al. [36]. They used SUV with 11.6 mM phospholipid. They showed that phase separation occurs in a range of 32–54 mM detergent concentration [36]. As shown in Fig. 3, freeze–fracture electron micrographs of the upper turbid phase revealed the existence of small vesicles about 30 nm in diameter, even though the original detergent-free LUV was about 200 nm in diameter. We named these small vesicles containing a large amount of detergent SUV* in order to distinguish them from conventional SUV prepared by ultrasonication. The formation of SUV* can elucidate the situation in which the OD and the size decrease but a lamellar state is maintained.

Nature of SUV*

In order to examine the nature of SUV* in detail, SUV* can be produced by adding an appropriate amount of detergent into SUV prepared by ultrasonication. The original detergent-free SUV were about 30 nm in diameter. The time dependence of the vesicles after addition of octylglucoside (1.0 molar ratio, R , in the membrane phase) or sodium cholate ($R = 0.3$) is shown in Fig. 4. As shown in Fig. 4 significant growth of SUV* after addition of octylglucoside was observed with incubation time and after about 2 days the size reached a maximum, indicating that the growth is a rather slow process. Freeze–fracture electron micrographs are shown in Fig. 5. The appearance of a transient state of fusion was observed even at 7 days after addition of detergent (Fig. 5b). On the other hand, detergent-free vesicles kept their original size 7 days after preparation (Fig. 5c). Next, the following cycle was devised. Octylglucoside was added to initially detergent-free SUV of

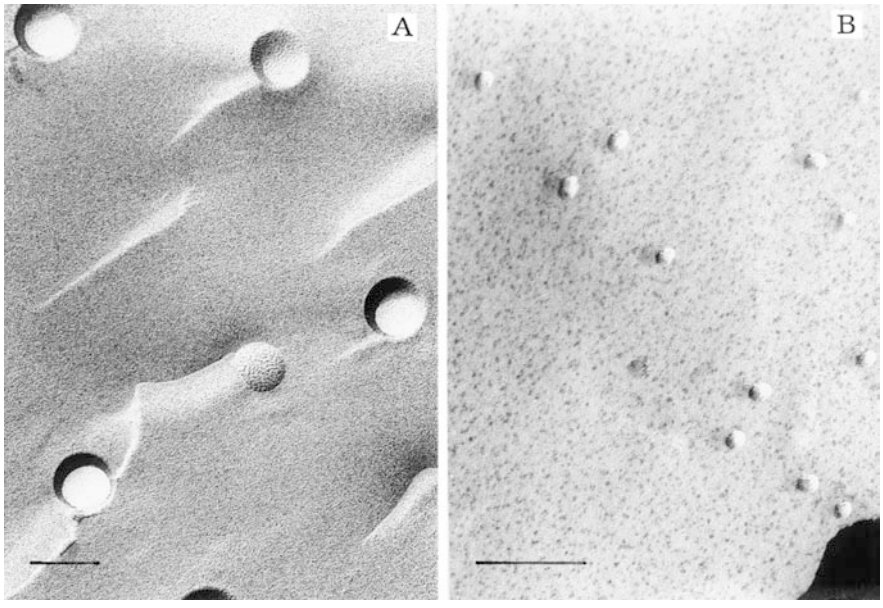


Fig. 3 Freeze–fracture electron micrograph of **a** original vesicles and **b** small vesicles in the upper turbid phase near the peak in Fig. 1b. The *bar* represents 200 nm

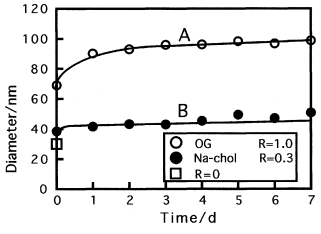
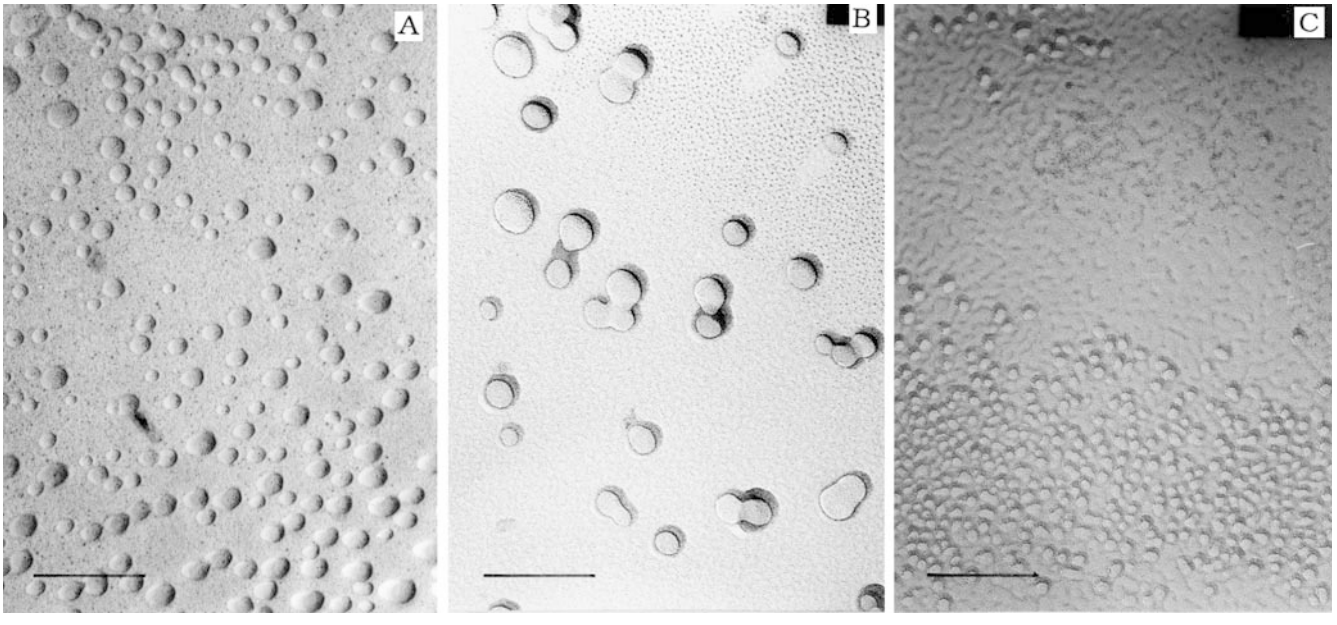


Fig. 4 Time course of vesicle size after addition of octylglucoside (A) and sodium cholate (B)

35-nm diameter at $R = 1.1$. The size of the vesicles increased from point E to point F in Fig. 6. Then, the vesicles were incubated for 6 h, and the size increased from point F to point G. Finally, the detergent was removed by dialysis against 1,000 ml buffer solution for 24 h, and the size decreased a little from point G to point H. Electron micrographs of points G and H are shown in Fig. 7. The image of the transient state of fusion (point G) completely disappeared in removing detergent (point H). In this cycle the vesicle size increased to about 3 times the original size.

Fig. 5A–C Freeze–fracture electron micrographs of vesicles after addition of octylglucoside at a molar ratio of 1.0. **A** Immediately, **B** 7 days after addition of detergent, **C** detergent-free vesicles 7 days after preparation. The *bar* represents 200 nm



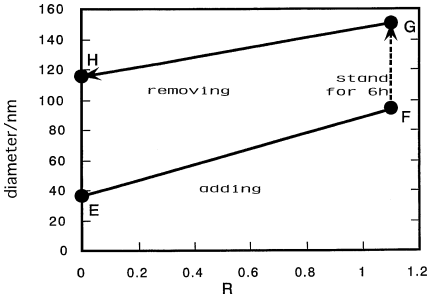


Fig. 6 Size change of liposomes in the process of adding detergent, standing, and removing detergent

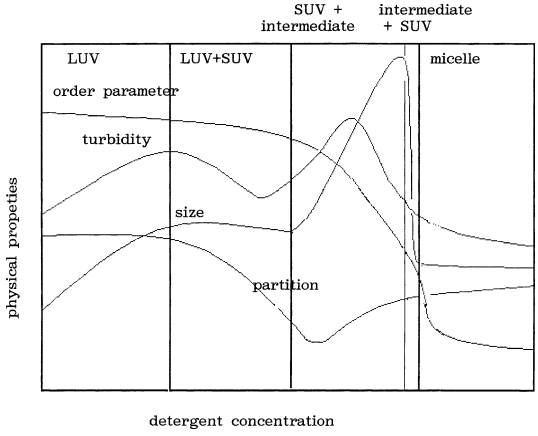


Fig. 8 Schematic representation of the solubilization process of LUV by a detergent

Discussion

The solubilization of LUV by detergent is shown schematically in Fig. 8. The scheme is characterized by insertion of SUV* in the second stage of the three-stage or four-stage model. The presence of SUV* can explain many phenomena observed in the micelle–vesicle transition, which appear, in some cases, to conflict with each other. The order parameter of the aggregate showed that the lamellar structure was maintained up to 20 mM octylglucoside, while the OD and the particle size abruptly decreased near 10 mM. These observations can be well explained by the formation of SUV* over 10 mM octylglucocide.

The partition behavior of octylglucoside observed in our previous study is very different from that reported by Almog et al. The apparent partition coefficient of octylglucoside for LUV showed a strong dependence on octylglucoside concentration as reported by Ollivon et al. In the very low concentration range, the partition coefficient was about 70 M^{-1}. In contrast to our results, Almog et al. reported that the partition coefficient for SUV remains almost constant at 33 M^{-1} over a wide range of concentration, which is about half the value obtained in our study. Interestingly, in a high concentration range our value approached that reported by Almog et al. [36]. The break down of LUV into SUV* with the addition of octylglucoside at a concentration of over 10 mM can well explain the discrepancy in partition behavior.

The size and turbidity changes with the increase in detergent concentration were dependent on the lipid concentration. The previously mentioned four distinctive states were observed in the phospholipid

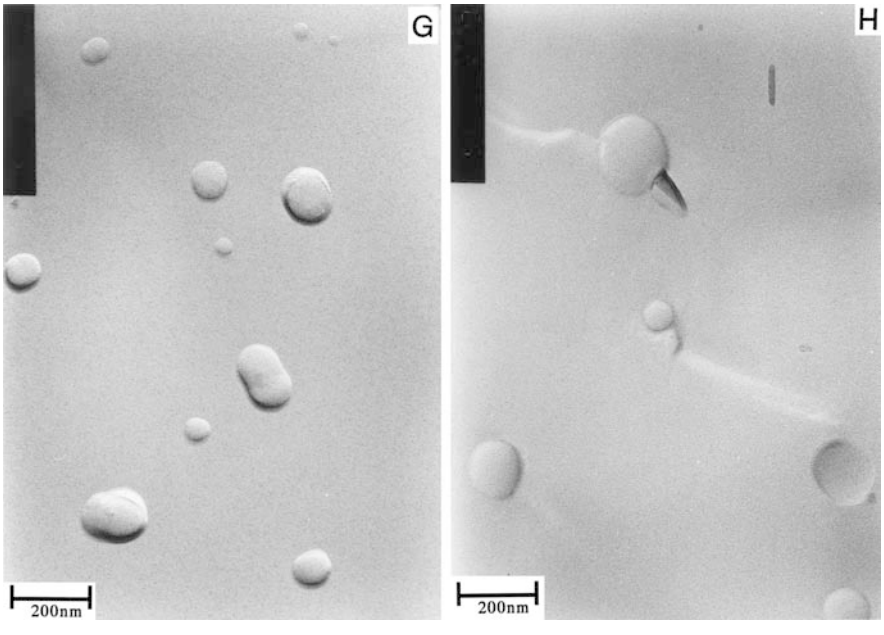


Fig. 7 Freeze–fracture electron micrographs at points G and H in Fig. 6

concentration range of 2.4–7 mM. At a lipid concentration of less than 2 mM, the second increases in OD and in size were not observed. On the other hand, at a lipid concentration of more than 7 mM, the first decreases in OD and in size were not observed [30]. With less than 10mM detergent, the OD and size simply increased as shown in Fig. 1. In this concentration range, if the growth of the vesicle size is only due to the extension of the bilayer by the detergent molecules incorporated into the vesicle membrane, the size of the vesicles containing detergent at D_x can be simulated using the ''effective'' cross-sectional area of the detergent molecules incorporated. If the ''effective cross-sectional area of the detergent is assumed to be half that of phospholipid by allowing for a single chain of the detergent and a double chain of the phosholipid, we obtain the following equation:

$$4\pi(D_x/2)^2 = 4\pi(D_0/2)^2(1+R/2). \quad (2)$$

Then

$$D_x/D_0 = (1+R/2)^{1/2}, \quad (3)$$

where R is the effective molar ratio of detergent to phospholipid in the membrane phase and is a function of the detergent concentration in the condition of constant phospholipid concentration. The broken line in Fig. 1b shows the theoretical line of Eq. (3). Below 10 mM octylglucoside, the theoretical curve coincides with the experimental data, indicating that in this concentration range the vesicles grow almost in accordance with the cross-sectional area of the detergent incorporated and that no special events, such as vesicle destruction or vesicle fusion, occur. This is not the case of Almog et al. [36]. They showed that the theoretical curve in a similar analysis as ours did not agree with experimental plots using SUV instead of LUV, even in the low detergent concentration range [36]. This discrepancy will be discussed later. Over 10 mM octylglucoside, the experimental plots (solid line in Fig. 1b) cannot be represented by a simple theoretical line (dotted line), suggesting that some special events occur in this concentration range.

Over 11 mM odetergent concentration, the OD and the size decreased, then increased at about 20 mM detergent, and abruptly decreased at a concentration of just less than 30 mM. This observation suggests that destruction of LUV occurs in two stages at least. The initial decrease in the OD and the size suggests that small particles form in the first stage and real vesicle destruction, i.e., solubilization, occurs in the second stage.

The result of our simulation of vesicle growth with increasing detergent concentration also differed from that of Almog et al. Our experimental plots agreed well with the theoretical curve up to 10 mM octylglucoside concentration as shown in Fig. 2. The result suggests that in this concentration range no special events occur for LUV, the vesicle size simply increasing by the corresponding cross-sectional area of the detergent incorporated in the membrane. On the other hand, Almog et al, in a similar analysis as ours, reported that the experimental plots did not agree with the theoretical curve even in the low concentration range. They used small vesicles of about 30 nm in diameter prepared by ultrasonication as the initial detergent-free vesicles. As the phosphatidylcholine molecule is almost cylindrical, the membrane surface is flat, i.e., larger vesicles are expected to be stable in the absence or in the presence of a small amount of detergent. Then a size of 30 nm is maybe far from the ''apparent equilibrium size'' [37,40], and addition of a small amount of detergent easily triggered vesicle growth on the experimental time scale, similar to that in Fig. 5c.

The experiment carried out regarding the growth of SUV* by addition of detergent to SUV does not involve the same process of vesicle growth in the micelle-to-vesicle transition. Nevertheless, these experimental results give us useful information; when the vesicle membrane is not so rigid, the size of the vesicles tends to increase to the ''apparent equilibrium size'' [37,40]. The findings that a transient fusion image was observed after addition of octylglucoside (Figs. 5b, 7, point G) and that the transient fusion image completely disappeared and spherical vesicles appeared (Fig. 7, point H), lead to the consideration of the mechanism of fusion that in the transient state local phase separation might occur.

In this report we have described that vesicle fusion is responsible for growth of small vesicles. Two mechanisms have been proposed: one is fusion and the other is phospholipid transfer. In this report electron microscopy images strongly suggest that vesicle fusion is the main process in the growth of small vesicles. Recently it has been found that egg phosphatidylcholine vesicles containing 5% of distearoyl phosphatidyl ethanolamine–poly(ethylene glycol) are small, only 70 nm in diameter, when the vesicles are prepared by octylglucoside removal by dialysis. This is to be compared to 200 nm for the size of conventional egg phosphatidylcholine vesicles prepared by the same method. This suggests that fusion is predominant in vesicle growth because a small amount of distearoyl phosphatidyl ethanolamine–poly(ethylene glycol) hardly interrupts phospholipid transfer, but easily interrupts vesicle fusion owng to steric hindrance by the great hydration of ethylene oxide.

Acknowledgements The authors are indebted to M. Ollivon and his colleagues, URA-CNRS Université Paris-Sud, for helpful discussion.

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