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Production of giant unilamellar vesicles by the water-in-oil emulsion-transfer method without high internal concentrations of sugars

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Giant unilamellar vesicles (GUVs) are large vesicles bounded by a single lipid bilayer, which have been used in various applications as artificial, cell-like compartments. The water-in-oil (w/o) emulsion-transfer method has been attracting attention as a method to prepare GUVs that can efficiently encapsulate macromolecules. For efficient GUV production by this method, non-physiological, high concentrations of sugars are usually required in the inner solution of the GUVs. These sugars limit the utility of the GUVs for a wide range of applications. In this study, we investigated various compositions of the inner and outer solutions to achieve efficient production without high concentrations of sugars through the w/o emulsion-transfer method. Firstly, we adjusted the osmotic pressure and density of the outer solution with NaCl and succeeded in increasing the proportion of GUVs and the absolute number in the prepared liposome population. Secondly, we increased the density of the inner solution with cytochrome *c*, but the proportion of GUVs and absolute number of vesicles did not increase. Thirdly, we increased the density of the inner and outer solutions with glycerol, which is membrane permeable and can be removed from GUVs, and succeeded in increasing the GUV proportion. These results provide useful information for the efficient preparation of GUVs that enclose a physiologically-relevant environment by the w/o emulsion-transfer method.

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[**Key words:** Liposome; Giant unilamellar vesicles; Transfer method; Water-in-oil emulsion-transfer method; Flow cytometry]

A liposome is an artificial cell-like compartment formed by a phospholipid bilayer membrane. Since Bangham et al. (1) reported their preparation in 1965, liposomes have been used extensively, such as carriers in drug delivery systems (2), micro-scale reactors that encapsulate particular biochemical reactions (3,4), and artificial cell models to simulate basic cellular functions (5–7). In addition, gene expression in giant liposomes has been used for the evolutionary engineering of enzymes and RNAs when combined with liposome sorting technology (8–10).

There are several methods for giant liposome preparation. In the hydration or the freeze drying methods (11), lipid films are prepared by evaporating solutions of lipid in organic solvent or freeze drying preformed liposomes, respectively, and then an aqueous solution is added to the lipid films to produce giant liposomes. The liposomes produced by these methods are mainly multilamellar vesicle (MLV), small unilamellar vesicles (SUV, smaller than 100 nm diameter), or large unilamellar vesicles (LUV, 100–250 nm diameter) (11–13). MLV, SUV, and LUV are not suitable for internal biochemical reactions because the reaction volumes of MLVs are uncertain due to their complex lamellar structures (14) and SUVs and LUVs are too small to encapsulate all components for complex biochemical reactions, such as gene expression.

For gene expression, giant unilamellar vesicles (GUVs), liposomes larger than 1 μm in size (15), have been used. As a simple method of preparing GUVs, the water-in-oil (w/o) emulsion-transfer method has been attracting attention (16–18). In this method, a w/o emulsion that contains water droplets bounded by single lipid leaflet is laid on an aqueous solution and centrifuged, during which, the w/o droplets obtain outer leaflets at the water–oil interface to become GUVs (shown schematically in Fig. 1). One of the advantages of this method is high encapsulation efficiency of macromolecules into the GUVs and it has therefore been used especially for encapsulation of gene expression systems (8,19–23) and the evolutionary engineering of genes in combination with GUV sorting technology (9,10). In addition, size controlling of GUVs (24) and the preparation of asymmetric lipid membranes (16,25) have been reported using this method.

Efficient GUV production by the w/o emulsion-transfer method normally requires strict control of the composition of the inner and outer solutions. The aqueous solution of the w/o droplets (i.e., the inner solution of the GUV) should be of high density to promote efficient passage through the oil-water interface and this is usually achieved in the recent literature by the additional incorporation of 0.1–0.5 M sugars into the inner solution that contains other solutes at physiological concentrations (9,10,21,22,24,25). Such unphysiologically high concentration of sugars limits the applicability of the GUVs prepared by this method because it may inhibit some biochemical reactions and induce rupture of GUVs under physiological conditions because of osmosis. Therefore, in this study, we investigated different compositions of the inner and outer solutions

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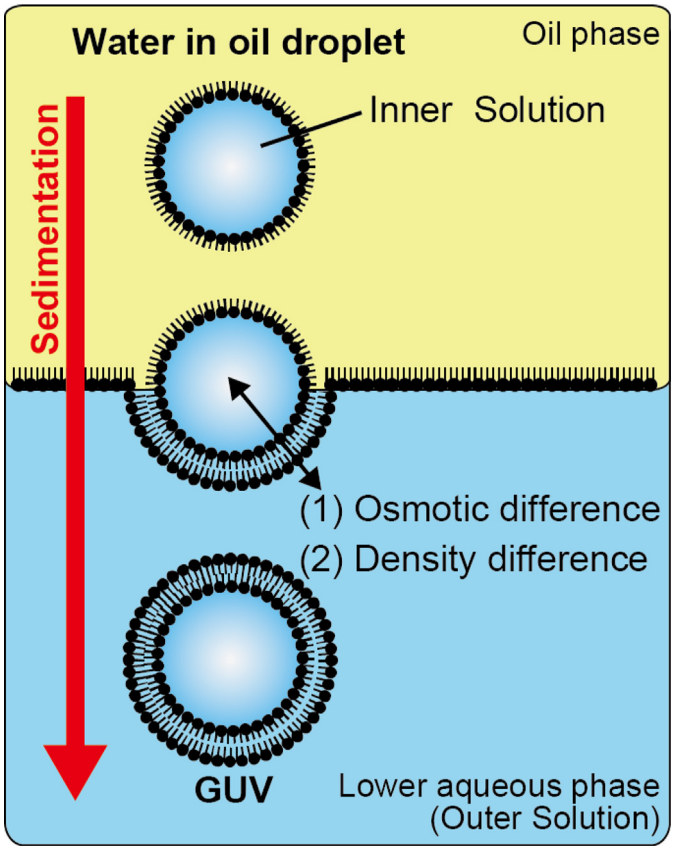

FIG. 1. Schematic diagram of the water in oil (w/o) emulsion-transfer method. A w/o droplet, with a lipid monolayer, settles in the oil phase and acquires an outer lipid monolayer at the oil-water interface. The resultant GUV further settles in the lower aqueous phase (outer solution) to the bottom of the tube. In this study, we focused on two factors that might affect GUV formation: 1, the osmotic difference between the inner and the outer solutions; 2, the density difference between the inner and the outer solutions.

to efficiently prepare GUVs by the w/o emulsion-transfer method without high concentrations of sugars. One of the intended applications of this method is gene expression and evolutionary engineering in physiologically relevant internal solutions.

MATERIALS AND METHODS

Materials 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-posphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cytochrome *c* (Cyt C) and potassium acetate were purchased from Nacalai Tesque (Kyoto, Japan). Liquid paraffin (0.86–0.89 g/mL at 20°C) and chloroform were purchased from Wako (Osaka, Japan).

Liposome preparation The w/o emulsion-transfer method was modified from the previous report (21). POPC was dissolved in chloroform at 100 mg/mL and then the lipid solution was mixed with liquid paraffin to produce a 5 mg/mL lipid solution. The lipid solution was heated at 80°C for 25 min to completely dissolve the lipids and evaporate chloroform. We then added 20 μL of the inner solutions described in Table 1 to 400 μL of the lipid solution. The solution was vortex mixed for 20 s and tapped for a further 20 s, three times, to form a w/o emulsion, and allowed to stand on ice for 10 min. Subsequently, 400 μL of the emulsion was gently placed by pipette on top of 200 μL of the outer solution described in Table 1 in a test tube and allowed to stand on ice for another 30 min. The test tube was centrifuged at 9000 × *g* for 60 min at 4°C to precipitate liposomes at the bottom of the tube. We opened the lid and made a hole pierced with a needle in the middle of the pellet in the bottom of the tube. The pellet was collected through the hole by closing the lid. If the pellet remained in the tube, we opened and closed the lid again carefully to push out the pellet. The obtained liposomes were mixed immediately with 400 μL of a dilution solution: 1 M glucose for the non-physiological condition, 0.22 M glucose for conditions 1–7, and 0.22 M glucose and 0.8 M glycerol for conditions 8–10 (Table 1). The liposomes were centrifuged at 18,000 × *g* for 5 min at 4°C. The supernatant was removed, and the precipitated liposomes were resuspended in 30 μL of the dilution solution. For the exchange of the outer solution to remove glycerol from the GUVs (Fig. 5), we centrifuged the liposomes again at 18,000 × *g* for 5 min at 4°C and the precipitated liposomes were suspended in the dilution solution without glycerol (0.22 M glucose). The compositions of the inner and outer solutions for the non-physiological condition was modified from our previous report (21). They contained 0.4 M potassium glutamate and 0.5 M sucrose for the inner solution and 0.4 M potassium glutamate and 1 M glucose for the outer solution.

Flow cytometry Forward and side scatter intensities (FSC and SSC) of 15,000 liposomes were analyzed with FACSAria II (BD, San Jose, CA, USA). The region corresponding to GUVs ($SSC \geq 158$ and $FSC \geq 0.0006 \times SSC^2$) was defined according to a previous report (26). In the literature, we analyzed membrane quantity and aqueous volume of each liposome and identified the region of FFC and SSC for GUVs. GUV ratios were calculated by dividing the numbers of liposomes in the GUV region by the total particle numbers (15,000). The total number of GUV was calculated by multiplying the GUV ratio by the total number of detected particles by the flow cytometer in a sample prepared as described above.

Calculation of osmotic pressure Osmotic pressures were calculated according to the van't Hoff equation, $\pi = MRT$, where M, R, and T are molar concentration (mol/L) of the solute particle, the gas constant (0.082 atm L/K/mol), and temperature (298.15 K), respectively.

RESULTS

Strategy The purpose of this study was to establish a method to prepare GUVs that do not contain high concentration of sugars by using the w/o emulsion-transfer method. In this method, a water-in-oil emulsion is laid on top of an aqueous phase (the lower aqueous phase), which becomes the outer solution of the GUVs following centrifugation (Fig. 1). The water-in-oil droplets contain the inner solution and have bounding lipid monolayers. During centrifugation, the droplets settle through the interface between the upper oil layer and the lower aqueous phase, where a lipid bilayer is formed. The resulting GUVs further sediment and are collected from the bottom of the tube. In this study, we focused on two factors that might affect GUV formation efficiency; (i) The difference in the osmotic pressure between the inner and the outer solutions. This osmotic difference causes movement of water molecules between the inner and the outer solutions at the oil-water interface and thereby changes the density of the inner solution and consequently changes the sedimentation rate of the droplets. (ii) The difference in the densities between the inner and the outer solutions, which directly affects the sedimentation rates of the droplets.

In order to investigate these two factors, we performed three experiments: (i) NaCl addition to the outer solution, which increases osmotic pressure as well as density, (ii) addition of a protein (Cyt C) to the inner solution to increase density, and (iii) glycerol addition to the inner and outer solutions to increase their densities with a minimum effect on osmotic pressure because glycerol freely permeates the lipid membrane (27).

The efficiency of GUV preparation was expressed by two values: the ratio of GUV numbers to total particles (GUV ratio) and the total number of GUVs in a liposome sample prepared by the w/o emulsion-transfer method described in the Materials and methods section (GUV number). To measure the GUV ratio, we analyzed liposome samples by flow cytometry, and counted the numbers of GUV and total particles, and then calculated the ratio of GUV to total particle. Additionally, in this assay, we measured the volume of the liposome sample used for the counting. To estimate the GUV number, the total GUV number in a liposome sample, we normalized the number of GUV counted above with the total volume of the liposome sample (30 μL). GUVs can be distinguished from other multi-lamellar vesicles or lipid aggregates based on the forward and side light scatter values (26). When we used inner and outer

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