Physical Properties of Phosphatidylcholine Vesicles Containing Small Amount of Sodium Cholate and Consideration on the Initial Stage of Vesicle Solubilization

Changqi SUN, Hiroshi KASHIWAGI,* and Masaharu UENO

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan. Received December 12, 2001; accepted May 7, 2002

The effects of sub-solubilizing concentrations of sodium cholate (Na-chol) on several physicochemical properties of phosphatidylcholine (PC) small unilamellar vesicles (SUV) were considered in connection with the initial stage of membrane solubilization. ESR spectra of 12-doxylstearic acid (12-DS) in phosphatidylcholine from egg yolk (EPC) or dimyristoylphosphatidylcholine (DMPC) SUV at low concentrations (insufficient to destroy the vesicles) of Na-chol were composed of two (a strongly immobilized and an additional weakly immobilized) immiscible components. The origin of the additional bands was phase separation which occurred in the hydrophobic parts of PC SUV in the presence of Na-chol. Differential scanning calorimetry measurements demonstrated that the mixed DMPC/Na-chol SUV possessed two (a sharp low-temperature and a broad high-temperature) endothermic peaks, which is consistent with the coexistence of two immiscible phases in the vesicular membranes. ζ Potentials of the EPC/Na-chol SUV revealed that high anionic densities appeared on the surfaces of the SUV at a Na-chol concentration slightly below the upper boundary of the vesicle region. Thus, the initial stage of the solubilization of PC SUV by Na-chol was caused by the aggregation of hydrophobic parts of PC membranes, followed by the occurrence of high anionic densities on the surfaces of the vesicles. The fact that removal of Na-chol from PC/Na-chol mixed systems preferentially resulted in the formation of small vesicles might originate from these anionic charges.

Key words sodium cholate; phosphatidylcholine vesicle; vesicle solubilization; ESR; differential scanning calorimetry; ζ potential

Sodium cholate (Na-chol) is a physiological detergent. Its solubilizing properties are important in several fundamental biophysical and biochemical processes. This includes disintegration of biomembranes to mixed micelles,[1] reconstitution of membrane proteins and lipids to functional supramolecular structures (vesicles in most cases)[2,3] and preparation of homogeneous lipid vesicles of controlled sizes.[4,5]

The vesicle destruction mechanism has been studied in connection with the reconstitution of membrane proteins after purification in detergent solutions. Concerning the disintegration from mixed vesicles to mixed micelles by Na-chol, many studies have concentrated on the variation in morphology and properties of mixed aggregates.[6,7] Consequently, the solubilization process of phosphatidylcholine (PC) vesicles is summarized as follows; addition of very small amounts of Na-chol to vesicles results in the partitioning of Na-chol between the water and lipid phases. At a further increased detergent concentration, vesicles containing a large amount of Na-chol are formed, whose behavior, such as fusion of small vesicles to larger ones, is found to be different from that of PC vesicles containing large amounts of nonionic detergents (*i.e.* octylglucoside).[5] At a still further increased detergent concentration, the Na-chol-containing vesicles disintegrate into intermediate structures, and finally into mixed micelles.[8,9] As far as intermediate structures are concerned, structural changes from vesicles to perforated vesicles, to discoidal sheets,[10] or to rod-like structures[11] have been reported. As described above, the incorporation of Na-chol into vesicular membranes remarkably influences the structures and properties of PC vesicles. However, little is known about the effects of the incorporation of Na-chol on the behavior of membranes at a molecular level, although it is important for further detailed studies on the mechanism of vesicle disintegration.

The present report deals with the influence of addition of small amounts (insufficient to destroy vesicles) of Na-chol on several physicochemical properties of PC small unilamellar vesicles (SUV). Similar to the previous reports,[12—14] ESR spectra of a spin label 12-doxylstearic acid (12-DS) are applied to monitor the variation in properties and structures of the mixed PC SUV. Differential scanning calorimetry (DSC) and ζ potentials measured with electrophoretic light scattering methods were also employed for observing the variation in the properties of PC SUV induced by Na-chol. The obtained results will be considered in connection with the initial stage of the solubilization of PC vesicular membranes.

Experimental

Materials Phosphatidylcholine isolated from egg yolk (EPC) (purity approximately 98.8%) was purchased from Nihon Yushi (Tokyo, Japan). Sodium cholate and cholesterol of guaranteed degree were obtained from Nacalai Tesque (Kyoto, Japan). Synthetic dimyristoylphosphatidylcholine (DMPC) (purity *ca.* 99%) as well as 5-, 12-, and 16-DS were purchased from Sigma (St. Louis, MO, U.S.A.). All reagents were used without further purification.

Preparation of Vesicle Suspensions A phospholipid (EPC or DMPC) and, if necessary, a given amount of cholesterol and a spin probe (5-, 12-, or 16-DS, about 1 mol% of the PC concentration) dissolved together in chloroform were evaporated under reduced pressure in a sample tube to yield a thin film. Then, the film was dispersed into *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (150 mM NaCl, and 20 mM TES, pH 7.0) with vortexing, yielding multilamellar vesicles (MLV). The MLV suspensions were frozen and thawed five times, then extruded through two stacked 0.6-μm-defined or 0.2-μm-defined pore polycarbonate filters (Nucleopore, Costar Co., U.S.A.) at least five times, producing extruded large unilamellar vesicles (LUV) possessing an average diameter of 200 nm. The repeated extrusion appeared to produce more uniform LUV. Small unilamellar vesicles (SUV) were prepared as follows: MLV suspensions of 4 ml×25 mM EPC were ultrasonicated three times for 20 min, with a rest time of 10 min, at an ice-bath temperature under a nitrogen atmosphere. Titanium frag-

* To whom correspondence should be addressed. e-mail: kasiwagi@ms.toyama-mpu.ac.jp

ments and multilamellar aggregates were removed by centrifugation at 100000×***g*** for 60 min at 4 °C. Here, SUV were found to have a uniform size (average diameter: 30 nm).

Measurement of the Molar Ratio of Na-chol to PC in the Membrane (Re) as a Measure of Detergent Concentration Na-chol solutions having given concentrations were added to the SUV or LUV suspensions with a syringe with stirring. The molar ratio of Na-chol to PC in the membrane (Re) was employed as a measure of detergent concentration.[15—18] The Re value was directly measured by equilibrium dialysis method. That is, the vesicle suspension was incubated with an appropriate amount of Na-chol for 1 d using a dialysis cell, which was composed of two compartments and separated by a dialysis membrane. The concentration of ^{14}C-labelled Na-chol was determined by monitoring its radioactivity using an LSC-5200 liquid scintillation system (Aloka, Japan). The phospholipid concentration was determined as phosphorous according to Ames.[19]

Turbidity and Size Measurement The molar turbidity of the mixed PC/Na-chol SUV was determined as absorbance at 350 nm with a UV spectrophotometer (UV-160, Shimadzu, Japan) at 25 °C. The mean diameter and size distribution were obtained by a quasielastic light scattering apparatus (LPA-3000/3100 laser particle analyzer, Otsuka Electronics, Japan) at 25 °C.

Measurement of ESR Spectra The vesicle suspensions containing 5-, 12-, or 16-DS were injected into glass capillary tubes (20 μl) after incubating at 37 °C for DMPC SUV or at 25 °C for EPC SUV. Measurement was performed with a JES-FE3X type X-band spectrometer (JEOL, Japan) at 28 °C. The conditions were set as follows: modulation amplitude, 100 kHz, 0.1 mT; microwave power, 8 mW; scan field, ±5 mT; scan speed, 4 min; response time, 0.1 s. The rotational correlation time (τ_c) due to an isotropic spectrum was calculated according to Cannon *et al*.[20]:

$$\tau_c = 6.5\times10^{-10}\, W_0\, [(I_0/I_{-1})^{1/2} - 1]$$

where W_0 is the maximum-to-minimum line width of I (nuclear spin angular momentum)=0 hyperfine splitting band; I_0 and I_{-1} are maximum-to-minimum heights of the I=0 and -1 hyperfine splitting bands, respectively.

DSC Measurement Approximately 60 μl of LUV or SUV suspension (lipid concentration: 25 mM) was equilibrated at 25 °C for 48 h. Measurements were made with a SSC/560 scanning calorimeter (Daini Seikosha, Japan) at a scan rate of 0.1 °C/min. The PC concentration was held constant (25 mM) in order for relative calorimetric enthalpies to be calculated simply by integrating obtained transition curves. As a check on reproducibility, at least two scans were performed on a given sample.

Measurement of ζ Potentials Electrophoretic light scattering apparatus (ELS-8000/6000, Otsuka Electronics, Japan) was used for measuring electrophoretic mobility of the Na-chol-containing EPC SUV. The measurements were performed in electric fields applied in the direction of and in the opposite direction of, gravity, at 25 °C. The mobility was calculated from the arithmetic mean of the values in the two directions. These electrophoretic mobilities were detected 3—5 times for each sample. The mobility was converted to ζ potential through Smoluchowski equation[21]:

$$\mathrm{Up} = \varepsilon\zeta/(4\pi\eta)$$

where ζ is ζ potential; Up is mobility; ε is dielectric constant; η is coefficient of viscosity.

Results

Change in Molar Turbidity with Molar Ratio of PC to Na-chol in Membrane (Re) Figure 1 shows the dependence of molar turbidity of 5 mM EPC or DMPC SUV on Re at 25 °C. These curves possess several breakpoints. No significant change occur in the turbidity curve of EPC SUV in the region of Re<0.23, whilst the turbidity for DMPC SUV increases considerably in the region of 0<Re<0.08, followed by a gradual increase in the region of 0.08<Re<0.16. The average sizes of DMPC SUV increased from 30 (Re=0) to 80—100 nm (Re=0.14) accompanied with this turbidity increase, which agreed with the results reported by Malloy and Binford.[22] There is a sudden increase in turbidity at Re=0.30 for EPC SUV and Re=0.18 for DMPC SUV. The previous study with freeze fracture electron microscopy demonstrated that no vesicle existed at these Re values.[11] Beyond these Re values, the turbidity decreased drastically,

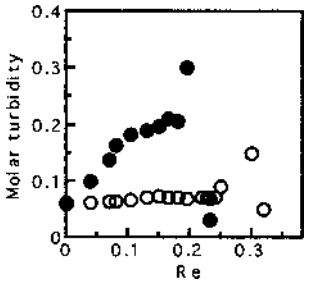

Fig. 1. Dependence of Molar Turbidity of 5 mM EPC SUV (○) and 5 mM DMPC SUV (●) on Re at 25 °C

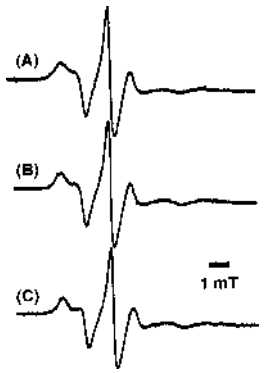

Fig. 2. ESR Spectra of EPC/5-DS SUV at Re=0 (A), 0.15 (B), and 0.20 (C) at 28 °C

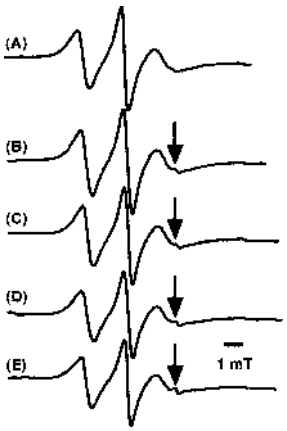

Fig. 3. ESR Spectra of EPC/12-DS SUV at Re = 0 (A), 0.07 (B), 0.15 (C), 0.22 (D), and 0.25 (E) at 28 °C

Arrows indicate the additional signals.

and the appearance of both EPC and DMPC SUV suspensions became clear, showing the formation of mixed micelles.[23,24] Thus, the vesicle regions for EPC or DMPC/Na-chol SUV are determined to be 0<Re<0.23 or 0<Re<0.16, respectively.[9,22]

Behavior of Nitroxide Spin Probes in PC SUV Containing Small Amounts of Na-chol Figures 2, 3, and 4 display the variation in ESR spectra of EPC/5-DS SUV, EPC/12-DS SUV, and DMPC/12-DS SUV, respectively, with Re of the vesicle region at 28 °C. There are some differences in the spectra of 5-DS (Fig. 2) and those of 12-DS (Fig. 3). In the absence of Na-chol, the spectrum of 5-DS is anisotropic, but that of 12-DS is isotropic. Isotropic triplet bands shown in Fig. 3A have been assigned to 12-DS incorporated into the

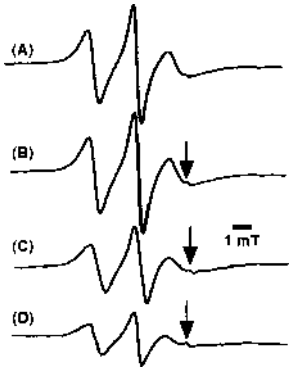

Fig. 4. ESR Spectra of DMPC/12-DS SUV at Re=0 (A), 0.05 (B), 0.07 (C), and 0.12 (D) at 28 °C

Arrows indicate the additional signals.

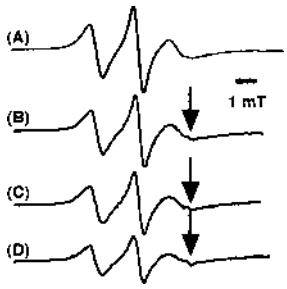

Fig. 5. ESR Spectra of 12-DS Incorporated into DMPC SUV Containing 0 (A), 13 (B), 15 (C), and 17 (D) mol% cholesterol at 28 °C

Arrows indicate the additional signals.

hydrophobic domain of EPC. A weak additional band appears at Re=0.07 for EPC or at Re=0.05 for DMPC SUV, as illustrated by arrows in Fig. 3 or 4, respectively. The intensity of the additional band increases with an increase in Re (Figs. 3C—E). The intensity of this additional band decreased at further increased Re, and became unobservable at an Re value greater than 0.30 for EPC SUV. On the other hand, no additional band appeared in the spectrum of 5-DS (Fig. 2) or 16-DS (data not shown) incorporated into EPC/Na-chol SUV. Further, the location of the additional band is scarcely influenced by the presence of the main band, but the total spectral width slightly decreases in the presence of the additional band.

Figure 5 represents ESR spectra of 12-DS dissolved in DMPC SUV containing cholesterol at 28 °C. Similar to the results of Na-chol-containing SUV systems (Fig. 4), the spectrum obtained when 15 mol% cholesterol is added (Fig. 5B) is explained as the superposition of strong triplet bands and a weak additional signal. The additional band did not appear in the absence of cholesterol. The intensity of the additional band increases with an increase in the amount of added cholesterol.

Figure 6 shows the dependence of the rotational correlation time (τ_c) of 12-DS added to EPC and DMPC SUV on Re values in the vesicle region at 28 °C. The τ_c value is frequently employed as a measure of the immobilization degree of a spin probe. When Na-chol is added, the τ_c values for EPC or DMPC SUV increase with Re values, almost leveling off at a further increased Re. Figure 7 shows the dependence of the τ_c values of 12-DS incorporated into EPC and DMPC

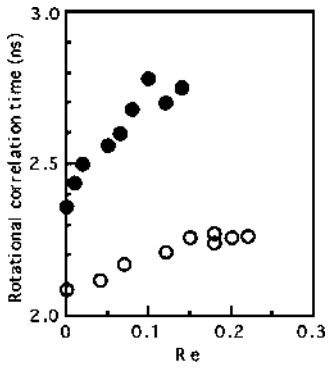

Fig. 6. Dependence of Rotational Correlation Times of 12-DS Incorporated into EPC SUV (○) and DMPC SUV (●) on Re at 28 °C

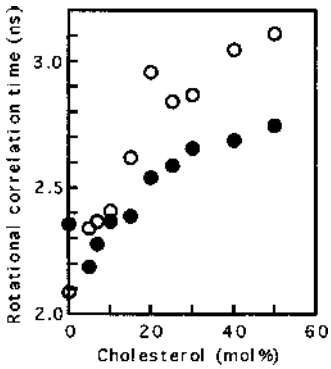

Fig. 7. Plots of Rotational Correlation Times of 12-DS Incorporated into EPC SUV (○) and DMPC SUV (●) against Cholesterol mol% at 28 °C

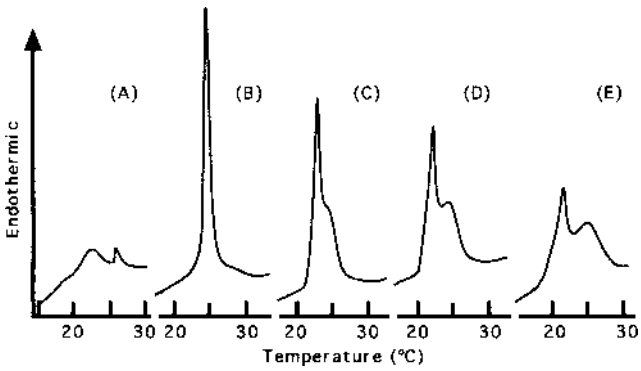

Fig. 8. DSC Transition Curves for the Ultrasonicated DMPC/Na-chol SUV at Re=0 (A), 0.025 (B), 0.061 (C), 0.073 (D), and 0.10 (E)

SUV on cholesterol mol% at 28 °C. The incorporation of cholesterol into the EPC SUV resulted in a steady increase in the τ_c values with cholesterol mol%. On the other hand, the τ_c values of DMPC SUV decreases at a cholesterol mol% less than 5%, followed by a steady increase at a further increased cholesterol mol%.

Differential Scanning Calorimetric Studies Figures 8 and 9 depict representative DSC heating thermograms obtained with ultrasonicated DMPC/Na-chol SUV and extruded DMPC/Na-chol LUV, respectively. The ultrasonicated Na-chol-free SUV have a broad low-temperature transition at 18 °C and a midpoint at about 24 °C (Fig. 8A), while the extruded Na-chol-free LUV have a single transition at 23.6 °C (Fig. 9A). Thus, the transition curves obtained in the absence

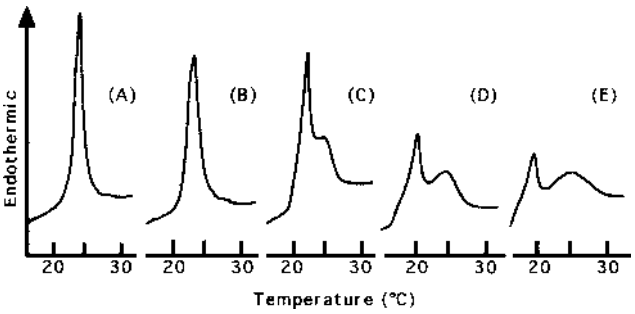

Fig. 9. DSC Transition Curves for the Extruded DMPC/Na-chol LUV at Re=0 (A), 0.04 (B), 0.10 (C), 0.15 (D), and 0.18 (E)

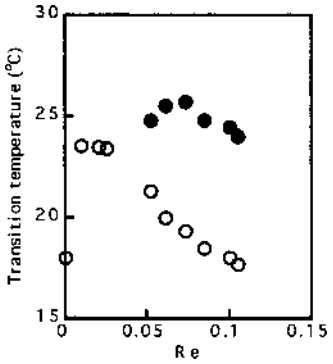

Fig. 10. Variation in Phase Transition Temperatures for Ultrasonicated DMPC/Na-chol SUV with Re

○, low-temperature transition; ●, high-temperature transition midpoint.

of Na-chol are dependent on their preparation methods. The addition of small amounts of Na-chol (Re=0.025) to ultrasonicated SUV (Fig. 8B) results in a single sharp transition with a large calorimetric enthalpy, which is similar to the curve obtained with extruded DMPC/Na-chol LUV (Fig. 9B). As shown in Figs. 8C—E and 9C—E, the DSC curves obtained at further increased Re values in the vesicle region exhibit two (a sharp low-temperature and a broad high-temperature) transitions. The DSC curves obtained in the presence of Na-chol were found to be reproducible, indicating DMPC/Na-chol mixed systems to be vesicles.[18]

Figure 10 displays the variation in phase transition temperatures of ultrasonicated DMPC/Na-chol SUV with Re. As can be seen from Figs. 8 and 10, both transition temperature (Tc) and relative enthalpy of the low-temperature transition curves decrease with an increase in Re. On the other hand, high-temperature transition curves become broader with an increase in Re. Their transition temperature midpoints (Tm) are high and relative enthalpy decreases gradually with increasing Re.

ζ Potentials of Na-chol-Containing EPC SUV Figure 11 displays the dependence of ζ potentials of EPC/Na-chol SUV on the Re values at 25 °C. As can be seen from Fig. 11, there is a gradual decrease in ζ potential in the $0<Re<0.15$ region, followed by a drastic drop in the $0.15<Re<0.25$ region. Thus, anionic densities on the surface of the SUV are the greatest at a Re value slightly below the initiation of the vesicle solubilization. These negative charges may play an important role in the initiation of PC SUV solubilization by Na-chol. Further increasing the value of Re results in a drastic increase in ζ potentials (or decrease in anionic densities).

Discussion

In order to investigate the membrane properties of PC/Na-chol mixed vesicles, we distinguished the region of Re for mixed vesicles from that for mixed micelles. The process of SUV solubilization has been well characterized by the dependence of molar turbidity on Re.[22,25] According to Fig. 1, vesicle solubilization was found to initiate at Re=0.23 for EPC SUV and Re=0.16 for DMPC SUV. As can be seen from Fig. 1, the molar turbidities of DMPC and EPC SUV show different relations with an increase in Re. The turbidity and size measurements shown in the present report were carried out at 25 °C, where EPC was liquid crystalline. On the other hand, the phase transition temperature of DMPC is about 24 °C (close to our experimental temperature), suggesting the coexistence of gel and liquid crystalline phases in the SUV, which results in the destabilization of SUV and a drastic increase in molar turbidities. Overall, solubilization of DMPC SUV was initiated at a considerably smaller Re value compared to that of EPC SUV.

The main purpose to obtain molar turbidities and particle sizes of EPC or DMPC/Na-chol SUV was to determine their vesicle regions rather than to obtain their physical properties. In contrast, ESR, DSC, and ζ potentials were employed mainly for investigating physical properties at small Re values in the vesicle region. As can be seen from Figs. 3 and 4, additional bands for 12-DS incorporated into PC SUV begin to appear at Re=0.07 for EPC SUV and Re=0.05 for DMPC SUV. These Re values are not only below the critical micelle concentration of Na-chol (8 mM), but also much smaller than the Re value for vesicle solubilization described above. Moreover, these additional bands did not appear in the ESR spectra of EPC and DMPC SUV containing both Na-chol and 5- or 16-DS at any Re value. After all, among the doxyl-stearic acids examined, only 12-DS was capable of producing such additional bands. Furthermore, no additional band was observed in spectra of EPC/12-DS SUV containing varying amounts of octylglucoside. Thus, neither probe decomposition nor micelle formation is responsible for the formation of the additional bands. As can be seen from Fig. 5, similar additional bands are obtainable with DMPC/cholesterol/12-DS SUV. The nitroxide group of 12-DS is known to locate very close to the rigid steroidal framework and to be easily subject to the influence of molecules with steroidal structures.[12,13] Therefore, analysis of the ESR spectra of 12-DS should provide useful information about the behavior of Na-chol in vesicular membranes.

Kawamura *et al*. reported the coexistence of two (strongly and weakly immobilized) immiscible components in dihydroxy and trihydroxy bile salt micelles by using 12-DS.[12] Similarly, we presumed from Figs. 3 and 4 that two (or more) immiscible sites for 12-DS might coexist in the membranes of EPC/Na-chol SUV at an appropriate region of Re. Generally speaking, fast rotational motion of a nitroxide spin probe or large fluidity of membranes results in narrow ESR spectral width accompanied by a remarkable increase in the intensity of I=−1 hyperfine splitting band locating at the highest magnetic field.[26] As can be seen from Figs. 3 and 4, the fact that only the hyperfine splitting bands near the highest magnetic field could be observed clearly and that total spectral width observed in the presence of the additional band (Figs. 3B—E) is slightly narrower than that obtained in the absence

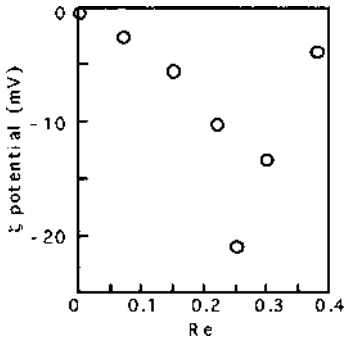

Fig. 11. Dependence of ζ Potentials of EPC/Na-chol SUV on Re at 25 °C

of it (Fig. 3A) indicates the production of more weakly immobilized components formed in the middle of the hydrocarbon chains of PC SUV. The small ratio of peak area of the additional band to that of the main band may indicate a small distribution of 12-DS into the newly appeared weakly immobilized phases.

As shown in Fig. 8A, DSC thermal transition for the ultrasonicated DMPC SUV possesses two small broad peaks. Suurkuusk *et al*. reported that calorimetric enthalpies of small vesicles made up of synthetic PC were smaller than those of large vesicles.[27] Malloy and Binford reported that about 50% DMPC were present in ultrasonicated centrifuged SUV.[22] Thus, the two peaks shown in Fig. 8A might be due to SUV and large multilamellar vesicles of DMPC.[22] The main transition temperature (Tc) of the extruded DMPC LUV (23.6 °C) shown in Fig. 9A was close to the 23.9 °C value reported by Mabrey *et al*.[28] Thus, the DSC transition curves of DMPC vesicles obtained in the absence of Na-chol vary with their preparation methods. The addition of small amounts of Na-chol (Re=0.025) to ultrasonicated DMPC SUV (Fig. 8B) results in a sharp transition peak with a large calorimetric enthalpy, which is similar to the one obtained with extruded DMPC LUV (Fig. 9B). According to Suurkuusk *et al*.,[27] DMPC SUV containing small amounts of Na-chol aggregate and fuse into large vesicles. As shown in Figs. 8C—E and 9C—E, sharp endothermic peaks of DMPC vesicles shift to lower temperatures, and broad peaks appear newly and shift to higher temperature with an increase in Re. As can be seen from Figs. 8 and 10, both Tc and relative enthalpies of the low-temperature transitions decrease with an increase in Re. On the other hand, high-temperature transition curves become broader with increase in Re. The Tm values are high and their relative enthalpies decrease gradually with increase in Re. DSC transition curves obtained with DMPC vesicles containing Na-chol were shown to be reproducible, indicating these systems to be vesicles.[18]

Spink *et al*. reported that the DSC transition curves of dipalmitoylphosphatidylcholine (DPPC)/taurocholate (TC) vesicles possessed a broad transition resulted from packing complexes induced by the steroidal skeleton of TC and a sharp transition caused by a pure DPPC or TC-poor DPPC phase.[18] Furthermore, Mabrey *et al*. and other researchers exhibited the presence of two (a sharp low-temperature and broad high-temperature) immiscible phases in DMPC/cholesterol mixed vesicles, which were attributed to a pure (or cholesterol-poor) DMPC and a cholesterol-rich DMPC phase, respectively.[28—30] Therefore, two immiscible peaks in the DSC transition curves of DMPC/Na-chol SUV (Fig. 8) or LUV (Fig. 9) might be due to the coexistence of two interior structures in the vesicular membranes. Furthermore, sharp low-temperature endothermic peaks are originated from pure (or Na-chol-poor) DMPC phases, while broad high-temperature peaks are from Na-chol-rich phases.

Malloy and Binford reported that the partition coefficient for Na-chol in an aqueous suspension of DMPC vesicles obtained at 20 °C, where DMPC was gel, was almost the same as that obtained at 27 °C, where DMPC was liquid crystalline.[22] Further, Schurtenberger *et al*. demonstrated that the partition coefficients obtained at temperatures above the phase transition depended only slightly on temperature and that the boundary for solubilization of DMPC vesicles was almost independent of temperature.[31] Thus, we thought it was possible to compare the membrane behavior obtained by DSC with that by ESR at the same Re. Thus, the strongly immobilized component may be attributable to 12-DS distributed in the bulky pure PC or Na-chol-poor domains, and the weakly immobilized component to the 12-DS distributed in Na-chol-rich phases. In addition, both the additional band in the ESR spectrum of 12-DS (Fig. 4) and the second peak of the DSC transition curve (Fig. 10) for DMPC/Na-chol SUV begin to appear at almost identical Re (*ca*. 0.05).

As can be seen from Fig. 6, the τ_c values for EPC or DMPC SUV significantly increase with the Re values of the vesicle region. The τ_c value increased even at a very small Re value where the additional band was lacking or very weak. It can be seen from Fig. 7 that the τ_c values of EPC/cholesterol SUV also increase with cholesterol mol%, which is similar to a previous report.[28] As can also be seen from Fig. 7, the τ_c value of DMPC SUV exceptionally decreases at a cholesterol content <5 mol%. This is probably caused by the fact that the phase transition temperature of DMPC (24 °C) is close to our experimental temperature, so both gel and liquid crystalline phases may be able to coexist in vesicular membranes. Although data were not shown, a similar increase in τ_c values was observed when 5-DS, instead of 12-DS, was incorporated into PC/Na-chol SUV. On the contrary, τ_c values decreased at Re values in the vesicle region when a nonionic detergent, such as octylglucoside, was added to the PC SUV (data not shown). Therefore, the steroidal structure of Na-chol or cholesterol "solidify" the membranes. Eventually, the aggregation of Na-chol in the hydrophobic domains of PC/Na-chol SUV induced the formation of the additional weakly immobilized phases at Re values of the vesicle region. However, the decrease in the membrane fluidity, which was induced by the steroidal structure of Na-chol or cholesterol, was found to initiate at much smaller Re values where the additional phases were lacking.

As can be seen from Fig. 11, there is a pronounced decrease in ζ potentials of EPC/Na-chol mixed SUV at Re values greater than 0.15, reaching a minimum near the upper boundary of the vesicle region, and increasing at a further increased Re in the non-vesicle region. We previously demonstrated that the removal of Na-chol from phospholipid/detergent mixed micelles usually gives rise to relatively small vesicles (*ca*. 50 nm), while that of octylglucoside to relatively large ones (100—200 nm) due to the time-dependent fusion of detergent-containing SUV.[5,9] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) is a zwitter-

ionic derivative of a trihydroxy bile salt, which has the same steroidal structure in the hydrophobic moiety as Na-chol, but no net charge. When CHAPS was removed from phospholipid/detergent mixed micelles, large vesicles (*ca*. 350 nm) were produced.[5,9] The present work demonstrated that the anionic charges produced on the surfaces of vesicles (Fig. 11) might induce electrostatic repulsion and inhibit the time-dependent fusion of Na-chol-containing SUV to larger vesicles, resulting in the formation of small vesicles. On the contrary, SUV containing CHAPS or octylglucoside might easily fuse and grow to large vesicles time-dependently.

To summarize the initial process in the solubilization of PC SUV by Na-chol, at a small Re, the steroidal structure of Na-chol, which is larger in size than the carboxyl and hydroxyl groups, may dominate the behavior of Na-chol in the membranes. At first, the addition of Na-chol gives rise to a decrease in membrane fluidity, and then to the aggregation of the Na-chol in the PC SUV, which results in the separation of the strongly and weakly immobilized phases. At further increased Re values of the vesicle region, anionic densities increase drastically on the surface of the SUV, which is probably induced by the carboxyl group of Na-chol. The tendency to produce relatively small vesicles on removal of Na-chol from PC/detergent mixed micelles might be due to the presence of these anionic charges. Furthermore, the added Na-chol aggregated and located at boundaries between the aqueous medium and the hydrophobic edges of PC membranes when either perforated vesicles with transient holes or intermediates such as discoidal and rod-like mixed micelles were formed.[7,10,33] We thought that this boundary formation may be caused by electrostatic repulsion between the anionic charges on the surfaces of the vesicles and that the boundary would protect the hydrophobic edges of the phospholipid from exposure to the aqueous medium. Eventually, the addition of Na-chol to PC SUV caused at first a decrease in membrane fluidity, followed by aggregation of Na-chol in the PC membranes and the separation of two immiscible phases. Further addition of Na-chol resulted in a drastic increase in anionic densities on the surfaces of the SUV, and finally in the solubilization of PC membranes.

References

1) Moller J. V., Maire M. Le, Andersen J. P., "Progress in Protein-Lipid Interactions," Vol. 2, ed. by Watts A., De Pont J. J. H. H. M., Elsevier, Amsterdam, 1983, pp. 147—196.
2) Sanders C. R., Landis G. C., *Biochemistry*, **34**, 4030—4040 (1995).
3) Rigaud J. L., Paternostre M. T., Bluzat A., *Biochemistry*, **27**, 2677—2688 (1988).
4) Schurtenberger P., Mazur N., Waldvogel S., *Biochim. Biophys. Acta*, **775**, 111—114 (1984).
5) Ueno M., Kashiwagi H., Hirota N., *Chem. Lett*., **1997**, 217—218.
6) Walter A., Vinson P. K., Kaplun A., Talmon Y., *Biophys. J.*, **60**, 1315—1325 (1991).
7) Hjelm R. P., Thiyagarajan P., Sivia D. S., Lindner P., Alkan H., Schwahn D., *Mol. Cryst. Liq. Cryst.*, **180A**, 155—164 (1990).
8) Meyuhas D., Lichtenberg D., *J. Biophys.*, **71**, 2613—2622 (1996).
9) Sun C., Ueno M., *Colloid and Polymer Sci.*, **278**, 855—863 (2000).
10) Mazer N. A., Benedek G. B., Carey M. C., *Biochemistry*, **19**, 601—615 (1980).
11) Hjelm R. P., Thiyagarajan P., Alkan-Onyuksel H., *J. Phys. Chem.*, **96**, 8653—8661 (1992).
12) Kawamura H., Murata Y., Yamaguchi T., Igimi H., Tanaka M., Sugihara G., Kratohvil J. P., *J. Phys. Chem.*, **93**, 3321—3326 (1989).
13) Taylor M., Smith L. C. P., *Biochim. Biophys. Acta*, **599**, 140—149 (1980).
14) Yamaguchi T., Kuroki S., Tanaka M., Kimoto E., *J. Biochem*. (Tokyo), **92**, 673—678 (1982).
15) Ueno M., *Biochemistry*, **28**, 5631—5634 (1989).
16) Almog S., Kushnir T., Nir S., Lichtenberg D., *Biochemistry*, **25**, 2597—2605 (1986).
17) Lasch J., *Biochim. Biophys. Acta*, **1241**, 269—292 (1995).
18) Spink C. H., Lieto V., Mereand E., Pruden C., *Biochemistry*, **30**, 5104—5112 (1991).
19) Ames B. N., *Methods Enzymol*., **8**, 115—118 (1966).
20) Cannon B., Polnaszek C. F., Butler K. W., Erikson L. E. G., Smith I. C. P., *Arch. Biochem. Biophys*., **167**, 505—518 (1975).
21) Ohshima H., Kondo T., *J. Colloid Interface Sci.*, **130**, 281—282 (1989).
22) Malloy R. C., Binford J. S., *J. Phys. Chem.*, **94**, 337—345 (1990).
23) Paternostre M. T., Roux M., Rigaud J. L., *Biochemistry*, **27**, 2668—2677 (1988).
24) Ollivon M., Eidelman O., Blumenthal R., Walter A., *Biochemistry*, **27**, 1695—1703 (1988).
25) Ueno M., *Membrane*, **18**, 96—106 (1993).
26) Knowles P. F., Marsh D., Rattle H. W. E., "Magnetic Resonance of Biomolecules," Wiley Interscience, London, 1976, pp. 189—197.
27) Suurkuusk J., Lentz B. R., Barenholz Y., Biltonen R. L., Thompson T. E., *Biochemistry*, **15**, 1393—1402 (1976).
28) Mabrey S., Mateo P. L., Sturtevant J. M., *Biochemistry*, **17**, 2464—2468 (1978).
29) Rubenstein J. L. R., Owicki J. C., McConnell H. M., *Biochemistry*, **19**, 569—573 (1980).
30) McMullen T. P. W., Lewis R. N. A. H., McElhaney R. N., *Biochemistry*, **32**, 516—522 (1993).
31) Schurtenberger P., Bertaniand R., Kanzing W., *J. Colloid Interface Sci.*, **114**, 82—87 (1986).
32) Rotenberg M., Lichtenberg D., *J. Colloid Interface Sci.*, **144**, 591—598 (1991).
33) Schubert R., *Proceedings of MoBBEL*, **4**, 1—17 (1989).