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Letters

Entrapping Desired Amounts of Actin Filaments and Molecular Motor Proteins in Giant Liposomes

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We have successfully prepared cell-sized giant liposomes encapsulating desired amounts of actoHMM, a mixture of actin filament (F-actin) and heavy meromyosin (HMM, an actin-related molecular motor), in the presence of 5 mM $MgCl_2$ and 50 mM KCl. We employed a spontaneous transfer method to prepare those liposomes. In the absence of HMM, F-actin was distributed homogeneously inside the liposomes. In contrast, when F-actin was encapsulated in liposomes together with HMM, network structures were generated. Such network structures are attributable to the cross-linking of F-actin by HMM.

1. Introduction

Cells and cellular organelles are compartmentalized by lipid bilayer membranes and have characteristic shapes related to their specific functions. Their morphologies can change in a flexible manner in response to environmental and physiological cues. Cytoskeletal networks of F-actin and/or microtubules (MTs) play essential roles in determining the membrane morphology.[1,2] To gain deeper insights into the mechanism, an artificial cell model that consists of those cytoskeletal proteins and giant liposomes has been developed using methodologies such as natural swelling and electroformation as a consequence of a reconstituting approach.[3−5] Giant liposomes, which are comparable in size to eukaryotic cells larger than several micrometers in diameter, have been actively studied for various applied and fundamental studies in physical, medical, and life sciences because of their simplicity and capability to be observed directly with optical microscopies.[2,6−8] The dynamic behaviors and changes in shape of liposomes driven by the assembly of cytoskeletal proteins encapsulated inside the liposomes have been visualized.[2] Those studies revealed that liposomes transform accompanied by the polymerization of encapsulated actin or by the growth of actin

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bundles or MTs.[8−10] The mechanics of the protrusions, microrheology, and buckling of liposomal membranes with encapsulated MTs or F-actin have also been reported.[11−14] These studies reported the encapsulation of actin (not more than 100 μM) into liposomes using monomer actin (G-actin) and introduced a metal ion (up to 5 mM of Mg^{2+}) into the system through the ionophore-mediated influx or electroporation technique.[5,9,12,13] It is to be mentioned that Miyata et al. have succeeded in preparing a giant liposome entrapping 200 μM G-actin in the absence of Mg^{2+}.[8]

In living cells, depending on the cell type and the intracellular location, actin is expressed up to about 300 μM and undertakes its functions with the cooperation of various myosin motor proteins under physiological salt conditions (with several of millimolars Mg^{2+} and several tens of millimolars of K^+ or Na^+).[15,16] However, to the best of our knowledge, by natural swelling or electroformation method, no one has previously succeeded in constructing giant liposomes that contain actin, myosin, and their fuel (Mg-ATP) while satisfying the above conditions simultaneously. This is due to the difficulty in preparing giant liposomes in the presence of salt, especially divalent cations such as Mg^{2+}, and under the highly viscous conditions of F-actin or actomyosin solutions. It should also be mentioned that most of the currently available methodologies for preparing giant liposomes cannot control the concentrations of encapsulated species because of the passive nature of the encapsulation of chemical species. Therefore, in order to make experimental conditions much closer to those in living cells, development of new methodologies for preparing giant liposomes, in addition to natural swelling and electroformation, has been awaited.

In the present study, we report the successful construction of giant liposomes encapsulating 200 μM F-actin in the presence of 5 mM $MgCl_2$ and 50 mM KCl using a spontaneous transfer method. This is a novel method adapting water-in-oil phospholipid-coated microdroplets (W/O droplets) as precursors of giant liposomes. Using W/O droplets as compartments, the interaction between F-actin (up to 30 μM) and the phospholipid layer in the presence of Mg^{2+} (up to 20 mM) was studied and showed that the characteristic transition of the morphology in F-actin depended on the concentration of Mg^{2+}.[17,18] In our system, the W/O droplets at an oil/water interface spontaneously transform into giant liposomes by crossing through the oil/water interface.[19−22] The diameter of liposomes obtained by this method is several tens of micrometers.

Moreover, using the spontaneous transfer method, we succeeded in encapsulating simultaneously desired amounts of heavy meromyosin (HMM) as well as F-actin into giant liposomes. HMM is a double-headed derivative of conventional myosin (myosin-II) (Figure 1A). The myosin motor is one of the most important partners of actin. Nowadays, approximately 20 classes of different types of myosins have been identified and can be classified into two groups according to their head structure, that is, double- or single-headed myosins.[23] Myosins belonging to the double-headed type, such as myosin-II, are able to transform actin bundles or actin gels, and HMM frequently has been studied as a representative double-headed myosin.[23]

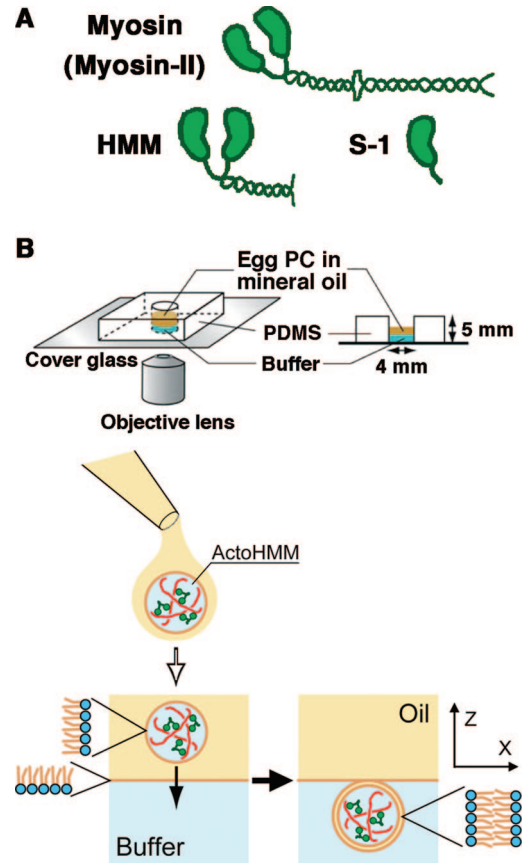

Figure 1. (A) Structural models of conventional myosin (myosin-II) and its derivatives (HMM and S-1). HMM is a double-headed derivative of myosin-II that consists of two heavy chains possessing an actin-sliding motor domain (head), an accompanying coiled-coil region responsible for the dimerization, and several light chains (not shown here). Therefore, HMM can cross-link F-actins into bundles. On the other hand, S-1 is a single-headed derivative of myosin-II, so that it cannot cross-link F-actins. (B) Top: Experimental setup for construction and observation of actoHMM-encapsulating giant liposomes using the spontaneous transfer method. Bottom: Schematic representation of the transformation from a W/O droplet in the oil phase (left) to a liposome in the aqueous phase (right). F-actin and HMM are illustrated with red and green, respectively. The interface of the droplet is depicted as a lipid monolayer, while a multilayered interface may be generated to some extent in our experiments.

This simple and very efficient system utilizing cell-sized giant liposomes containing both F-actin and HMM might represent the first critical step for developing a motile artificial cell model.

2. Materials and Methods

2.1. Proteins. Actin and myosin were obtained from rabbit skeletal muscles, and HMM and S-1 (subfragment 1) (Figure 1A) were obtained by digestion of myosin with chymotrypsin as previously

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detailed.[23] Actin was polymerized in F-buffer (2 mM Tris-HCl pH 8.0, 30 mM KCl, and 0.2 mM adenosine 5′-triphosphate (ATP)) and then used for the experiments. To visualize F-actin entrapped within liposomes, rhodamine-phalloidin (R-415, Molecular Probes, Eugene, OR) was added to the actin or actoHMM solution (the molecular ratio against actin monomer was approximately 1/40).

2.2. Preparation and Observation of Liposomes. Phosphatidylcholine (EggPC) was purchased from Sigma (St. Louis, MO). The preparation and observation of liposomes were performed as previously reported (Figure 1B).[22] Briefly, 5 μL of an aqueous solution (buffer A: pH 7.5 with 25 mM imidazol-HCl, 5 mM $MgCl_2$, 50 mM KCl, and 10 mM DTT in the presence or absence of 1 mM EGTA) containing F-actin or actoHMM was emulsified in 100 μL of oil containing EggPC (0.5 or 1.0 mM) to obtain W/O droplets through the pipetting procedure. The oil containing the W/O droplets was then situated on an oil phase (10 μL, containing 0.5 or 1.0 mM EggPC) that had been placed above an aqueous phase (10 μL, up to twice the concentration of buffer A or buffer A with sucrose, to regulate the osmolarity). The W/O droplets in the oil gradually fell down onto the oil/water interface because of gravity. Interestingly, the droplets then spontaneously moved through the interface into the aqueous solution keeping their spherical shape. In our experimental conditions, the transferred droplets, or liposomes, are anchored onto the interface as schematically depicted at the bottom of Figure 1B. Although it is possible to transfer the liposomes further into the bulk aqueous phase using centrifugation, we performed the observations on liposomes anchored to the interface, since we could then monitor the full process of the transfer on each specific liposome. As for the transformation of a droplet in oil into a liposome in water, we have already discussed the full details of the process.[22] To confirm the formation of the liposomal membrane, we visualized that by adding an NBD-conjugated lipid (NBD-PC, Molecular Probes) to the phospholipid at a ratio of 1/100−1/1000 (mol/mol)(data not shown). Observations were performed using a Zeiss Axiovert 100 inverted microscope equipped with a LSM 510 module for confocal microscopy.

3. Results

3.1. Formation of Liposomes Encapsulating F-Actin. Figure 2 shows giant liposomes encapsulating the desired amounts of F-actin up to 200 μM in the presence of 5 mM $MgCl_2$. Note that 200 μM is comparable to the actin concentration expressed in living cells[15,16] and is the upper limit concentration of F-actin for handling such as pipetting due to its very high viscosity. By evaluation of fluorescence intensity, the encapsulated concentration of F-actin inside the liposomes is almost constant and F-actin exists in a homogeneous manner. Most liposomes are spherical under these conditions, and no protrusions develop.

The small spherical objects situated on the surfaces of liposomes in the transmission images are attributed to oil droplets in the water phase.[22]

3.2. Effect of HMM. Figure 3 shows liposomes entrapping F-actin together with HMM. In contrast to the images with F-actin only, in the presence of HMM, F-actin assemblies, such as bundles and networks, are generated. We have also confirmed the appearance of similar assemblies of F-actin in aqueous solution in control experiments. As exemplified in Figure 3C and D, with increased HMM concentration, deformed liposomes tend to appear.

3.3. Comparison between HMM and S-1. As shown in Figure 4, giant liposomes coencapsulating F-actin and S-1, instead of HMM, did not show nonuniform distribution of actin or nonspherical shape, even under conditions where a molar excess of S-1 was coencapsulated. Since HMM, but not S-1, can cross-link F-actins into bundles (Figure 1),[23] it seems likely that the actin bundling is a motive force for organizing the actin networks and the liposomal morphogenesis.

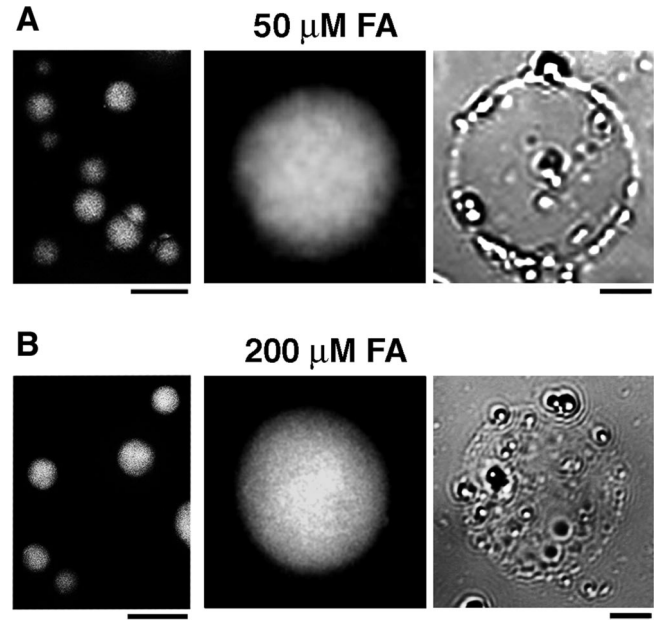

Figure 2. Confocal microscopy images of giant PC liposomes encapsulating F-actin obtained by the spontaneous transfer method (left and center, fluorescence; right, transmission). The concentrations of F-actin (FA) inside the liposomes are 50 μM (A) and 200 μM (B). The fluorescence images show the distribution of rhodamine-phalloidin-labeled F-actin. Center and right show enlarged images of individual F-actin-encapsulating liposomes. Transmission images show the existence of small oil droplets around the liposome, which were squeezed out from the oil phase. Scale bars are 50 μm (left) and 10 μm (center and right).

4. Discussion

In previous studies using the natural hydration method, the highest concentration of actin that could be encapsulated in liposomes was 200 μM, when gel-filtrated G-actin was encapsulated and then polymerized into F-actin with KCl introduced by an electroporation method after the formation of liposomes.[8] Although such studies are interesting, it has been impossible to efficiently encapsulate desired amounts of F-actin inside liposomes using the natural swelling method as described above. This study showed that G-actin-encapsulating liposomes grow protrusive structures as the actin polymerizes. On the other hand, liposomes encapsulating only already polymerized F-actin show no transformation (Figure 2). Altogether, the assembly of G-actins at the end of the membrane-pushing F-actin, that is, the mechanical force generated during actin polymerization and elongation, should be required for liposomal transformation.[2,8,24] In this study, we show that not only the polymerization of G-actin but also the bundling and redistribution of F-actin, that is the result of interfilament physical interactions such as cross-linking by HMM, can induce the morphological changes of liposomes (Figure 3C and D). Especially when 50 μM F-actin and 5.0 or 7.5 μM HMM are coencapsulated, nonspherical irregular-shaped liposomes are generated in a reproducible manner, although a large fraction of liposomes remain spherical. Since each HMM has two actin-binding motor domains (Figure 1A) and those domains are roughly 5 times larger than an actin monomer, the molecular ratio of 10/1 (actin monomer/HMM) may be the most effective to cause cross-linking among F-actins and, as a result, the marked transformation of the liposome shape is generated. The remaining important problem is how to control the reaction between F-actin, HMM,

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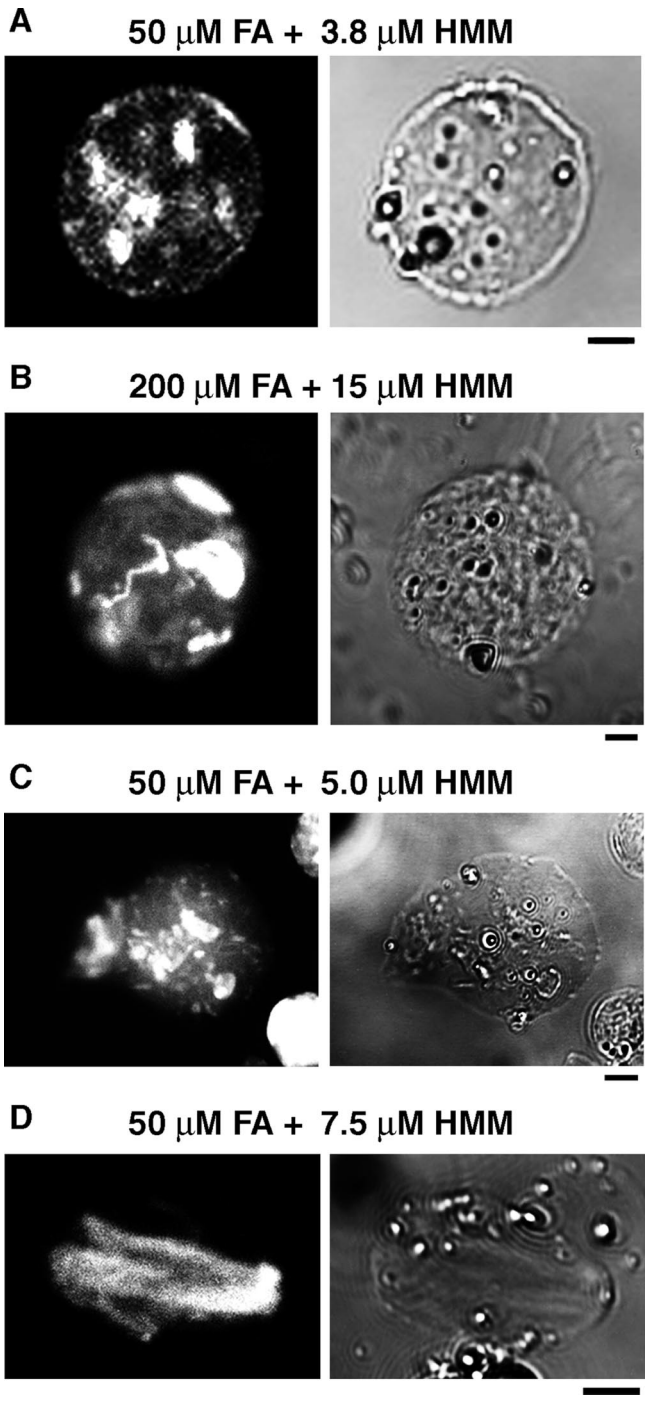

Figure 3. Confocal microscopy images of actoHMM-encapsulating giant liposomes obtained by the spontaneous transfer method (left, fluorescence; right, transmission). The concentrations of encapsulated F-actin (FA) and HMM are indicated at the top of each panel. The fluorescence images show the distribution of F-actin. In (C) and (D), liposomes possessing nonspherical irregular shapes are shown. Transmission images indicate the squeezed out oil droplets outside the liposome. Bars = 10 μm.

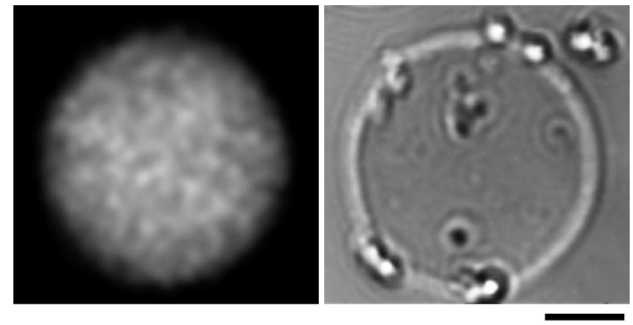

Figure 4. Confocal microscopy images of actoS-1-encapsulating giant liposomes obtained by the spontaneous transfer method (left, fluorescence; right, transmission). The concentrations of encapsulated F-actin and S-1 are 50 and 60 μM, respectively. Fluorescence images show the distribution of F-actin. Bar = 10 μm.

and ATP. In addition, it may be essential to develop a methodology to supply a sufficient amount of ATP to the actoHMM existing inside the liposomes. In relation to this, recently, we have succeeded in developing a system for the controlled fusion of two droplets with one containing a substrate and the other an enzyme.[25] Thus, a next step may be to perform the experiments by merging a couple of droplets or liposomes where F-actin, HMM, or ATP are encapsulated separately.

In this study, (i) we successfully constructed giant liposomes encapsulating both F-actin and HMM at high concentrations in the presence of 5 mM $MgCl_2$ and 50 mM KCl, using the spontaneous transfer method, (ii) the encapsulated actoHMM formed self-organized actin-network-like structures, and (iii) nonspherical liposomes were obtained in a reproducible manner. This study serves as the first step in developing motile giant liposomes containing actoHMM and in generating spontaneous motion in a system similar to but much simpler than living cells.

Further development of the spontaneous transfer method to construct an artificial model cellular system is needed, including the physicochemical characterization of the liposomes formed. We are now trying to compare the membrane structure of the giant liposome obtained by the spontaneous transfer method with the vesicle obtained by the natural swelling or electroformation method.[26]

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