PRODUCTION OF LIPID-CORE/MULTILAMELLAR-SHELL HYBRID LIPOSOMES UTILIZING NON-EQUILIBRIUM MICROFLUIDIC DROPLETS

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ABSTRACT

A microfluidic process has been newly proposed to synthesize highly unique micrometer-size hybrid liposomes having multi-lamellae structure. The process includes (1) formation of monodisperse droplets of a polar solvent containing phospholipids at a microchannel confluence, (2) dissolution of the solvent into the continuous water phase, and (3) simultaneous reconstitution of large multilayer membranes and formation of a lipid core. We successfully prepared monodisperse hybrid liposomes composed of a lipid core and a multilamellar shell, and demonstrated the applicability as carriers for both hydrophilic and hydrophobic compounds.

KEYWORDS: Liposome, Non-equilibrium droplet, Droplet microfluidics, Multilamellar vesicle

INTRODUCTION

Lipid vesicles are functional soft materials used as cell membrane models, carriers for DDS, and so forth. Liposomes are classified into two groups; unilamellar and multilamellar vesicles (MLV). The former is suitable for incorporating hydrophilic compounds inside its aqueous core, whereas the latter can carry hydrophobic compounds within the lipid layers. Attempts have been made to produce monodisperse liposomes. For example, microfluidic technologies have been applied to form monodisperse unilamellar vesicles from monodisperse oil-in-water droplets [1]. On the other hand, MLVs are usually produced by swelling dried phospholipid films [2], which results in the formation of polydisperse MLVs (diameter of 0.05-100 μm), and thus, a technique for size-based separation should be employed to produce monodisperse MLVs [3].

Here we propose a simple but highly versatile strategy to produce monodisperse hybrid liposomes having multi-lamellae structures (Fig. 1), utilizing non-equilibrium O/W droplets temporary formed in microchannels [4,5]. Droplets of a polar solvent (ethyl acetate; EA) containing phospholipids are formed in the continuous water phase at the microchannel confluence, which gradually shrink during flowing through the microchannel because of the solvent dissolution into the continuous phase. By further diluting and completely removing the solvent, phospholipid vesicles are generated.

EXPERIMENTAL

PDMS-glass microdevices were fabricated by using standard soft lithography and replica molding techniques (Fig. 2 (a)). We employed a flow-focusing microchannel having an orifice structure to generate monodisperse O/W droplets. The width of the orifice and other microchannel segments were 50 and 100/200 μm, respectively, and the depth was uniform, ~100 μm. To reproducibly form O/W droplets in the microchannel, the channel surface was rendered to be hydrophilic; PDMS and glass plates were bonded via O_2 plasma treatment and subsequently silanized, just before conducting experiments. We used DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) as the phospholipid with 0.1mol% of fluorescent phospholipid (NBD C_{12}-HPC, dissolved with ethanol). The concentration of DOPC was changed from 1 to 5% (w/v). EA with a purity of >99.9% was used as the water-soluble organic solvent. PBS (×1 or ×5 concentration) or distilled water was used as the continuous phase. In addition to the phospholipid solution, EA was introduced into the microchannel on both side of the phospholipid solution, which prevented the microchannel clogging by the precipitation of lipid molecules near the confluence. The phospholipid solution, EA, and the continuous phase (water or PBS) were continuously introduced into the microchannel by using syringe pumps at flow rates of 2, 2, 20 μL/min, respectively. Also, additional continuous phase was introduced at 500 μm downstream from the confluence, to prevent the formed droplets from

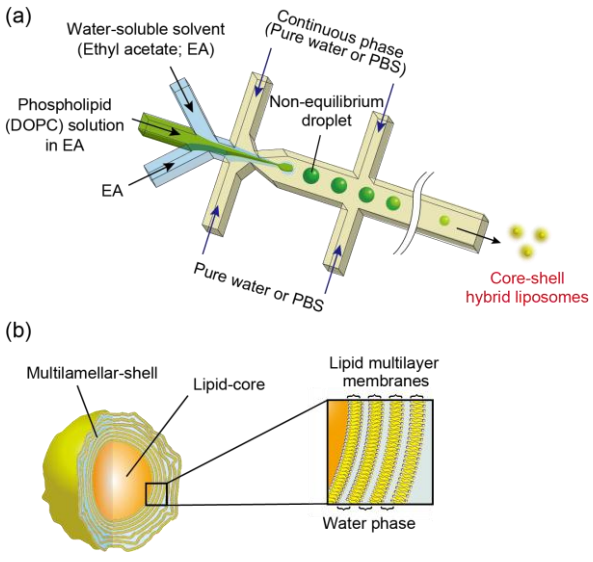

Figure 1: (a) Basic principle of synthesis of hybrid liposomes in microchannels using non-equilibrium droplets. The water-soluble organic solvent in the O/W droplets is gradually dissolved into the continuous phase, while the phospholipid molecules are precipitated, forming hybrid liposomes. (b) Schematic image showing the core-shell hybrid liposomes and the enlarged figure of the oriented phospholipid molecules on the core. This hybrid liposome is consisted of a lipid-core and the multilammellar-shell, in which phospholipid molecules formed lipid bilayer membranes.

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coalescing with each other, at a flow rate of 120 μL/min. After the formation of the core-shell liposomes, the polar solvent was removed via further dilution in PBS or water, and morphologies of the liposomes were observed by using an optical/fluorescence microscope.

RESULTS AND DISCUSSION

We first examined if the non-equilibrium O/W droplets containing phospholipid molecules are actually formed in the microchannel. As shown in Fig. 2, we observed that the phospholipid solution of EA formed monodisperse O/W droplets (diameter of 90 μm, CV value of 5.2%), which gradually shrunk during flowing through the microchannel. Near the exit of the microchannel, the droplet diameter was 58 μm (CV value of 8.7%, shown in Fig. 2 C). Fig. 3 (a) shows the formed liposomes after completely removing the solvent, which were prepared by using pure water as the continuous phase. We found that the obtained liposomes exhibited unique morphologies with a core (diameter of ~17 μm, CV value: 6.9%) and an outer shell of multilamellar lipid layers, which swelled and spread around the lipid core (diameter of ~56 μm, CV value of 18%). It was possible to control the liposome sizes by changing the DOPC concentration (Fig. 4) and/or the formed droplet sizes. When PBS of a higher ionic strength was used as the continuous phase, the swelling of the outer shell was suppressed, and smaller spherical liposomes were obtained (Fig. 3b,c). We assumed that the core was made of the lipid particles and the origin of this lipid core as the agglomerate of precipitated phospholipid (DOPC) molecules, which was formed during the dissolution of EA into the continuous phase and the aggregation of concentrated phospholipids (Fig. 5). Multilamellar shells would be derived from lipid bilayers formed on the droplet surface during the droplet dissolution, which consequently swelled in the aqueous environment to form complex shape phospholipid vesicles.

Next, we demonstrated the encapsulation of hydrophilic and/or hydrophobic compounds into the core and the shell of the hybrid liposomes, respectively (Fig. 6a-c). When a hydrophilic dye, nile red or sudan black was dissolved in the phospholipid solution, these molecules were incorporated into both the multilamellar shell and the lipid core. When an aqueous solution of sodium fluorescein was used as the continuous phase, this molecule was incorporated into the core-shell liposome on the surface of the core. These lipid-based vesicles have unique properties; for example, we found that the outer lipid shells sometimes transformed into multilamellar lipid tubes (Fig. 6d), because the lipid tube is one of the stable morphologies of the aggregates of phospholipid molecules with bilayer orientation. In addition, it was possible to separate lipid cores from the shells by sonication or by surfactant treatment such as Triton X-100 and SDS (Fig. 6e), showing the possibility of preparing monodisperse lipid particles that contain both hydrophilic/hydrophobic compounds.

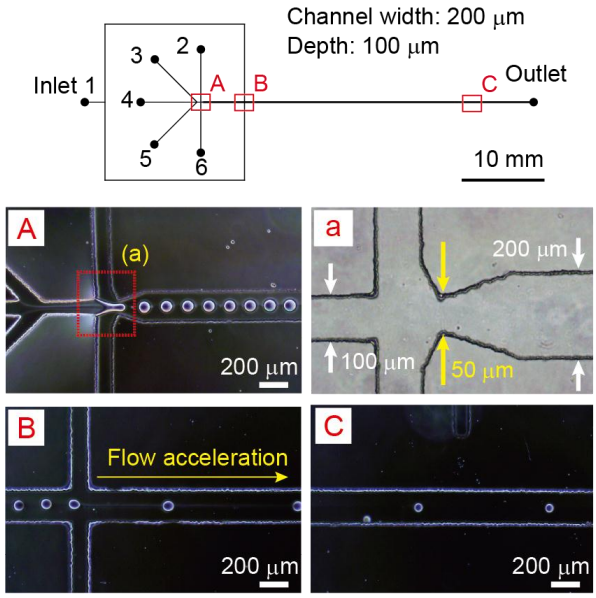

Figure 2. Droplet formation inside the PDMS microchannel. Flow rates from Inlets 1, 2, 3, and 4 were 120, 20, 2, and 2 μL/min, respectively. Area A, first confluence; area B, second confluence, and Area C, near the outlet.

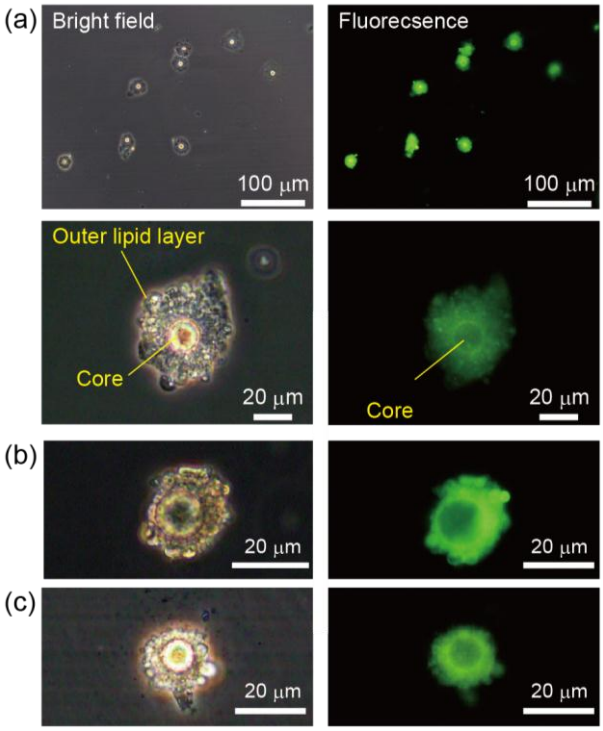

Figure 3. Micrographs of liposomes produced by using (a) pure water, (b) 1×PBS, and (c) 5×PBS as the continuous phase, respectively. The average liposome sizes were 56, 28, and 22 μm, respectively.

CONCLUSIONS

A simple microfluidic system was presented for preparing highly-unique monodisperse liposomes having lipid-core and multilamellar-shell structure, which are advantageous in terms of the effective hydrophobic molecule

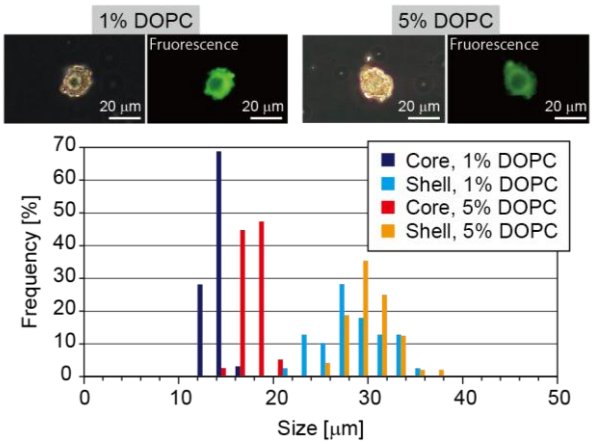

Figure 4. Size distributions of the core and shell of the obtained liposome, when DOPC conc. was changed as indicated. PBS was used as the continuous phase.

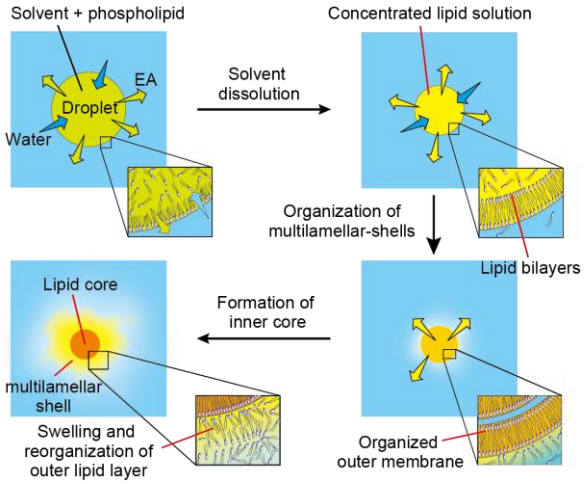

Figure 5. Schematic image showing the formation process of the lipid-core multilamellar-shell liposomes. The formed droplet gradually shrunk because of the dissolution of EA into the continuous water phase, resulting in the increased concentration of lipid molecules. Phospholipids at the droplet interface were organized to form outer shells. In contrast, inner lipid molecules were precipitated and aggregated to form lipid core.

encapsulation and transportation compared to unilamellar vesicles. The presented vesicles are useful as functional lipid-based materials for pharmaceutical, food, and cosmetic applications, and would be available as the high-performance carriers for DDS because we can precisely control the particle size and easily conduct surface modification or functionalization by incorporating functional molecules such as antibody or magnetic particles in the multilamellar shells.

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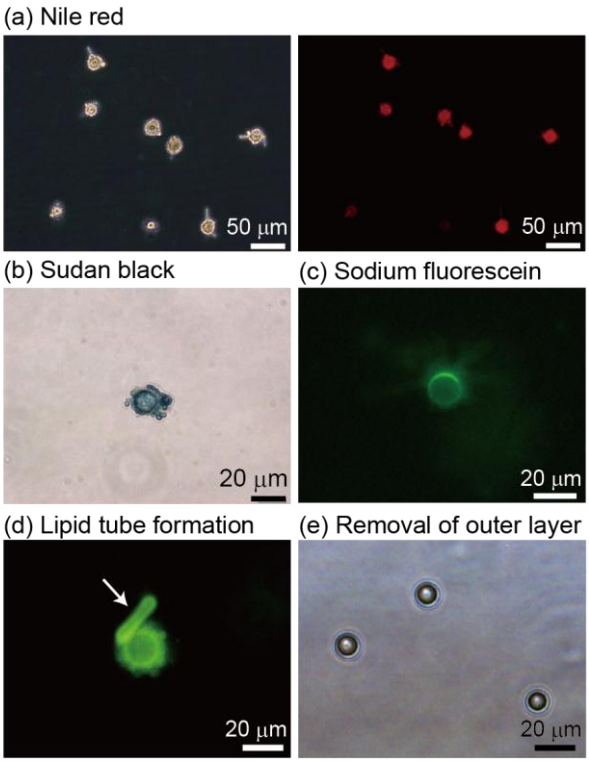

Figure 6. (a-c) Incorporation of various hydrophobic/ hydrophilic compounds into the hybrid liposomes. Nile red or sudan black was dissolved in the EA solution together with DOPC, whereas sodium fluorescein was dissolved in the continuous water phase. (d) Formation of lipid tubes on the liposome surface, and (e) separation of the lipid core from the outer multilamellar shell by sonication.