Spontaneous Transfer of Phospholipid-Coated Oil-in-Oil and Water-in-Oil Micro-Droplets through an Oil/Water Interface

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We studied the evolution of oil-in-oil (O/O) and water-in-oil (W/O) phospholipid-coated micro-droplets at an oil/water interface. We found that, in both cases, micro-droplets spontaneously transferred from the oil phase to the water phase. O/O micro-droplets transformed into oil-in-water micro-droplets, while W/O micro-droplets led to the formation of liposomes.

Introduction

Since Bangham et al.[1] discovered that phospholipid molecules,[2] the main component of cytomembranes, spontaneously form closed bilayer vesicles referred to as liposomes in an aqueous solution, there have been many investigations on liposomes.[3] In particular, cell-sized liposomes, i.e., with a size of 10−100 μm, have been actively studied as cell models[4−8] or microreactors for biochemical reactions,[9,10] and several methodologies for their preparation and characterization have been proposed.[11−18] However, many challenging problems, such as controlling the size of liposomes[19] or making liposomes with an asymmetric membrane[20] have not been fully solved.

Water-in-oil (W/O) micro-droplets coated by phospholipids or chemical surfactants have also been studied as a model of living cells.[21−23] By taking advantage of microfluidic techniques, micro-droplets with a controlled size and shape can be easily prepared and manipulated.[24−26] Consequently, there have been several attempts to prepare liposomes or polymerosomes from W/O micro-droplets or double emulsions.[20,27−30] In this case, liposomes were prepared by transferring W/O droplets coated by phospholipids from an oil phase to a water phase by using an external force such as centrifugation. However, by using centrifugation, it is difficult to follow the evolution of the transferring droplets at the oil/water interface, and the mechanism of such transfer is far from understood.

Therefore, we studied, for the first time to our knowledge, the spontaneous behavior of cell-sized micro-droplets (oil-in-oil (O/O) and W/O) coated by phospholipids at an oil/water interface, i.e., without applying any external driving force. Remarkably, O/O and W/O micro-droplets spontaneously transferred across the oil/water interface from the oil phase to the water phase. During this process, it was found that O/O micro-droplets transform into O/W micro-droplets, while W/O micro-droplets form liposomes.

Experimental Section

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) was obtained from Wako Pure Chemicals. Egg yolk L-α-phosphatidylcholine (egg PC) 100 mg/mL in chloroform and fluorescent dye Fura 2 were from Sigma. Mineral oil was purchased from Nacalai Tesque. Fluorescent phospholipid 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was from Avanti Polar Lipids.

Preparation of O/O and W/O Micro-Droplets. First, a phospholipid solution was prepared (10 mM in a chloroform/methanol

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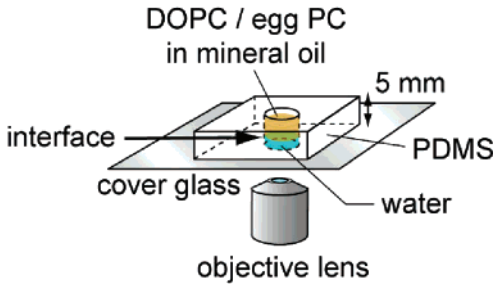

Figure 1. Schematic illustration of the experimental setup. The observation chamber is made of poly(dimethylsiloxane) (PDMS) on a microscope cover glass slide. By using phase-contrast and fluorescence microscopy, we observed the interface between a water phase and an oil phase containing 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) or egg yolk L-α-phosphatidylcholine (egg PC) phospholipids. We observed the behavior of O/O and W/O micro-droplets initially in the oil phase and coming to the oil/water interface.

(2:1, v/v) mixture for DOPC, 100 mg/mL in chloroform for egg PC) and poured into a glass test tube. For fluorescence microscopy observations of droplet membranes, fluorescent phospholipid NBD-PC was mixed with DOPC at a molar ratio of 1/1000. The organic solvent was then evaporated under nitrogen flow and dried under vacuum to make a dry film at the bottom of the test tube. Mineral oil was then added to the test tube prior to ultrasonication for 60 min at 50 °C and vortex mixing (final lipid concentrations in oil were between 0.1 and 1 mM). This procedure resulted in the spontaneous formation of O/O micro-droplets for lipid concentrations higher than 0.5 mM. To obtain W/O droplets, we added 5 vol % of aqueous solution (pure water or 10 mM Fura 2) to the oil phase containing phospholipid and then emulsified the mixture by pipetting up and down with a micropipet.

Observation of Micro-Droplets at the Oil/Water Interface. To observe the behavior of micro-droplets at the oil/water interface, we used the experimental setup sketched in Figure 1. The observation chamber consisted of a cylindrical hole (ca. 4 mm in diameter) in a PDMS (poly(dimethylsiloxane)) sheet (ca. 5 mm thick) on a microscope glass slide previously cleaned by 1 h of baking at 500 °C. A thin layer of water phase was introduced at the bottom of the cylinder and then topped by a thin layer of the oil phase containing phospholipid. For experiments with W/O droplets, a third layer of W/O emulsion was added right after emulsification (within a few seconds) above the first two layers. Observations of micro-droplets were made around the oil/water interface by using a Nikon TE300 inverted microscope equipped with a 100× oil immersion lens, a Zeiss Axiovert 200 inverted microscope equipped with a 10× lens,

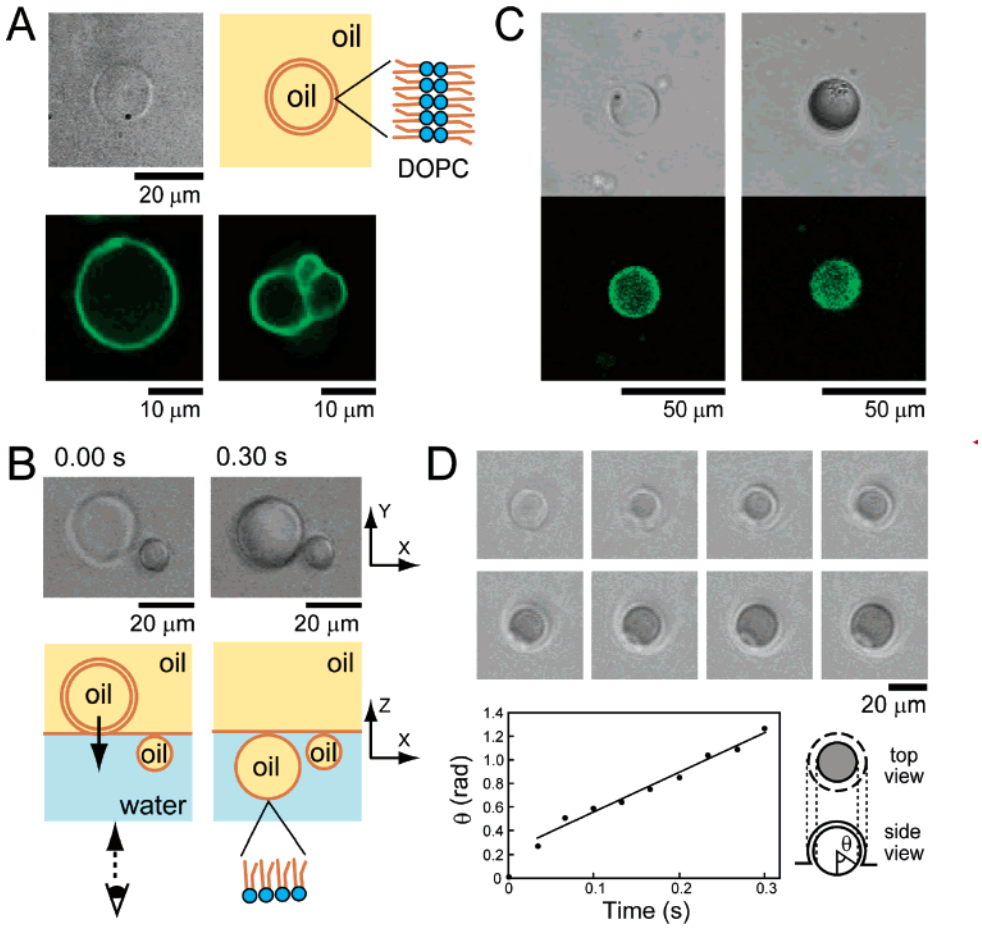

Figure 2. (A) Top left: phase-contrast microscopic image of an O/O micro-droplet. Top right: estimated structure of the O/O micro-droplet and its phospholipid membrane. Bottom: Fluorescent confocal microscopy images of an O/O micro-droplet (left) and a small aggregate of O/O micro-droplets (right). All observations were made in mineral oil containing 1 mM DOPC + 1 μM NBD-PC in the absence of the water phase. (B) Phase-contrast microscopy image (top) and schematic representation (bottom) before ($t = 0$ s) and after ($t = 0.3$ s) the spontaneous transfer of an O/O micro-droplet from the oil phase to the water phase. The phospholipid is DOPC at a concentration of 1 mM in mineral oil. The focal plane is slightly below the oil/water interface. (C) Confocal microscopy observation (top: transmission, bottom: fluorescence) of the transfer of an O/O micro-droplet before (left) and after (right) the transfer. Same experimental conditions as (B) with NBD-PC mixed to DOPC at a 1/1000 molar ratio. (D) Top: Phase-contrast image sequence of an O/O droplet spontaneously crossing the oil/water interface. The phospholipid is DOPC at a concentration of 1 mM in mineral oil. The focal plane is exactly at the oil/water interface. The time between two consecutive snapshots is 33 ms. Bottom: angular parameter θ measured from the observation of the cross-section of the O/O droplet at the interface as a function of time. The solid line is the linear fit of the experimental points between 0.03 and 0.30 s.

and a Zeiss Axiovert 100 inverted microscope equipped with a LSM 510 module for confocal microscopy.

Results

Spontaneous Formation of O/O Micro-Droplets. First, we characterized the oil phase containing phospholipids prepared as described in the Experimental Section in the absence of the water phase. We observed the presence of spherical self-assembled structures with a diameter of 10−50 μm. Figure 2A (top left) shows a typical phase contrast microscopic image of such an object, which appears as an oil droplet in the oil phase. Since these spherical structures were not observed in the oil without phospholipids and had a fluorescent membrane when fluorescent phospholipid NBD-PC was mixed to DOPC (Figure 2A bottom), we conclude that these self-assembled microstructures are O/O micro-droplets, where the interface is composed of phospholipid. Figure 2A (top right) shows a schematic representation of the possible organization of phospholipid molecules at the interface.

Spontaneous Transfer from O/O Droplets to O/W Droplets across the Oil/Water Interface. Next, the oil phase containing O/O droplets was placed above a thin water layer in the observation chamber, as shown in Figure 1. By using phase-contrast microscopy, we examined the area around the obtained oil/water interface. We observed that when an O/O droplet came to the oil/water interface it spontaneously and abruptly transferred into the lower water phase. Figure 2B shows typical phase-contrast images around the interface just before (t = 0 s) and after (t = 0.3 s) such transfer. In this figure, at 0 s, the image is focused on an already formed O/W droplet just below the interface. To the left of this droplet, the slightly blurred (unfocused) image of an O/O droplet can be seen just above the interface. At t = 0.3 s, the O/O droplet has already been transferred. It appears as a dark droplet on a light background. The resulting structure is thus interpreted as an O/W micro-droplet, where the contrast is due to the difference in the refractive index between oil and water. Then, we observed this transfer by confocal microscopy, where the membrane of O/O droplets was labeled by NBD-PC. Figure 2C shows typical transmission and fluorescence images before and after the transfer. The resulting O/W droplets have a fluorescent membrane, which indicates the presence of phospholipids coating the surface of the droplets, as sketched in Figure 2B (bottom right). When we focused on the interface at a high sensitivity, we observed the release of fluorescent phospholipids from the droplets upon the transfer through the interface (Supporting Information). This suggests that an O/O droplet loses its outermost phospholipid layer during the transfer process. To characterize the dynamics of this transfer, observations were made by focusing exactly on the bulk interface during droplet transfer. Figure 2D (top) shows the image sequence of a ~20 μm droplet crossing the oil/water interface. In this image sequence, the dark disk corresponding to the cross-sectional area of the droplet grows as a function of time. Figure 2D (bottom) shows the angular parameter, θ, as a function of time where t = 0 s is taken just before transfer (θ = 0 at t = 0 s). It shows that θ increases as a function of time in a nearly linear fashion except at around t = 0 s.

Spontaneous Transfer from a W/O Droplet to a Liposome across the Oil/Water Interface. As in the study on O/O micro-droplets, we sought to characterize the behavior of W/O micro-droplets at the oil/water interface. For this purpose, oil containing W/O micro-droplets was placed above the water layer in the observation chamber, and we characterized the behavior of W/O micro-droplets around the oil/water interface by fluorescence and phase-contrast microscopy. In this case, we observed that

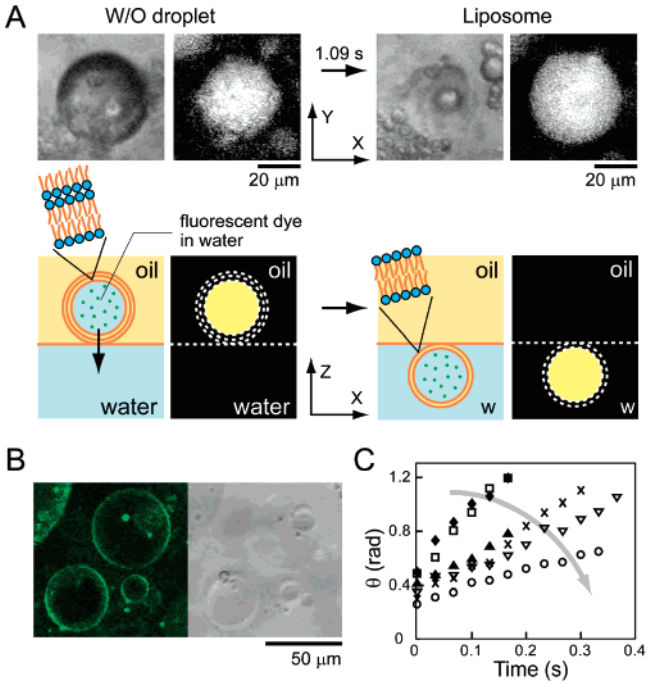

Figure 3. (A) Top left: phase-contrast (left) and fluorescence (right) microscopy images of a W/O micro-droplet containing 10 mM of the fluorescent dye Fura 2 in water just above the oil/water interface. Top right: after 1.09 s, the droplet has spontaneously transferred into the water phase to form a liposome in the water phase (phase-contrast and fluorescence microscopy). Phospholipid is DOPC at a concentration of 1 mM in mineral oil. The focal plane is slightly below the oil/water interface. Bottom: Schematic representation of the transformation before (left) and after (right) the transfer from the oil phase into the water phase. (B) Fluorescence (left) and transmission (right) confocal microscopy of the micro-droplets obtained after transfer. Phospholipid is DOPC mixed to NPB−PC (0.1%) at a concentration of 0.1 mM in mineral oil. The focal plane is slightly below the oil/water interface. (C) Angular parameter θ (as defined in Figure 2D) of W/O droplets crossing the interface as a function of time for various droplet sizes. The arrow indicates the increase in droplet size: 14.8 (diamond), 27.7 (square), 32.8 (triangle), 33.5 (cross), 36.1 (inverted triangle), and 54.2 μm (circle). Phospholipid is DOPC at a concentration of 0.5 mM in mineral oil.

the micro-droplets coming to the interface spontaneously transferred from the oil phase to the water phase.

Figure 3A shows typical phase-contrast and fluorescence microscopy images of a droplet with DOPC before (left) and after (right) transfer, while focusing slightly below the interface (water phase). To distinguish the aqueous part, which is originally contained in the W/O droplet, from the bulk water phase below the interface, fluorescent dye Fura 2 was added to the W/O droplet during preparation of the W/O emulsion. Before transfer, Figure 3A (top left) shows slightly blurred (unfocused) pictures of the W/O droplet above the interface. After 1.09 s, Figure 3A (top right) shows that the droplet has transferred into the water phase. The fluorescence microscopy image reveals that molecules of Fura 2 stayed within a spherical microvolume during transfer since the intensity of the emitted fluorescent light is nearly equal to that of the initial W/O droplet. The transferred droplet thus has a membrane that prevents the inner medium from diffusing spontaneously into the outer medium. In the corresponding image by phase-contrast microscopy, the initial dark color of the droplet has vanished, which indicates that the inner and outer media have a similar refractive index, i.e., that of water. To characterize the composition of the membrane, we used fluorescent phospholipid NBD-PC mixed to DOPC and observed by confocal fluorescence microscopy the transferred water-in-water (W/W)

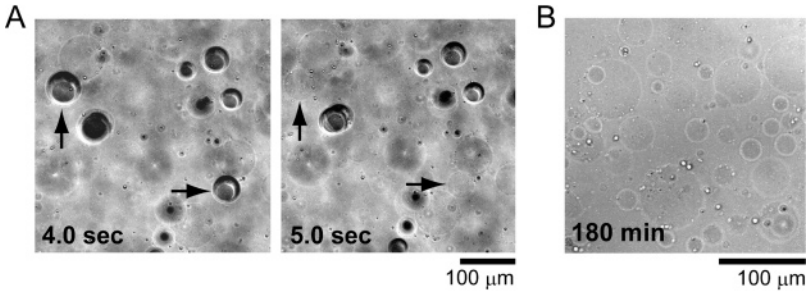

Figure 4. (A) Microscopy images focused on the water phase slightly below the oil/water interface, at $t = 4.0$ s and 5.0 s after the introduction of W/O micro-droplets to the oil phase. W/O micro-droplets and liposomes coexist in the two images and appear as dark gray and light gray disks, respectively. The arrows indicate W/O micro-droplets that are transferring from the oil phase ($t = 4.0$ s) to the water phase to form liposomes ($t = 5.0$ s). The phospholipid is egg PC at a concentration of 0.5 mM in mineral oil. (B) Same conditions as in (A) at 180 min after the introduction of W/O micro-droplets.

micro-droplets. Figure 3B shows typical images of transferred micro-droplets. All transferred W/W micro-droplets have a fluorescent membrane, which denotes the presence of phospholipids composing the membrane. All these observations demonstrate that W/O micro-droplets have been transformed into W/W micro-droplets with a membrane composed of phospholipid molecules, i.e., into liposomes.

By using a technique similar to that for O/O droplets (Figure 2D), we measured the angular parameter θ as a function of time as the W/O droplet was crossing the interface. Figure 3C shows that, regardless of droplet size, θ increases as a function of time in a nearly linear fashion, which is similar to the case of O/O droplets. Moreover, it shows that the velocity of the transfer increases with a decrease in droplet size.

Obtaining Stable Liposomes from W/O Droplets. To investigate the general nature of the spontaneous transformation of W/O micro-droplets, we conducted the same experiment as described above but with egg PC as a phospholipid instead of DOPC. By varying the concentration of egg PC in mineral oil, we found that 0.5 mM was the optimal final concentration of egg PC in oil to obtain a neat oil/water interface and the formation of stable liposomes in the water phase. Moreover, it is important to add a thin layer of oil containing phospholipids and then wait about 2 h for stabilization before adding the W/O emulsion. Under these experimental conditions, it is possible to observe the transformation of a large number of W/O droplets into stable liposomes. Figure 4A shows phase-contrast microscopy images obtained by focusing slightly below the oil/water interface at 4.0 and 5.0 s after the addition of W/O droplets into the chamber, respectively. At $t = 4$ s, we observed the coexistence of previously formed liposomes in the water phase (below interface) with W/O micro-droplets in the oil phase (above interface, slightly unfocused). At $t = 5$ s, some W/O droplets visible at $t = 4$ s (indicated by arrows) have clearly transferred into the water phase and have been transformed into liposomes (indicated by arrows). We also observed that almost all the W/O droplets transferred into the water phase within a few minutes. If the W/O emulsion was added directly on the water phase, liposomes were not stable and disappeared almost immediately after they were formed. In contrast, by using the procedure described above (a stable oil/water interface is formed before the W/O emulsion is added), liposomes were stable for hours. This is illustrated in Figure 4B, where a very large number of liposomes can be observed 3 h after the droplet-to-liposome transformation.

Discussion

We observed that a phospholipid-coated O/O micro-droplet at an oil/water interface spontaneously transferred into the water phase to form a phospholipid-coated O/W micro-droplet. We also observed that this transfer was accompanied by the release of phospholipids from the micro-droplet surface. Therefore, we expect that O/O micro-droplets lose their outermost phospholipid layer upon transfer, as depicted in Figure 2B (peeling model). When the O/O droplet comes into contact with the interface and a small part of the outermost layer fuses with the phospholipid layer at the oil/water interface, the driving force that pushes the droplet into the water phase is expected to be surface tension. Moreover, using a peeling model, one should expect a constant transfer velocity as a function of θ, except at $\theta \approx 0$ and π,[31] which is in agreement with our experimental observations (Figure 2D).

Furthermore, we observed that a phospholipid-coated W/O micro-droplet at an oil/water interface spontaneously transferred into the water phase to form a liposome. This phenomenon can be compared to a recent method used to produce liposomes, which consists of forcing the transfer of W/O micro-droplets from oil to a water phase by centrifugation.[20,27,28] In this case, the mechanism proposed to explain the droplet-to-liposome transformation was that when a W/O micro-droplet coated by a phospholipid monolayer crosses the oil/water interface, it is coated by a second monolayer coming from the phospholipids at the oil/water interface. However, as shown in a recent study, it is difficult for a W/O droplet to be coated by a new layer of phospholipids at an oil/water interface without trapping some oil within the droplet membrane.[30] Therefore, we consider another mechanism, which would be analogous to that of the transformation from O/O droplets to O/W droplets. The analogy between the transformation from O/O to O/W micro-droplets and that from W/O micro-droplets to liposomes is confirmed by the similar kinetic behavior of the transfer. In both cases, the transfer proceeds at a nearly constant velocity (Figures 2D and 3C). Moreover, O/O and W/O droplets of similar size and coated by the same phospholipid (here, DOPC) transferred at a similar speed. Therefore, by analogy with the O/O-to-O/W transformation, it may be expected that a W/O micro-droplet has in fact a multilayered membrane and loses its outermost layer(s) when it crosses the oil/water interface to form a liposome. Further experimental and theoretical developments should clarify which of the two proposed mechanisms (gain of a new layer or loss of outermost layer(s)) is the most plausible to describe the droplet-to-liposome transformation.

Conclusions

By direct microscopic observations, we studied the evolution of O/O and W/O phospholipid-coated micro-droplets at an oil/water interface. We found that, in both cases, micro-droplets spontaneously transfer from the oil phase to the water phase. O/O and W/O micro-droplets transform into phospholipid-coated

O/W micro-droplets and liposomes, respectively. On the basis of this spontaneous transfer phenomenon, stable liposomes with a typical diameter of 10−100 μm were prepared in large quantity. Further combined with microfluidics technology for the controlled generation of W/O micro-droplets, this transfer method may provide a novel route for the preparation of liposomes of controlled size and composition.

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Supporting Information Available: Typical fluorescence and transmission confocal microscopy images of an oil droplet right after its transfer to the water. This material is available free of charge via the Internet at http://pubs.acs.org.

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