Formation and Physicochemical Properties of Liposomes Using a Supercritical Reverse Phase Evaporation Method

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ABSTRACT: L-a-dipalmitoylphosphatidylcholine (DPPC) liposomes were prepared using supercritical reverse phase evaporation (scRPE) method. The effects of phospholipid structure, preparation pressure, and amount of ethanol on their physicochemical properties were examined using measurements of trapping efficiency, cloud point measurements, dynamic light scattering, and osmotic water transport studies. The maximum trapping efficiency of DPPC liposomes prepared by the scRPE method were 22.5 %. The phase behavior of DPPC/ethanol/CO_2 mixture before introducing water strongly influenced their physicochemical properties. The sizes of liposomes prepared at pressures below 200 Bar seem to be larger than those prepared at > 200 Bar. The minimum ethanol concentration to obtain water in CO_2 emulsion at 200 bar is 6.8 wt% which corresponds well to the optimum ethanol concentration to obtain large unilamellar vesicles with the highest trapping efficiency. On the other hand, Multilamellar vesicles (MLV) seem to be formed when the ethanol concentration added to the system is less than 4.5 wt%. The scRPE method makes it possible to control the physicochemical properties of liposomes like particle size, size distribution, trapping efficiency and lamellarity in a single step.

I. INTRODUCTION

Liposomes are self-enclosed lipid bilayer encapsulating an inner solution phase, initially found by Bangham *et al.* in 1974 [1]. They have long been used as models for biological membranes [2-5]. Recently liposomes have proven to be promising carriers for drugs, gene therapy, and many other medical and cosmetic applications [6-14]. There are several methods for the formation of liposomes such as Bangham method, freeze-thawing method, supersonic irradiation method, reverse-phase evaporation method, and detergent dialysis method. All of these methods require large amounts of organic solvents that are harmful to the environment and human body, and it is very difficult to obtain liposomes having a high trapping efficiency for water-soluble substances without organic solvents. When liposomes are used as drug carriers, use of organic solvents must be avoided as much as possible in their preparation because complete removal of the remaining organic solvent is required. Further, as these methods consist of many steps, they are not suitable for mass production. Because of these reasons, few examples of the effective use of liposomes are known even though their potential advantages have long been widely recognized.

Supercritical carbon dioxide ($scCO_2$) is an environment-friendly alternative solvent that can replace organic solvents since it has similar solvent properties to non-polar organic solvents, has low critical temperature (T_c= 31 ^oC) and a pressure (P_c=73.8 bar), and is non-toxic, inflammable, and cheap. With $scCO_2$, we developed a new method, supercritical reverse phase evaporation (scRPE) method, for the preparation of liposomes of a high trapping efficiency for water-soluble substances using $scCO_2$. The basic concept of the scRPE method is

that form lipid interface between water and $scCO_2$ by way of the formation of emulsions, and inverses the phase by depressurization. [15-17]

II. EXPERIMENTAL

MATERIALS: L-a-dipalmitoylphospha-tidylcholine (DPPC, 99.8 %) was supplied by Nippon Oil & Fats Co., and used without further purification. D-(+)-glucose (98.0 %) purchased from Showa Chemical Co. was used as supplied to determine the trapping efficiency of liposomes for water-soluble substance. Ethanol (EtOH, 99.5 %) was purchased from Wako Pure Chemical Industries, Ltd. and used as a co-solvent. Injection grade water was purchased from Otsuka Co. Ltd., and used as received. Carbon dioxide (CO_2, 99.99 %) was purchased from Tomoe Shokai CO., Ltd., and used without further treatment.

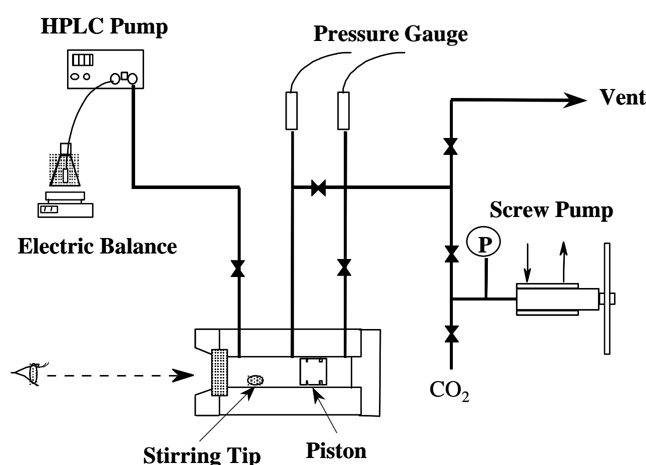

Figure 1. Schematic representation of experimental apparatus.

APPARATUS: Figure 1 shows a schematic diagram of the experimental apparatus used for liposome preparation and cloud point measurements. It consists mainly of three parts, a variable volume view cell (Model HP-1, Tama Seiki Co.), HPLC pump (Shodex DS-4) for feeding aqueous solution into the view cell, and a high pressure pump (Model HP-1, Tama Seiki Co.) for $scCO_2$ feed and pressure control. Temperature of the view cell was measured and maintained with a temperature controller equipped with a Pt resistance thermometer. Pressures on the front and backside of the cell were measured with two strain gauges (PG-500KU, Kyowa Co.). The accuracies of temperature and pressure were within $\pm 0.1^{o}C$ and $\pm 0.1 kgf/m^2$, respectively. The amount of aqueous solution fed into the cell was measured with an electric balance (BX 4200H, Shimadzu Co.). The volume of the view cell was ca.50 cm^3.

LIPOSOMES PREPARATION: After phospholipid (0.3 wt% against CO_2 (wt/wt)) and ethanol (0~24wt% against CO_2 (wt/wt)) were sealed in the view cell, CO_2 (11.87g) was introduced into the cell, and the cell temperature was then raised to $60^{o}C$, a temperature higher than the phase transition temperature ($41^{o}C$) of DPPC, while the pressure was maintained at the designed pressure (120~300Bar). After 20 minutes for equilibration, an aqueous D-(+)-glucose solution (0.2 mol/L) was slowly (0.05 ml/min) introduced into the cell via a HPLC pump until the desired amount of solution is attained. The pressure was then reduced to release CO_2, thereby giving a homogeneous liposomal dispersion. The contents of the cell were continuously mixed with a magnetic stirrer during the experiments. Efficiency of the liposome formation was judged by the trapping efficiency witch given by the ratio of the amount of water phase trapped inside the liposome. Detailed description of experimental procedure is described in the previous paper [15].

TRAPPING EFFICIENCY MMEASUREMENTS: The liposome dispersion was dialyzed against water using a cellophane tube (Viscase Scales Corp.) to remove the non-encapsulated glucose remaining in the dispersion medium. The liposomes inside the tube were then destroyed by addition of ethanol. The amount of glucose in the solution was determined by the mutarotase GOD method [24,25] using a spectrophotometer (MPS-2000, Shimazu). The trapping efficiency was calculated according to the equation.

$$Trapping\ efficiency\,[\%] = \frac{glu\cos e\ excapsulatedinliposome}{glu\cos einliposomalsolution} \times 100$$

OSMOTIC SHURINKAGE VELOCITY MEASUREMENTS: There is a linear relationship between the volume change and the difference in osmotic pressure of the outer and inner solution of liposome as shown bellow:

$$\frac{dV}{dt} = K_W SRT\left(c_i^0 - c_i^i\right)$$

where dV/dt the volume change with time, K_W the permeability constant for water, S the area occupied by the membrane, R the gas constant, T the absolute temperature and c_i^0 and c_i^i are the concentrations of the impermeable solutes outside and inside the liposomes, respectively. If the concentration change was measured by a photometer, one can measure the volume change as a change in absorbance. Since the relative initial change in the absorbance is directly proportional to the initial volume change, the following relation holds:

$$d\left(\frac{1}{A}\right)\Big/dt\,[\%] = k\frac{dV}{dt} = kK_W SRT\left(c_i^0 - c_i^i\right)$$

where $d(1/A)/dt\,[\%]$ the initial shrinkage velocity given by $(\Delta At/\Delta t)\cdot(100/A_0)$, and $\Delta(1/A)[\%] = (1/A_0 - 1/A_{max})A_0 \cdot 100$ $D(1/A)$ being a measure for the extent of shrinkage. The liposomes (15mM) were prepared in an aqueous D-(+)-glucose solution (0.2 mol/L) as previously described. For the osmotic shrinkage measurements the liposomes were diluted to 1mM in the medium in which they were prepared (D-(+)-glucose solution 0.2 mol/L). After temperature equilibration, an osmotic shock was given by rapidly injecting a small volume (0.1ml) of a concentrated D-(+)-glucose solution (2M) preincubated at the same temperature. Changes in the turbidity were determined with a spectrophotometer (MPS-2000, Shimazu) at 450 nm, and initial shrinkage velocity was determined.

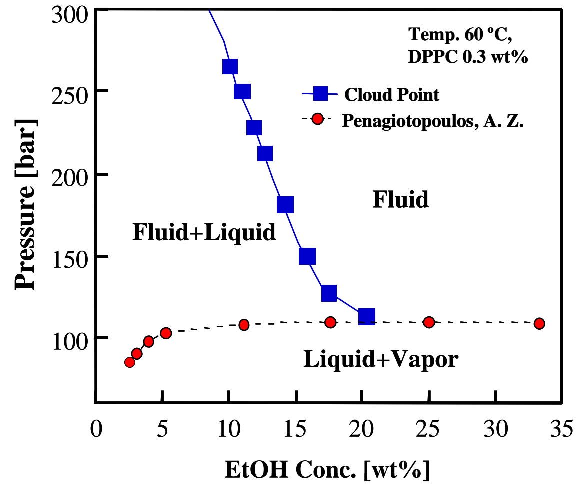

Figure 2. Phase diagram of DPPC/scCO$_2$/EtOH system at 60°C.

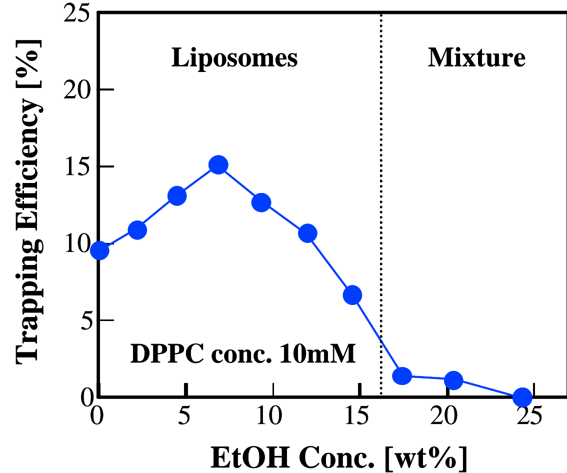

Figure 3. EtOH concentration dependence of the trapping efficiency at 200bar.

III. RESULTS AND DISCUSSION

Figure 2 shows the effect of EtOH on the cloud-point behavior of DPPC/scCO$_2$ at 60 °C. The cloud-points pressure was defined as pressure at which the mixture becomes so

opaque that it is no longer possible to recognize the stirring tip inside the cell. Cloud points were obtained at a fixed DPPC concentration of 0.3 wt%, which is identical to that used in liposome preparation. The figure also shows the vapor-liquid equilibrium data for binary $scCO_2$/EtOH mixtures measured by Penagiotopoulos [18] at 64.2 °C. The cloud point curve takes on the appearance of a typical lower critical solution temperature (LCST) boundary of $scCO_2$/EtOH mixtures. The cloud point curve intersects a liquid → liquid + vapor (LV) curve at EtOH concentration 20.4 wt%. The amount of EtOH required to obtain a single phase under the conditions of liposome preparation, 200 bar and 60 °C, was estimated to be about 14 wt%.

Liposomes were prepared at constant pressure (200bar) with changing the EtOH concentration and at constant EtOH concentration (14wt%) with changing the pressure. **Figure 3** shows the EtOH concentration dependence of the trapping efficiency of liposome prepared at 60 °C and 200 bar. The trapping efficiency increased and then decreased to zero with increase in the amount of EtOH. The turbidity of the liposome suspension became low, i.e. transparent, with an increase in the amount of EtOH added to the system. As EtOH is an inhibitor of liposome formation and its excess amount destroys the liposomes formed.

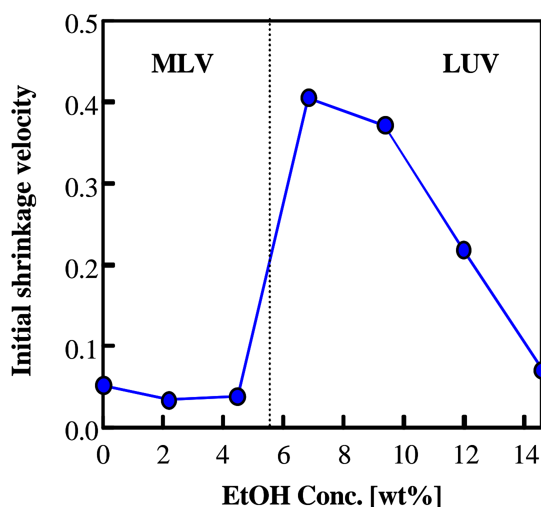

Figure 4. EtOH dependence of the initial shrinkage velocity of liposomes.

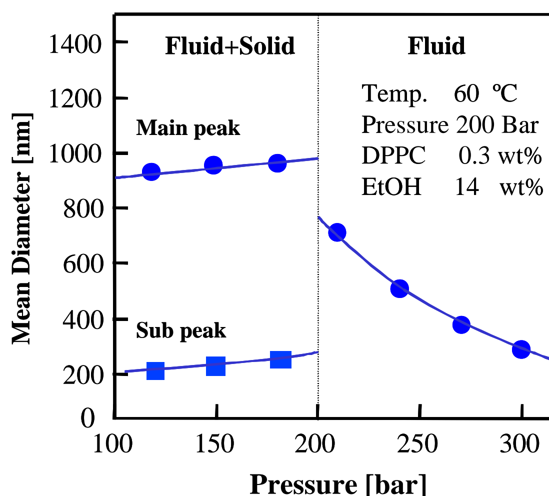

Figure 5. Pressure dependence of the mean diameter of liposomes.

It is well known that liposomes behave as a perfect osmometers. **Figure 4** shows EtOH concentration dependence of initial shrinkage velocity of liposomes. In general, the osmotic shrinkage velocity and trapping efficiency of liposomes depends on their lamellarity (number of bilayers). Liposomes consisting of a smaller number of bilayers have higher velocity, and higher trapping efficiencies. From the Figs. 3 and 4, it is clear that the lamellarity of liposomes can be controlled by varying the amount of EtOH. The liposome formed at the maximum trapping efficiency condition was large unilamellar vesicle [15].

Figure 5 shows the pressure dependence of the mean diameter of liposome prepared at DPPC 10mM and EtOH 14wt%. From the figure, it is clear that the liposomes prepared at pressures higher than 200 Bar have smaller size and size distribution compared with those of the liposome prepared at pressures lower than 200 Bar. These differences of size and size distribution will be the cause of the pressure dependence of the trapping efficiencies difference. The trapping efficiencies of polydispersed liposomes are higher than those of homodispersed liposomes since polydispersed liposomes are packed higher in the solution than that of homodispersed liopsomes. As mentioned above, the mean diameters of the liposomes prepared

at pressures higher than 200 Bar are homodisperse, and decrease with an increase in the pressure. These results clearly show that the desired particle size of liposome could be prepared by varying the preparation pressure.

We have already reported that a water-in-scCO_2 (water/CO_2) emulsion is formed before liposome formation inside the cell [15-17]. Visual observations of water-in-scCO_2 emulsion at pressures higher and lower than 200 bar revealed that the emulsion is more turbid at lower pressures, implying that the droplet size in water-in-scCO_2 emulsion at the lower pressures is larger than that at the higher pressures. The difference in the droplet size in water-in-scCO_2 emulsion would result in that in the particle size of liposomes prepared at various pressures. These results demonstrate that the phase behavior of phospholipid/scCO_2/EtOH mixtures before introducing water influences the trapping efficiency and size distribution of liposomes. In other words, with changing the phase condition of precursor phospholipids/EtOH/ scCO_2 solutions, size and lamellarity of the liposomes could be controlled.

Figure 6 shows the trapping efficiency of the DPPC liposome prepared at the maximum condition (60°C, 200bar, EtOH 7wt%, DPPC 0.3wt%). That of liposomes prepared by the Bangham method is also shown for comparison. The trapping efficiency is 5 times higher than that obtained by the Bangham method.

Figure 7 shows the freeze fracture TEM image of the liposomes obtained by the scRPE method. From the figure, it is clear that, as mentioned above, liposomes prepared by the scRPE have only single lipid bilayer.

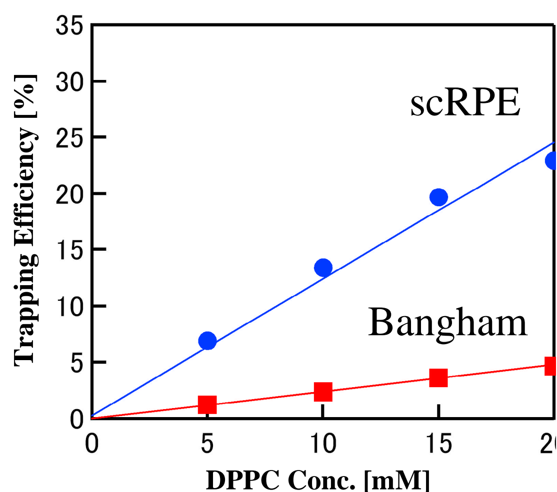

Figure 6. Trapping efficiency of the DPPC liposome.

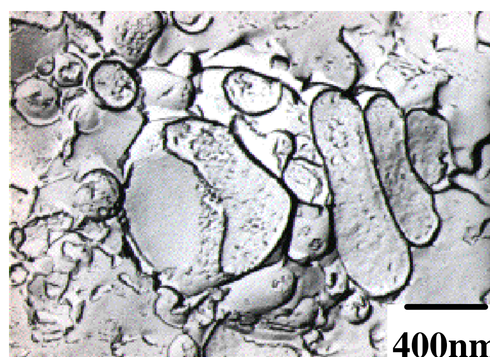

Figure 7. TEM image of the liposome obtained by the scRPE method.

Figure 8. Outlook of the semi batch type liposome production apparatus.

VI. CONCLUSION

Liposomes are thermodynamically unstable, and the method of their preparation directly determines their physicochemical properties. But the new scRPE method makes it possible to

control the physicochemical properties such as particle size, size distribution, trapping efficiency, and lamellarity by varying the preparation pressure and the amount of ethanol added to the system. Because this method can make liposomes by one step, and uses no toxic organic solvents, scale-up is very easy. Figure 8 shows the semi-batch type liposome preparation apparatus constructed in our laboratory [19]. With the apparatus, 50cc of liposomal solution was obtained in 100 minutes. Liposomes entrapping Vitamin C instead of glucose were prepared. Liposomes entrapping oil-soluble substances such as cholesterol, Ceramide 3, and Vitamin A derivative were also successfully prepare with system. The scRPE method enables the preparation of liposomes with a high trapping efficiency for not only water-soluble but also oil-soluble substances without using any toxic organic solvents.

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