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Phospholipid vesicles were prepared by the nonsolvent method using high-pressure CO₂/water systems. The membrane properties of vesicles prepared at different pressures and temperatures were mainly characterized based on analysis of the membrane fluidity and membrane polarity, using the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene and 6-dodecanoyl-N,N-dimethyl-2-naphthylamine, respectively. The CO₂(liquid)/water(liquid) and the CO₂(supercritical)/water(liquid) two-phase (heterogeneous) systems resulted in the formation of vesicles with high yield (ca. 85%–88%). The membrane fluidity and polarity of the vesicles were similar to those of liposomes prepared by the conventional method. It is suggested that high-pressure CO₂ can be used to form an appropriate hydrophobic–hydrophilic interface where phospholipid molecules as a self-assembled membrane.

I. INTRODUCTION

Water and carbon dioxide (CO₂) are the most abundant materials on earth. The solvent properties of liquid or supercritical CO₂ (scCO₂) (T_c = 31.1 °C, P_c = 7.4 MPa) are tunable by pressure and temperature, and scCO₂ is a functional solvent that is nontoxic and nonflammable. One of the advantages of using scCO₂ is the easy removal of the solvent, and it can be used as a solvent in chemical processes, such as extraction, crystallization, reaction, and so on. Amphiphilic molecules (e.g., surfactant and lipid) in the solvent play crucial roles in the liquid–liquid extraction process, and they also form a self-assembled structure in the solvent. Because of the low polarizability per volume of CO₂, the surfactant often forms self-assembled aggregates, such as micelles, reversed micelles, vesicles, and microemulsions, in the CO₂/water/surfactant ternary system. Because CO₂ is easily removed by decomposition of the CO₂/water/surfactant system, such systems can be regarded as nonsolvent methods. Recently, nonsolvent methods to prepare self-assemblies have attracted much attention to overcome the operational drawbacks of the conventional method to prepare liposomes (vesicles).

Emergent properties arising from the membrane of the liposome (vesicle) have been introduced, where the vesicle (liposome) membrane can selectively interact with various types of molecules under particular conditions. The important functions of vesicles include (1) interaction with proteins (enzymes) and nucleotides, (2) storage of encapsulated molecules, and (3) fusion with membranes. Vesicle membranes are dynamic, for example, vesicles composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) possess phase transition temperatures (T_m) at 23.6 and 41.6 °C, respectively. Based on vesicle characterization protocols, a variety of vesicles prepared by several methods have been investigated. The membrane properties, such as the phase state, membrane fluidity, and membrane polarity, are possible factors to recruit the guest molecules on the membrane surface. However, it is assumed that a specific assembled state (i.e., interdigitated membrane) might form in the liquid–liquid–surfactant ternary system. Therefore, it is important to estimate the physicochemical properties of vesicles, such as the membrane fluidity and polarity.
In the case of the thin-film hydration method, phospholipids are first dissolved in organic solution. After the solvent is removed, the phospholipids are dispersed in water and then form a self-assembled bilayer. Otake et al. reported the scCO$_2$ reverse phase evaporation method using a scCO$_2$/water fluid solvent. In this method, the components are mixed in a “homogeneous” fluid system (scCO$_2$/water). On the other hand, the microchannel method requires heterogeneous liquid systems, such as isopropyl alcohol (IPA)/water. Although such liquid–liquid two phase (heterogeneous) systems have advantages from the viewpoint of energy loss, organic solvents in the product must be removed when the vesicles are used in medical applications, cosmetics, and food. Therefore, the nonsolvent CO$_2$ method is an ideal method to provide environmentally friendly vesicles. However, the details of vesicle bilayer formation and the physicochemical membrane properties are still unclear.

The aims of this study are to clarify the key step in formation of vesicle aggregates in high-pressure CO$_2$/water systems, and to analyze the membrane properties of the vesicles. By controlling the temperature and pressure, CO$_2$/water can take the following phase states: CO$_2$(gas)/water(liquid), CO$_2$(liquid)/water(liquid), and CO$_2$(supercritical)/water(liquid) (Fig. 1). In the present study, the phospholipid vesicles were prepared in high-pressured CO$_2$/water systems. DMPC and DPPC are well-known phospholipids that are used to model biomembranes and drug encapsulating carriers. At the interface of high-pressure CO$_2$ and water, phospholipid molecules might act as a surfactant and form a self-assembled membrane in the water phase after decompression of the systems. We describe the physicochemical properties of vesicles prepared at different temperatures and pressures. Finally, the possible mechanism for the membrane formation is discussed.

II. EXPERIMENT

A. Materials

DPPC, DMPC, and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH), 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical (Osaka Japan) and were used without further purification. Distilled water was purified with the Millipore Milli-Q system (EMD Millipore Co./Direct-Q UV3). CO$_2$ gas was purchased from Ueno Gas Co. (Mie, Japan).

B. Vesicle preparation by nonsolvent CO$_2$ method

The phospholipid vesicles were prepared by using the stainless batch reactor, TSC-CO$_2$-08 [inside volume of 80 ml, $\phi = 40$ mm, Taiatsu Techno Co. (Tokyo, Japan), see Fig. 2]. A phospholipid powder (4.5 mg), distilled water (3 ml), and CO$_2$ (gas or liquid) were incubated in the reactor with stirring at various temperatures (25, 50°C) and pressures (5, 10 MPa). After 20 min-incubation for equilibration, the sample solutions were decompressed by releasing CO$_2$. The lipid concentration was measured by using phospholipid C-test Wako (Wako Pure Chemical Industries, Ltd.).

C. Liposome preparation by conventional thin-film hydration method

A chloroform solution of phospholipids was dried in a round-bottomed flask by rotary evaporation under vacuum. The resulting lipid films were dissolved in chloroform once...
more, and the solvent was evaporated. This operation was repeated at least twice. The obtained lipid thin film was kept under high vacuum for at least 3 h and then hydrated with distilled water at room temperature. The obtained vesicle suspension was frozen at \(-80^\circ\text{C}\) and then thawed at 60 \(^{\circ}\text{C}\) to enhance the transformation of small vesicles into larger multilamellar vesicles (MLVs). This freeze-thaw cycle was repeated five times. MLVs were used to prepare large unilamellar vesicles by extruding the MLV suspension 11 times through two layers of a polycarbonate membrane with a mean pore diameter of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada). Vesicles with different compositions were also prepared by the same method.

D. Size distribution of vesicles

The average diameter and size distribution of prepared vesicles (lipid concentration: 20 mM) were analyzed with a laser diffraction system (MT3300 II, NIKKISO CO., Tokyo, Japan) equipped with the TRI-BLUE laser (780 nm). Scattering angle was varied (0\(^{\circ}\)–180\(^{\circ}\)) to examine the polydispersity (PDI) of the vesicles. The average diameters were calculated based on a number-average diameter. PDI values were calculated based on the literature.\(^{30}\) All measurements were performed at 25 \(^{\circ}\text{C}\).

E. Evaluation of the membrane properties of vesicles

The fluidity in the interior of the liposomes was evaluated based on the previous reports.\(^{21,27}\) Fluorescent probe DPH was added to a vesicle suspension with a molar ratio of lipid/DPH = 250/1; the final concentrations of lipid and DPH were 100 and 0.4 \(\mu\text{M}\), respectively. The fluorescence polarization of DPH (Ex. = 360 nm, Em. = 430 nm) was measured using a fluorescence spectrophotometer (FP-6500; JASCO, Tokyo, Japan) after incubation at 30 \(^{\circ}\text{C}\) for 30 min. The sample was excited with vertically polarized light (360 nm), and emission intensities both perpendicular \((I_\perp)\) and parallel \((I_\parallel)\) to the excited light were recorded at 430 nm. The polarization \((P)\) of DPH was then calculated by using the following equations:

\[
P = (I_\parallel - GI_\perp)/(I_\perp + GI_\perp),
\]

\[
G = I_\perp/I_\parallel.
\]

where \(I_\perp\) and \(I_\parallel\) are emission intensity perpendicular and parallel to the horizontally polarized light, respectively, and \(G\) is the correction factor. The membrane fluidity was evaluated based on the reciprocal of polarization, \(1/P\).

Fluorescent probe Laurdan is sensitive to the polarity around itself, which allows the surface polarity of lipid membranes to be determined.\(^{21,22,31}\) Laurdan emission spectra exhibit a red shift caused by dielectric relaxation. Thus, emission spectra were calculated by measuring the general polarization \((GP_{340})\) for each emission wavelength as follows:

\[
GP_{340} = (I_{440} - I_{490})/(I_{440} + I_{490}),
\]

where \(I_{440}\) and \(I_{490}\) are the emission intensities of Laurdan excited with 340 nm light. The final concentrations of lipid and Laurdan in the test solution were 100 and 1 \(\mu\text{M}\), respectively.

III. RESULTS AND DISCUSSION

A. Characterization of DMPC vesicles prepared by the nonsolvent CO\(_2\) method

DMPC vesicle suspensions were prepared at different temperatures and pressures, and the size distributions were evaluated using laser diffraction analysis (Fig. 3). DMPC aggregates were obtained with average diameters of 3.5–9.9 \(\mu\text{m}\) and PDIs of 0.014–0.357. It is notable that self-assemblies were formed at different incubation conditions, such as CO\(_2\)(gas)/water(liquid), CO\(_2\)(liquid)/water(liquid), and CO\(_2\)(supercritical)/water(liquid). Because the light scattering
analysis might not distinguish solid particles (lipid aggregates) and vesicle aggregates, Raman spectroscopic analysis was performed (Fig. S1, supplementary material\textsuperscript{32}). In our previous report, the ratio of Raman peak intensity ($I_{2940}/I_{2930}$) was shown to reflect the phase states of the vesicles in aqueous solution, while the ratio indicated constant value in the case of lipid powder (solid).\textsuperscript{33} Based on Raman analysis, the phase transition occurred in the case of the DMPC aggregates and its tendency is similar to that in the case of DMPC liposomes prepared by the thin-film hydration method.\textsuperscript{33} In addition, analysis of the physicochemical membrane properties of the DMPC aggregates revealed the formation of vesicle membranes (see Sec. III C). These results suggest that the nonsolvent CO$_2$ method is useful to prepare phospholipid vesicles. However, the size distribution could not be completely controlled, and also, a small amount of lipid aggregates could still remain in the batch reactor, indicating that the phospholipid solubility depends on the preparation conditions. In order to evaluate the quantity and quality of the vesicles obtained in this method, the yield of the obtained vesicle and its membrane properties were furthermore evaluated.

B. Lipid concentration of the vesicles prepared at different temperatures and pressures

Because the $T_m$ value of DPPC is 41.6°C, the dispersibility of DPPC molecules in CO$_2$/water systems could be different at 25°C (below $T_m$) and 50°C (above $T_m$). Therefore, DPPC vesicle suspensions were prepared at different temperatures and pressures as shown in Fig. 1. From mass balance, the phospholipid yields were estimated for the following systems: (I) CO$_2$(gas)/water(liquid), 25°C and 5 MPa; (II) CO$_2$(gas)/water(liquid), 50°C and 5 MPa; (III) CO$_2$(liquid)/water(liquid), 25°C and 10 MPa; and (IV) CO$_2$(supercritical)/water(liquid), 50°C and 10 MPa. To compare the dispersibility of DPPC in water, the vesicle yield was evaluated by the following equation:

$$\text{Yield} \ (\%) = \frac{C_{\text{vesicle}}}{C_{\text{ideal}}} \times 100,$$

where $C_{\text{vesicle}}$ and $C_{\text{ideal}}$ are the obtained lipid concentration and the initial lipid concentration before CO$_2$ treatment. The relationship between the vesicle preparation conditions and the yield was shown in Fig. 4. The yields for systems (II), (III), and (IV) were as good as that of the conventional vesicle preparation method (i.e., the yield of DPPC vesicles using the thin-film hydration method\textsuperscript{22,34} was 92.8 ± 3.0%; although a small amount of lipid film existed in the round bottom flask in our experiment). In contrast, the yield for system (I) was lower than the other conditions, and an insoluble lumpy lipid aggregate was observed in the sample. At 25°C, the yield for system (III) was higher than that for system (I), indicating that high-pressure CO$_2$(liquid) could assist the dispersion of DPPC molecules in the water phase. Because of the lower dielectric constant of high-pressure CO$_2$(liquid, supercritical)\textsuperscript{9,10} phospholipid molecules could be soluble in the CO$_2$ phases, and then the membrane could form at the interface of the CO$_2$–water phases. Although the conditions for system (I) and (II) were single liquid (water) phase systems, the vesicle yield for system (II) was higher than that for system (I). This indicates that the dispersibility of phospholipid in water above $T_m$ is higher than that below $T_m$. In the conventional vesicle preparation process, organic solvents are used to prepare a lipid thin-film, which improves the dispersibility of lipid molecules in aqueous solution. The lipid powder is usually difficult to disperse in water because of the strong van der Waals forces between lipid molecules. In the high-pressure CO$_2$(liquid, supercritical)/water(liquid) systems, the liquid (fluid)–liquid interface could act as a platform for organization of the phospholipid thin layer, resulting in a higher yield of DPPC vesicles like the conventional method. It is therefore suggested that the CO$_2$–water interface plays an important role in the formation of phospholipid assemblies in the nonsolvent method.

C. Characterization of the physicochemical properties of the vesicles

To estimate the physicochemical properties of the vesicles, the membrane fluidity and polarity were analyzed using DPH and Laurdan, respectively (Fig. 5), based on our previous reports.\textsuperscript{21,22} As a reference, conventional vesicles were prepared by the thin-film method. It was found that the membrane fluidity and polarity of DPPC vesicles prepared by the nonsolvent CO$_2$ method were almost the same as those of conventional DPPC liposomes. Based on Cartesian diagram analysis,\textsuperscript{22} the vesicles (liposomes) appeared in the second quadrant (low membrane fluidity and high membrane polarity) could be in ordered phases. Because the $T_m$ of DPPC is 41.6°C, a drastic increase in the membrane fluidity of all of the DPPC vesicles was observed at 50°C, together
with a decrease in the membrane polarity. The conventional liposomes also showed such a phase transition tendency.

These results clearly demonstrate that the quality of the vesicles prepared by the nonsolvent CO2 method (heterogeneous systems) is almost the same with that of the liposomes prepared by the conventional method. For the DOPC vesicles prepared by the nonsolvent CO2 method, analysis of the membrane properties was carried out (Fig. S2, supplementary material). In Cartesian diagram, the DOPC vesicles and the conventional DOPC liposomes appeared in the fourth quadrant for 20–50°C, indicating that DOPC vesicles possess high fluidity and low membrane polarity. The Tm of DOPC is −17.2°C, and the DOPC vesicles are estimated to be in liquid disordered phases. At the liquid–liquid interface (II and III) in the CO2–water–phospholipid system, formation of the thin lipid layer could be promoted, which can enhance the self-assembly of lipid molecules (Fig. 6). In addition, the nonsolvent CO2 method can be applied to prepare other types of vesicles, such as DMPC (Fig. S3, supplementary material) and DOPC/DPPC binary vesicles (data not shown). Comparing these CO2/water systems (gas–liquid, liquid–liquid, and supercritical–liquid), it is suggested that the interface regions in the CO2/water systems play a crucial role on the organization of phospholipid membrane, and the organized membrane converts to vesicles after decompression.

IV. SUMMARY AND CONCLUSIONS

In the present study, phospholipid vesicles were prepared by the nonsolvent CO2 method, and they were then characterized focusing on the physicochemical membrane properties. DMPC vesicle formation was verified by laser diffraction and Raman analysis. Phospholipid vesicles were efficiently formed in liquid (fluid)–liquid systems. The characteristics of the vesicles prepared by this method were almost the same as those of conventional liposomes. The nonsolvent process and one-step quick preparation are regarded as the advantages of this method, although the vesicle yield and the diameter control must be improved. It is expected that vesicles with desired size can be prepared by controlling the microstructure of the self-assembly and the characteristics of its interface.
during the shift of the pressure and temperature above the critical point of the system. A continuous vesicle preparation method using microfluidics also requires a liquid–liquid interface (e.g., IPA/water) to form a membrane, although the prepared vesicle suspension must be heat treated to remove IPA. Nonsolvent methods for vesicle preparation are preferred in the research fields of biochemistry, drug delivery systems, food, and cosmetics. Introduction of high-pressure CO2/water systems to such continuous systems would improve self-assembled vesicle preparation and provide vesicles with desired membrane properties.

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