

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 ($W_1/O/W_2$) multiple emulsions after the removal of organic solvent by evaporation under ambient condition. The primary W_1/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 $W_1/O/W_2$ multiple emulsions were formed through microchannel (MC) emulsification by introducing the dispersed phase, i.e., (W_1/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 W_1/O emulsion (192 nm), indicating that the vesicle size reflects the water droplet size of the primary W_1/O emulsion. High entrapment yields for hydrophilic molecules, namely $89.3 \pm 4.2\%$ for calcein and $41.1 \pm 3.3\%$ 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 ($W_1/O/W_2$) multiple emulsions as vesicle templates [5,6]. In this vesicle preparation method, the internal water droplets (W_1 , 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. $W_1/O/W_2$ emulsion is composed of internal (W_1) and external (W_2) water phases, which are chemically alike, with an intermediate immiscible oil (O) phase that physically separates the

two like-phases. For the preparation of $W_1/O/W_2$ multiple 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 homogenous 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

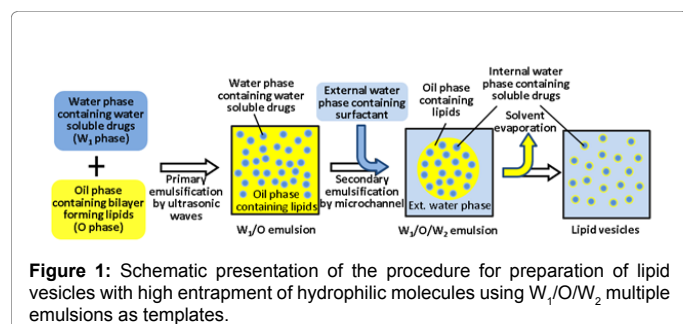
The schematic presentation of the procedure for preparation of lipid vesicles using multiple emulsions as templates is depicted in (Figure 1). The primary W_1/O emulsion was prepared by ultrasonically mixing the dispersed phase, W_1 with the continuous oil phase (O phase) in the ratio of 1:3 in a glass vial. The W_1 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/L 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 instrument (obtained from EP-Tec, Ibaraki, Japan) was used to prepare $W_1/O/W_2$ emulsions. The device consists of a silicon MC plate, a glass plate, a stainless module, a glass liquid chamber to feed the W_1/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 57 μm and a channel depth of 11 μm. The MC module was initially filled with the continuous water (W_2) phase containing sodium caseinate or Pluronic F68 as surfactant. The primary W_1/O emulsion, which was pressurized by the head difference of the liquid chamber, entered into the microchannel, and oil droplets containing dispersed W_1 phase were formed from the MC. The prepared $W_1/O/W_2$ emulsion was recovered in a glass sample bottle by flow of the continuous phase.

To obtain the lipid vesicles from the $W_1/O/W_2$ emulsion, solvent evaporation of the $W_1/O/W_2$ emulsion was conducted under atmospheric pressure and room temperature for 20 h.

Analytical methods

The sizes of the W_1/O emulsion droplets and the lipid vesicles were



measured by dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Worcester, UK). The size of the $W_1/O/W_2$ 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 using a fluorescence spectrophotometer (FP-6500, JASCO, Tokyo, Japan). The fluorescence from 3 mL of the sample was measured before (F_{total}) and after (F_{inside}) addition of 0.03 mL of $CoCl_2$ solution (10 mmol/L). F_{total} corresponds to the fluorescence from all materials composing the sample and F_{inside} corresponds to the fluorescence from the internal water phase plus the fluorescence from lipid vesicles themselves (F_{blank}). The entrapment yield of calcein was calculated from the equation,

$$\text{Entrapment yield [\%]} = 100 \times \frac{(1.01 \times F_{inside} - F_{blank})}{(F_{total} - F_{blank})} \quad (1)$$

Where, the value of 1.01 is the dilution volume factor accounting for addition of $CoCl_2$ solution.

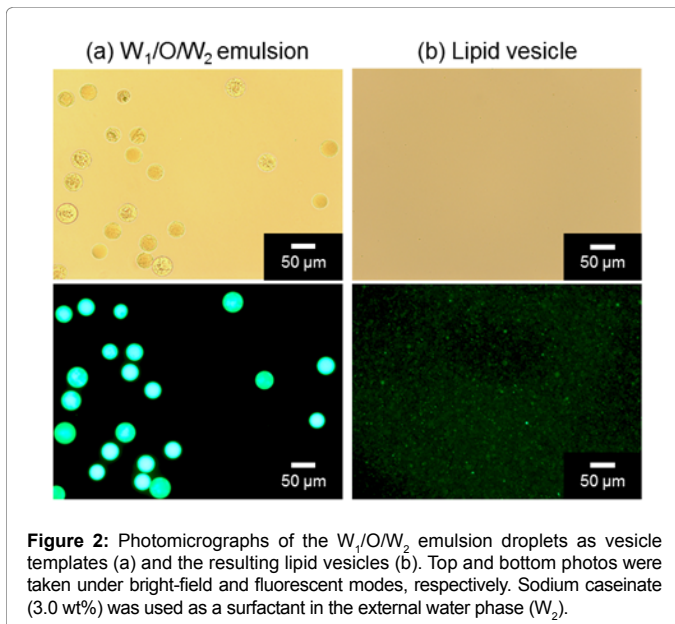
The entrapment yield of 5-fluorouracil into the lipid vesicles was determined by high performance liquid chromatography (HPLC). Asahipak ODP-50 4D column (4.6 mm × 150 mm, Showa Denk, Tokyo, Japan) was used. The mobile phase was a mixture of water and acetonitrile, in the ratio of 90:10 (v/v). The column 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-970, JASCO, Tokyo, Japan) at a wavelength of 265 nm. In order to determine the concentration of 5-fluorouracil in the outer water phase (C_{out}), the vesicle suspension was centrifugally ultrafiltrated for 10 minutes at 5,100 rpm using Centrisart I (MWCO 10,000, Sartorius AG, Goettingen, Germany). Lipid vesicles are rejected by the ultrafiltration membrane, therefore the permeate contains only non-entrapped 5-fluorouracil (C_{out}). To determine the total concentration of 5-fluorouracil in the vesicle suspension (C_{total}), 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).

$$\text{Entrapment yield [\%]} = \frac{C_{total} - C_{out}}{C_{total}} \times 100 \quad (2)$$

Results and Discussion

Preparation of lipid vesicles in a few hundred nanometer size using $W_1/O/W_2$ multiple emulsions as templates

Ultrasonic treatment of the mixture of internal aqueous phase and oil phase produced the W_1/O emulsion, which contains to-be-entrapped material (calcein or 5-fluorouracil) in the dispersed water



droplets. This primary W_1/O emulsion was used as a to-be-dispersed phase in the secondary microchannel emulsification. Figure 2 shows the photomicrographs of the $W_1/O/W_2$ emulsion droplets prepared by MC emulsification (Figure 2a) and the resulting lipid vesicles (Figure 2b). Green fluorescent emission of calcein from the multiple water (W_1) droplets in oil (O) droplets was observed in a dark external continuous water (W_2) phase (Figure 2a bottom). Sodium caseinate or Pluronic F68 (3.0 wt% each) was used as a surfactant in the W_2 phase to obtain $W_1/O/W_2$ 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 CoCl_2 to the external water (W_2) 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 W_1/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, the casein micelles were removed before the preparation of $W_1/O/W_2$ emulsion in this experiment. The sodium caseinate solution (0.5 wt%) was filtrated by mixed cellulose ester membranes with pore sizes of first 200 nm, then 100 nm (Advantec Toyo Kaisha, Ltd., Tokyo, Japan) and 50 nm (Merck Millipore, Darmstadt, Germany). These filtration processes lead to the removal of the casein micelles resulting in the size distribution shown in (Figure 3). The size of the prepared particles was comparable to that of the water droplets of the W_1/O emulsions, indicating that the small suspended particles after solvent evaporation were formed from the internal water droplets of the $W_1/O/W_2$ multiple emulsion.

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.

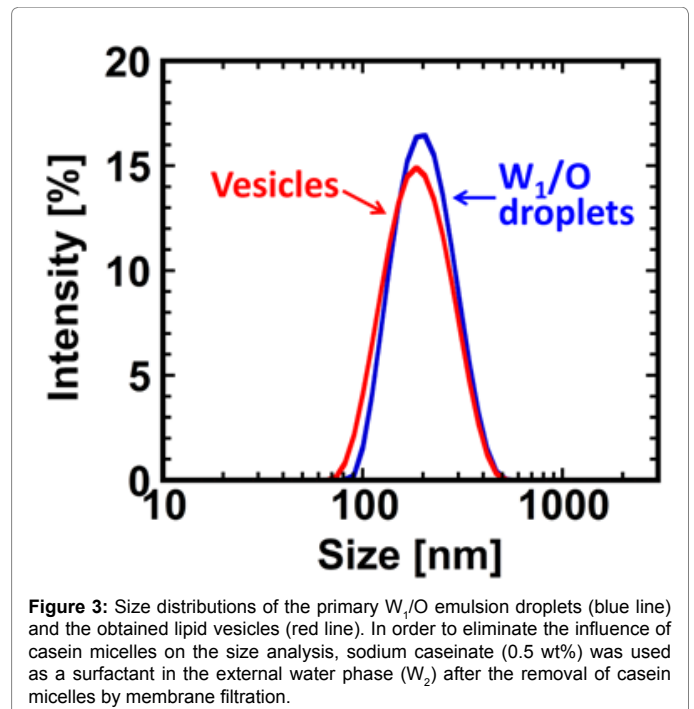


Figure 3: Size distributions of the primary W_1/O emulsion droplets (blue line) and the obtained lipid vesicles (red line). In order to eliminate the influence of casein micelles on the size analysis, sodium caseinate (0.5 wt%) was used as a surfactant in the external water phase (W_2) after the removal of casein micelles by membrane filtration.

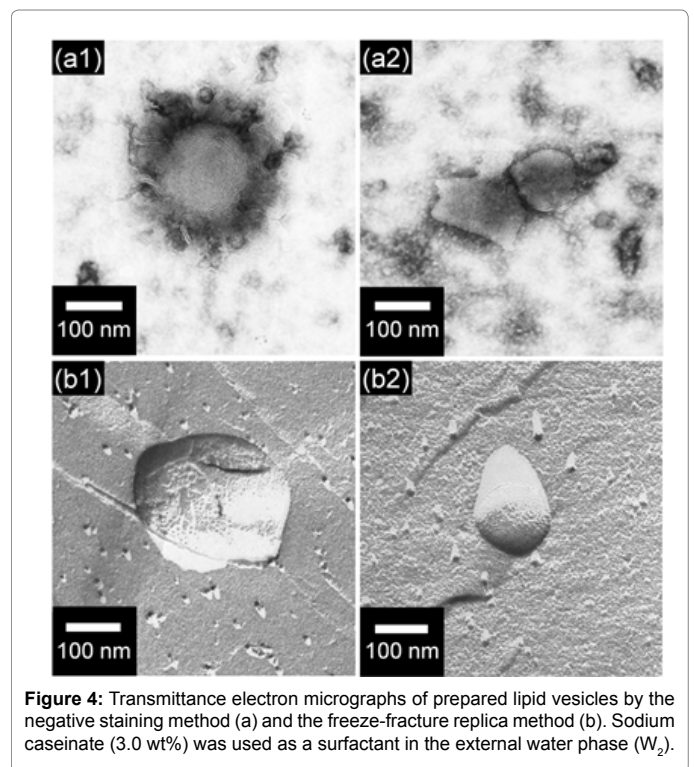


Figure 4: Transmittance electron micrographs of prepared lipid vesicles by the negative staining method (a) and the freeze-fracture replica method (b). Sodium caseinate (3.0 wt%) was used as a surfactant in the external water phase (W_2).

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 W_1/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 W_1/O

emulsion with average droplet size ($1.7 \pm 0.32 \mu\text{m}$) comparable to the final vesicle sizes ($1.7 \pm 0.46 \mu\text{m}$). 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 $89.3 \pm 4.2\%$. With Pluronic F68 (average M.W. ca. 8,700) as a block copolymer surfactant, the entrapment yield of calcein was $58.5 \pm 24.7\%$ (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 W_2 phase, resulting in a low entrapment yield below 20% for calcein. The entrapment yields for the vesicles were found to be lower than those for the $W_1/O/W_2$ emulsions [6], suggesting that the leakage of hydrophilic molecules from internal water (W_1) phase to the external water (W_2) 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 $89.3 \pm 4.2\%$ 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 $41.1 \pm 3.3\%$, 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.

Compounds	Surfactant in the external water (W_2) phase	Entrapment yield (%)
Calcein (M.W. 622.5)	Sodium caseinate (3.0 wt%)	89.3 ± 4.2
Calcein (M.W. 622.5)	Pluronic F68 (3.0 wt%)	58.5 ± 24.7
5-Fluorouracil (M.W. 130.1)	Sodium caseinate (3.0 wt%)	41.1 ± 3.3

Table 1: Entrapment yield of hydrophilic compounds into lipid vesicles prepared by multiple emulsion method.

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 $W_1/O/W_2$ 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 multilamellar 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 W_1/O emulsion (192 nm), indicating that the vesicle size reflects the water droplet size of the primary W_1/O emulsion. High entrapment yields for hydrophilic molecules, namely $89.3 \pm 4.2\%$ for calcein and $41.1 \pm 3.3\%$ 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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