Preparation of Lipid Vesicles Having Suitable Size for Drug Delivery with High Entrapment of Hydrophilic Molecules Using Multiple Emulsions

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Abstract

A method for preparing lipid vesicles having suitable size for drug delivery (in a few hundred nanometer size) with high entrapment efficiency of hydrophilic molecules was developed. The lipid vesicles containing hydrophilic molecules in their internal compartments were formed from water-in-oil-in-water (W1/O/W2) multiple emulsions after the removal of organic solvent by evaporation under ambient condition. The primary W1/O emulsions were formed via homogenization by sonication of mixture of oil phase containing bilayer forming lipids, and the water phase containing hydrophilic molecules to be entrapped. The W1/O/W2 multiple emulsions were formed through microchannel (MC) emulsification by introducing the dispersed phase, i.e., W1/O emulsion, into the MC device in the presence of polymeric surfactants in the external water phase. The average size of the lipid vesicles formed, measured using dynamic light scattering and observed by transmittance electron microscopy, was 182 nm, and comparable with the size of the initial water droplet of the primary W1/O emulsion (192 nm), indicating that the vesicle size reflects the water droplet size of the primary W1/O emulsion. High entrapment yields for hydrophilic molecules, namely 89.3 ± 4.2% for calcein and 41.1 ± 3.3% for 5-fluorouracil, were achieved.

Keywords: Lipid vesicles; Multiple emulsion; Microchannel emulsification; Drug encapsulation, Drug carrier; Electron microscopy

Introduction

Lipid vesicles (also called liposomes) are compartments enclosing aqueous phase separated from an external water phase by membrane phospholipids. They are formed by the molecular assembly of lipids capable of forming bilayer structure. Extensive studies have been carried out on the formulation of lipid vesicles as carriers for the efficient delivery of bioactive molecules in pharmaceutical industries, food, cosmetics, and other related fields. Many efforts have been made to improve on the properties of nano- and micro-dispersions, especially the entrapment efficiency, size, and stability, which are still major problems standing in the way of new advances in this field of research [1]. It is usually difficult to entrap hydrophilic molecules in the internal aqueous phase due to their solubilization to internal and external water phases of a vesicle suspension, with low volume ratio of the internal water phase to the external water phase [2].

The size of lipid vesicles is of great importance for their use in delivery of therapeutic agents, since it has been reported to influence their behavior in biological systems [3]. For the transfer of the lipid vesicles from the blood vessels into the diseased site, the size ought to be about submicron level [4]. To attain the therapeutic levels of the drugs, efficient entrapment is required or a large amount of lipids is needed. Therefore, high entrapment efficiency is an important issue for vesicle preparation.

Recently we developed a novel method for preparing lipid vesicles in sub- to few micrometer size with high entrapment efficiency and controlled size using water-in-oil-in-water (W1/O/W2) multiple emulsions as vesicle templates [5,6]. In this vesicle preparation method, the internal water droplets (W1, with hydrophilic materials) of multiple emulsions are converted to internal water phases of lipid vesicles, providing a platform for the realization of high entrapment efficiencies of hydrophilic materials and size control by controlling the emulsion droplet size. W1/O/W2 emulsion is composed of internal (W1) and external (W2) water phases, which are chemically alike, with an intermediate immiscible oil (O) phase that physically separates the two like-phases. For the preparation of W1/O/W2 emulsions as templates for vesicles, microchannel (MC) emulsification technique [7,8] was used. The MC emulsification does not cause a high shear field during emulsification process, and is being driven by interfacial droplet formation mechanism. As a result, there is lower rate of breakdown and deformation of multiple droplets, as reported previously [9-11]. Thus, MC technique is advantageous in minimizing the leakage of encapsulated materials and a more homogeneous size distribution of the resulting vesicles.

In this study, we applied our new vesicle preparation method for preparing lipid vesicles having suitable size for drug delivery, namely in a few hundred nanometer size, with potentials for high entrapment efficiencies of hydrophilic molecules, i.e., calcein as a fluorescent marker and 5-fluorouracil as a cancer drug. Furthermore, polymeric surfactants were used in the external water phase. These would help to stabilize the template multiple emulsions against leakage [12,13], and also help to avoid the solubilization of lipid bilayers of vesicle by surfactant micelles.

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Materials and Methods

Chemicals

Phosphatidylcholine (PC) from egg yolk (PC, COATSOME NC-50, purity >96%) was purchased from NOF Corporation, Tokyo, Japan. Cholesterol (Chol), oleic acid (OA), sodium caseinate (M.W. ca 24,000), and n-hexane were obtained from Wako Pure Chemical Industries, Osaka, Japan. Polyoxyethylene polyoxypropylene glycol (also called Pluronic), of the type Pluronic F68 (average M.W. ca 8,700) was kindly gifted from NOF Corporation (Tokyo, Japan). Calcein and 5-fluorouracil were purchased from Sigma (St Louis, MO). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), and were of extra pure grade.

Preparation of lipid vesicles – primary and secondary emulsification, solvent evaporation

The schematic presentation of the procedure for preparation of lipid vesicles using multiple emulsions as templates is depicted in (Figure 1). The primary W1/O emulsion preparation was by ultrasonically mixing the dispersed phase, W1 with the continuous oil phase (O phase) in the ratio of 1:3 in a glass vial. The W1 phase consists of calcein (0.4 mmol/L), or 5-fluorouracil (7.7 mmol/L in a 50 mmol/L tris-HCl buffer at pH 8). n-Hexane containing PC, Chol and OA (26 mmol/mL each) was used as a continuous phase (O phase). The mixture was emulsified by sonication. The sonication was carried out at 71 W power with a 1:1 pulse ratio using the probe-type sonicator (UD-201, Tomy Seiko Co. Ltd., Tokyo, Japan). During the sonication process, the glass vial containing the mixture was kept at below 25°C and sonicated for 10 min.

A MC emulsification unit (obtained from EP-Tec, Ibaraki, Japan) was used to prepare W1/O/W2 emulsions. The device consists of a silicon MC plate, a glass plate, a stainless module, a glass liquid channel to feed the W1/O emulsion as the to-be-dispersed phase, and a microscope video system to observe the emulsification process. The MC used in this study had a channel width of 16 µm, a terrace length of 11 µm. The MC module was initially filled with the continuous water (W2) phase containing sodium caseinate or 5-fluorouracil (7.7 mmol/L in a 50 mmol/L tris-HCl buffer at pH 8). The MC used in this study had a channel width of 16 µm, a terrace length of 11 µm. The MC module was initially filled with the continuous water (W2) phase containing sodium caseinate or 5-fluorouracil (7.7 mmol/L in a 50 mmol/L tris-HCl buffer at pH 8). The MC module was initially filled with the continuous water (W2) phase containing sodium caseinate or 5-fluorouracil (7.7 mmol/L in a 50 mmol/L tris-HCl buffer at pH 8). The MC module was initially filled with the continuous water (W2) phase containing sodium caseinate or 5-fluorouracil (7.7 mmol/L in a 50 mmol/L tris-HCl buffer at pH 8). During the sonication process, the glass vial containing the mixture was kept at below 25°C and sonicated for 10 min.

To obtain the lipid vesicles from the W1/O/W2 emulsion, solvent evaporation of the W1/O/W2 emulsion was conducted under atmospheric pressure and room temperature for 20 h.

Analytical methods

The sizes of the W1/O and W1/O/W2 emulsion droplets and the lipid vesicles were measured by dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Worcesters, UK). The size of the W1/O/W2 emulsions was determined by analyzing images of at least 150 droplets taken with the microscope video system. The image-analyzing software WinRoof (Mitani Co. Ltd., Tokyo, Japan) was used to analyze the captured images.

Transmission electron micrographs of the vesicles were obtained on a JEM2000EX (JEOL, Tokyo, Japan) using the negative staining method with phosphotungstic acid as a staining reagent, and the freeze fracture replica method.

The proportion of calcein entrapped into lipid vesicles was determined fluorometrically by modifying the method used for determining the trapped volume of liposomes [2]. The calcein content in lipid vesicles was determined from fluorescence intensities before and after cobalt (II) ions were added, which quench fluorescence of calcein. Fluorescence intensity was measured as the fluorescence spectrophotometer (FP-6500, JASCO, Tokyo, Japan). The fluorescence from 3 mL of the sample was measured before (Ftotal) and after (Faux) addition of 0.03 mL of CoCl2 solution (10 mmol/L). Ftotal corresponds to the fluorescence from all materials composing the sample and Faux corresponds to the fluorescence from the internal water phase plus the fluorescence from lipid vesicles themselves (Faux). The entrapment yield of calcein was calculated from the equation,

\[ \text{Entrapment yield} \% = 100 \times \left( \frac{F_{\text{total}} - F_{\text{aux}}}{F_{\text{total}} - F_{\text{aux}}} \right) \]

Where, the value of 1.01 is the dilution volume factor accounting for addition of CoCl2 solution.

The entrapment yield of 5-fluorouracil into the lipid vesicles was determined by high performance liquid chromatography (HPLC). Asahipak ODP-50 4D column (4.6 mm × 150 mm, Showa Denk, Tokyo, Japan) was used. The mobile phase was a mixture of water and acetonitrile, in the ratio of 90:10 (v/v). The column temperature was kept at 30°C and a flow rate of the mobile phase was 0.6 ml/min. The 5-fluorouracil was detected using a UV-vis detector (UV-9700, JASCO, Tokyo, Japan) at a wavelength of 265 nm. In order to determine the concentration of 5-fluorouracil in the outer water phase (Cout), the vesicle suspension was centrifugally ultrafiltrated for 10 minutes at 5,100 rpm using Centrisart I (MWCO 10,000, Saltorius AG, Goettingen, Germany). Lipid vesicles are rejected by the ultrafiltration membrane, therefore the permeate contains only non-entrapped 5-fluorouracil (Caux). To determine the total concentration of 5-fluorouracil in the vesicle suspension (Ctotal), methanol (5 mL) was added to 5 mL of vesicle suspension and mixed well. The addition of methanol leads to the destruction of the lipid membranes of vesicles releasing their contents into the medium. The mixture was then centrifugally ultrafiltrated to remove a precipitate for HPLC analysis as the same manner described above. The entrapment yield of 5-fluorouracil was calculated by Eq. (2).

\[ \text{Entrapment yield} \% = \frac{C_{\text{total}} - C_{\text{aux}}}{C_{\text{total}}} \times 100 \]

Results and Discussion

Preparation of lipid vesicles in a few hundred nanometer size using W1/O/W2 multiple emulsions as templates

Ultrasonic treatment of the mixture of internal aqueous phase and oil phase produced the W1/O emulsion, which contains to-be-entrapped material (calcein or 5-fluorouracil) in the dispersed water.
droplets. This primary W₁/O emulsion was used as a to-be-dispersed phase in the secondary microchannel emulsification. Figure 2 shows the photomicrographs of the W₁/O/W₂ emulsion droplets prepared by MC emulsification (Figure 2a) and the resulting lipid vesicles (Figure 2b). Green fluorescent emission of calcein from the multiple water (W₁) droplets in oil (O) droplets was observed in a dark external continuous water (W₂) phase (Figure 2a bottom). Sodium caseinate or Pluronic F68 (3.0 wt% each) was used as a surfactant in the W₂ phase to obtain W₁/O/W₂ emulsion droplets having diameters of ca. 34 µm.

Evaporation of n-hexane in oil (O) phase resulted in the formation of very small particles emitting green fluorescent emission of calcein (Figure 2b bottom) dispersed in the aqueous phase. Calcein was found to be entrapped inside the dispersed particles (Figure 2) since its fluorescent could not be quenched by the addition of CoCl₂ to the external water (W₂) phase. This indicates that the particles contain hydrophilic portions in their structure separated from the external water phase, suggestive of lipid vesicles.

The size distribution of the W₁/O emulsion droplets and the resulting particles are shown in Figure 3, having average sizes of 192 nm and 182 nm, respectively. In order to eliminate the influence of casein micelles on the size analysis, sodium caseinate (0.5 wt%) was used as a surfactant in the external water phase (W₂) after the removal of casein micelles by membrane filtration.

Evaporation of n-hexane in oil (O) phase resulted in the formation of very small particles emitting green fluorescent emission of calcein (Figure 2b bottom) dispersed in the aqueous phase. Calcein was found to be entrapped inside the dispersed particles (Figure 2) since its fluorescent could not be quenched by the addition of CoCl₂ to the external water (W₂) phase. This indicates that the particles contain hydrophilic portions in their structure separated from the external water phase, suggestive of lipid vesicles.

The transmission electron microscopy (TEM) images of the obtained particles are shown in Figure 4. TEM images shown in Figure 4a were observed by negative staining method. Small particles having a few hundred nanometer size were observed rounded by lipid lamellae as a typical structure of lipid vesicles. The images by freeze-fracture replica method (Figure 4b) show the fractured surface of particles.

Spherical particles of a few hundred nanometer size were observed, similar to images reported in the previous studies [11,12]. These TEM observations and size distribution (Figure 3) indicate that the water droplets of primary W₁/O emulsion were directly converted into the internal water phases of the resultant lipid vesicles. The corresponding result was also demonstrated in our previous report [6], where the use of a different primary emulsification method resulted in primary W₁/O
emulsion with average droplet size (1.7 ± 0.32 µm) comparable to the final vesicle sizes (1.7 ± 0.46 µm). With the preparation process of lipid vesicles in this study, the final size of the vesicles is controllable by the primary emulsification step.

**Entrapment yields of hydrophilic compounds into lipid vesicles**

Table 1 shows the entrapment yields of calcein and 5-fluorouracil into lipid vesicles prepared by the multiple emulsion method. Two different surfactants, i.e., sodium caseinate and Pluronic F68, were used at 3.0 wt% in the external water phase. Using sodium caseinate (M.W. ca. 24,000) as a protein surfactant, the entrapment yield of the fluorescent calcein into lipid vesicles was 89.3 ± 4.2%. With Pluronic F68 (average M.W. ca. 8,700) as a block copolymer surfactant, the entrapment yield of calcein was 58.5 ± 24.7% (Table 1). These results suggest that surfactant with high molecular weight could give high entrapment efficiency of hydrophilic molecules into lipid vesicles by reducing the leakage of the hydrophilic material as reported in our recent study [6] where Tween 80 (M.W. 1,310) was used as the surfactant in W1 phase, resulting in a low entrapment yield below 20% for calcein. The entrapment yields for the vesicles found to be lower than those for the W1/O/W2 emulsions [6], suggesting that the leakage of hydrophilic molecules from internal water (W1) phase to the external water (W2) phase partly occurred during the solvent evaporation process. Use of polymeric surfactants, for example proteins, has been reported [13-15] to minimize the leakage of the hydrophilic molecules from internal to external water phase.

The entrapment yield 89.3 ± 4.2% of the fluorescent calcein for the resulting vesicles is quite high for a hydrophilic molecule when compared with other lipid vesicle preparatory methods [16,17]. The encapsulation of 5-fluorouracil as an anti-cancer drug into the lipid vesicles was investigated previously. The reported entrapment yields were 19% by Wang and coworkers [4] and in the range from 12 to 15% by Nii and Ishii [18]. In our study, the entrapment yield of 5-fluorouracil reached 41.1 ± 3.3%, indicative of the suitability of our method for the formulation of lipid vesicles with high entrapment of water-soluble drugs.

The entrapment yield of calcein (M.W. 622.6) was about two times higher than that of 5-fluorouracil (M.W. 130.1). The observed difference could be attributed to the molecular weight difference of the to-be-entrapped molecules. Since the molecular weight of 5-fluorouracil is lower than that of calcein, the leakage of 5-fluorouracil would occur more frequently during the lipid vesicle formation process. In addition, 5-fluorouracil molecules are more likely to be incorporated into water pools of reverse micelles in the oil phase, those that have negatively charged surroundings caused by partly ionized oleic acid, than calcein having a negative charge. Therefore, 5-fluorouracil would be more easily transported from the internal water phase to the external water phase by reverse micelles.

**Conclusions**

Lipid vesicles having suitable size for drug delivery (in a few hundred nanometer size) with high entrapment efficiency of hydrophilic molecules were successfully prepared using W1/O/W2 multiple emulsions as vesicle templates. This multiple emulsion method is a combined process of ultrasonic emulsification homogenization, MC emulsification, and the subsequent removal of organic solvent by evaporation. Formation of multimellar lipid vesicles was confirmed by TEM observations. The average size of the lipid vesicles formed was 182 nm, and comparable with the size of the initial water droplet of the primary W1/O emulsion (192 nm), indicating that the vesicle size reflects the water droplet size of the primary W1/O emulsion. High entrapment yields for hydrophilic molecules, namely 89.3 ± 4.2% for calcein and 41.1 ± 3.3% for 5-fluorouracil, were achieved. On the basis of these features, this multiple emulsion method could be potentially useful in the fields of pharmaceutical, food, and cosmetic industries.

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