Reconstruction of Motile Actin Networks in Giant Liposome

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Abstract:
To construct a simple model cellular system exhibiting the property of self-propelled motion, 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), have been prepared. We adapted the methodology of spontaneous transfer of a water droplet through oil/water interface in the presence of phospholipid and successful obtained stable giant liposome with the inner physiological biopolymer solution. We introduced ATP to the bathing solution of liposome encapsulating actoHMM, in which bilayer membrane α-hemolysin, a bacterial membrane pore-forming toxin, is embedded. In this system, ATP is supplied into the inner volume of liposome through the protein pores in a passive manner. Accompanied by the ATP supply, actin networks or bundles that have encapsulated in the liposomes exhibited specific morphological change, being attributable to the active sliding between F-actin and HMM. Remarkable difference in the behavior of F-actins is found; *i.e.*, inside the liposome, almost all the F-actins situate around the inner periphery of the liposome, whereas, in the bulk solution, actin bundles form an aster-like structure.

1. INTRODUCTION

Cytoskeletal networks of actin filament (F-actin) play essential roles in determining the morphology and propelling movement of living cells [1-8]. To investigate the principal mechanism, as an artificial cell model giant liposome encapsulating cytoskeletal proteins has been developed using the methodologies such as natural swelling and electro-formation, as a consequence of a reconstituting approach [9-17]. Cell-sized giant liposomes 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 [18, 19]. The dynamic behaviors and changes in shape of liposomes driven by the assembly of cytoskeletal proteins encapsulated inside the liposomes have been visualized. Those studies revealed that liposomes transform accompanied by the polymerization of encapsulated actin, or by the growth of actin bundles. In living cells, however, actin is undertaken its functions with the cooperation of various myosin motor proteins under physiological salt conditions (with several millimolar of Mg^{2+} and several tens of millimolar of K^+ or Na^+). To the best of our knowledge, there has been no report in successful construction of giant liposomes encapsulating actin, myosin and their fuel (Mg-ATP) with satisfying the above conditions simultaneously.

Recently, there have been several attempts to employ water-in-oil (W/O) droplets coated by phospholipids as a precursor of liposomes [20-26]. The W/O droplets are easily prepared by emulsifying an aqueous solution together with oil containing phospholipids. The process enables us to encapsulate biomolecules at a controlled concentration under any salt strength, into cell-sized compartments covered with a monolayer of phospholipids. Consequently, spontaneous transfer method, the methodology to obtain liposomes by transferring phospholipid-coated W/O droplets from an oil phase to an aqueous phase through their interface has been developed [23-25]. With the method, one can obtain liposomes with sizes of 10-100 μm containing desired amounts of molecules. Using the method, we have successfully constructed giant liposomes encapsulating 200 μM of F-actin in the presence of 5 mM $MgCl_2$ and 50 mM KCl [25, 27]. Note that 200 μM is comparable to the actin concentration expressed in living cells [28, 29], and is the upper limit of concentration that allows handling due to its very high viscosity. Moreover, this method enabled us to succeed in encapsulating simultaneously desired amounts of F-actin and HMM into giant liposomes [25]. The motor domain of myosins that is required and sufficient for generating actin-sliding movement is termed “head”. HMM is a double-headed derivative of conventional myosin (myosin-II), and is able to crosslink F-actins into bundles or gels and moreover to transform actin bundles or actin gels [30, 31]. When F-actin was encapsulated in liposomes together with HMM, network structures were generated, while F-actin was distributed homogeneously inside the liposomes in the absence of HMM. This 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.

Remaining important problem is to develop the methodology in constructing an open system using closed giant liposome to control continuously the reaction between F-Actin, HMM and ATP. Especially, how to supply ATP, the fuel of actomyosin, into our developed system is essential for constructing a motile system consists of liposome and cytoskeletal proteins. In this study, to further develop the spontaneous transfer method to construct an artificial motile model cell that could generate spontaneous motion similar to but much simpler than living cells, we adopted α-hemolysin, a bacterial membrane pore-forming toxin, which can spontaneously penetrate in lipid bilayer membrane, assemble as heptamer and form channel larger

than 2 nm in diameter [22, 26, 32, 33], to introduce ATP into the system.

2. METHODS

2.1 Chemicals

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), phosphatidylcholine isolated from native source (egg yolk l-a-phosphatidylcholine, eggPC), and cholesterol were purchased from Sigma (St. Louis, MO, USA) or Avanti Polar Lipids (Alabaster, AL, USA). Mineral oil was purchased from Nacalai Tesque (Kyoto, Japan). Rhodamine-phalloidin (R-415) was purchased from Molecular Probes (Eugene, OR, USA). These chemicals were used without further purification. Methylcellulose (1,500 cP) was purchased from Wako Pure Chemical (Osaka, Japan), and was dissolved in and dialyzed at least overnight against Milli-Q water to make stock solution (2%, properly final 5 mM NaN_3 was added).

2.2 Proteins

Actin and myosin were obtained from rabbit skeletal muscles, and HMM and S-1 (subfragment 1) were obtained by digestion of myosin with chymotrypsin as previously detailed [30, 31, 34]. Actin was polymerized in F-buffer (2 mM Tris-HCl, pH 8.0, 30 mM KCl and 0.2 mM ATP) and then used for the experiments. To visualize F-actin entrapped within liposomes, rhodamine-phalloidin was added to the actin or actoHMM solution (the molecular ratio against actin monomer was approximately 1/40). Alpha-hemolysin was purchased from Sigma.

2.3 Preparation and observation of liposomes

The preparation and observation of liposomes were performed as previously reported [25, 27]. The observation chamber consisted of a cylindrical hole in a poly(dimethylsiloxane) (PDMS) sheet (ca. 5 mm thick), which was obtained by mixing the base solution and a curing agent of Silpot 184 W/C (Dow Corning Toray, Tokyo, Japan), on a glass microscope slide (0.12-0.17 mm thick).

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) containing F-actin or actoHMM was emulsified in 100 μl of oil containing lipids (0.5 or 1.0 mM) to obtain W/O droplets through the pipetting procedure. For the case of the encapsulation of actoHMM bundles that are formed with inert high polymer into giant liposomes, actoHMM solution was lastly very gently added with final 0.3% methylcellulose, and incubated for several minuets [30, 31, 35]. Then, 5 μl aliquot was emulsified in the lipid-containing oil to form W/O droplets as described above. The oil containing the W/O droplets was then situated on an oil phase (10 μl, containing 0.5 or 1.0 mM lipids that had been placed above an aqueous phase (10 μl, buffer A, up to twice the concentration of buffer A or buffer A with sucrose, to regulate the osmolarity). The lipid compositions we used are eggPC, DOPC or DOPC/DPPC/Cholesterol (4:4:2, molar ratio). We properly selected the lipid concentration and composition to obtain the optimal liposome formation, and the lipid composition did not affect the obtained results. The W/O droplets in the oil gradually fell down onto the oil/water interface due to gravity. Interestingly, the droplets then spontaneously moved through the interface into the aqueous solution keeping their spherical shape (Figure 1). In our experimental conditions, the transferred droplets, or liposomes, are anchored onto the interface [25]. 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 [23]. After the formation of actoHMM-containing liposomes was confirmed, α-hemolysin (final 12.5 μg/ml) solved in the solution that is same as the aqueous phase (5 μl, with or without final 10 mM ATP) was added to the bottom aqueous phase using a narrow pipette tip (Catalogue No. 010, Quality Scientific Plastics, Kansas City, KS, USA) (Figure 1). Observations were performed using a Zeiss Axiovert 100 inverted microscope equipped with a LSM 510 module for confocal microscopy. Unless particularly denoted, the samples for observation were prepared and the observations were performed at 25°C. The recorded images were analyzed using ImageJ software.

3. RESULTS

3.1 Spontaneous supply of ATP by α-hemolysin into the actoHMM-containing giant liposomes

Figure 1 shows the formation of actoHMM-containing giant liposomes by the spontaneous transfer method, and supply of ATP into the liposomes by addition of α-hemolysin. Alpha-hemolysin is soluble monomer protein, but once interacts with a lipid bilayer membrane, spontaneously penetrates into the membrane and forms heptamer to open channel with about 2 nm diameters. Although the kinetic process has not been fully clarified yet, α-hemolysin has been utilized to construct an open system consists of membrane vesicles. The diameter of membrane channel is suitable to supply ATP into actoHMM-containing liposomes because the size is large much enough to through ATP from the external solution but too small to allow the encapsulated actin and HMM proteins to leak to the external solution.

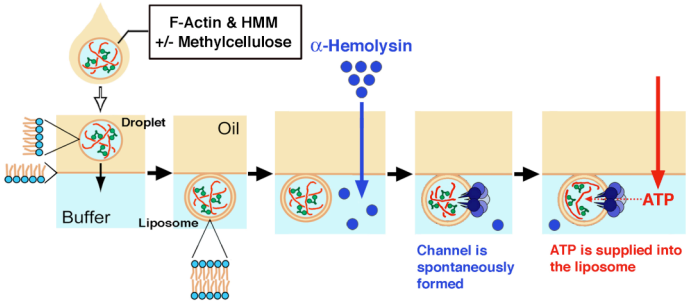

Figure 1. Schematic representation of the transformation from a W/O droplet in the oil phase (left) to a liposome in the aqueous phase (left to center). F-actin and HMM are illustrated with red and green, respectively. The interface of the droplet is depicted as a lipid monolayer, while a multilayered interface may be generated to some extent in our experiments. After construction of actoHMM-encapsulating giant liposomes, a solution containing α-hemolysin and/or ATP is added to the bottom aqueous solution. Alpha-hemolysin is soluble monomeric protein, but once comes across with a lipid bilayer membrane, it will spontaneously penetrates into the membrane and opens large channel larger than 2 nm in diameter. The external ATP flows into the liposome by passive diffusion through the channel (right).

3.2 The effect of ATP supply on the actoHMM that have encapsulated in the liposomes

Figure 2A shows giant liposomes encapsulating F-actin and HMM in the presence of $MgCl_2$. In the absence of ATP, the strong binding takes place between each myosin head and F-actin (rigor state). As the result, HMM strongly crosslinks F-actins. Inside of the liposomes, robust actin bundles and networks are efficiently observed before ATP supply [25, 27]. In the condition that F-actin and HMM concentrations are 50 and 5.0 μM, respectively, non-spherical irregular-shaped liposomes were generated in a reproducible manner, although a large fraction of liposomes remains spherical [25]. We have confirmed the appearance of similar assemblies of F-actin in bulk aqueous solution.

Once Mg-ATP is supplied, myosin head escapes from the rigor state and starts sliding motion along F-actin. Therefore, by adding α-hemolysin, as ATP in the outside solution was introduced into the internal of liposomes, actin bundles resulted from the crosslinking of F-actins by double-headed HMM should be deformed. As long period incubation up to for overnight, actin bundles and networks gradually dispersed (Figure 2B). Finally, F-actins inside the liposomes redistributed in uniformly.

3.3 Formation of liposomes encapsulating actoHMM bundles that have formed with methylcellulose

When actoHMM is mixed with inert high polymer, such as methylcellulose, a large number of F-actins closely align in parallel with overlaps to form long and thick bundles with several tens of micrometers long [35]. This alignment is generated by the depletion effect of the high polymer [35, 36]. Frequently they are narrow spindle-like shapes (Figure 3A) [30, 31, 35].

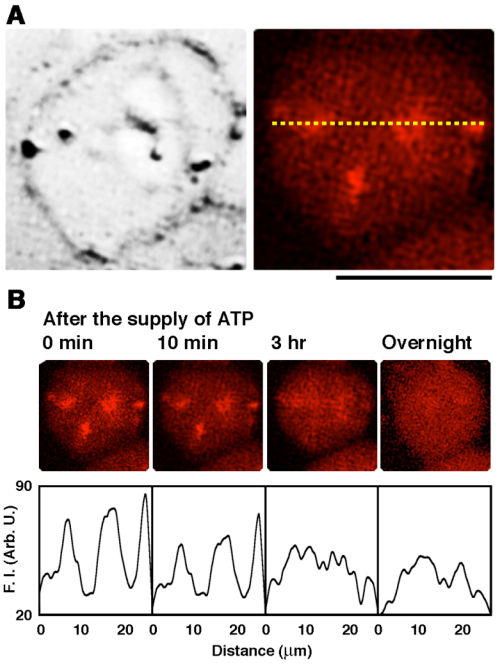

Figure 2. Cell-sized liposome encapsulating actoHMM. (A) Confocal microscopy images of actoHMM-encapsulating giant liposomes with DOPC bilayer membrane by the spontaneous transfer method. The concentrations of encapsulated F-actin and HMM are 50 and 5.0 μM, respectively (left: transmission, right panel: fluorescence). Fluorescence image shows the distribution of F-actin. Bar = 20 μm. The small spherical objects situated on the surfaces of liposomes in the transmission images are attributed to oil droplets in the water phase. (B) Top: Fluorescence images showing disassembly process of the crosslinked F-actins that have formed with HMM in the liposome shown in right panel of (A). Time after the ATP supply by the addition of α-hemolysin is indicated in top of each panel. Bottom: Relative fluorescence intensity profiles of the top panel at the position indicated by yellow dotted line in right panel of (A). F.I. = fluorescence intensity. The sample was observed for 3 hrs at 25°C, then left overnight at 4°C. Twelve hrs later, the same sample was observed again after it was returned to 25°C.

Figure 3B shows giant liposomes encapsulating the actoHMM bundles that have formed with methylcellulose. The result clearly shows that the spontaneous transfer method enable us to encapsulate desired amounts of not only F-actins but also actin bundles that have the larger size into cell-sized giant liposomes. Even bundles are encapsulated, most liposomes are spherical and no protrusions develop under the conditions we examined.

3.4 The effect of ATP supply on the actoHMM bundles that have formed inside of the liposomes

In the presence of molecular motor possessing the double-headed structure such as HMM, when F-actin bundles that have formed with inert high polymer such as methylcellulose were exposed to Mg-ATP, they shortened, while becoming thicker, that is result of their contraction caused by the active sliding motion between F-actins and HMM [30, 31]. Subsequently, each bundle was split

longitudinally into several bundles in a stepwise manner, while the newly formed ones remained associated together at one of the two ends. The product, an aster-like assembly of actoHMM, was morphologically quiescent; that is, individual bundles never contracted upon second exposure to ATP. Thus, as shown in Figure 3C, when the actoHMM bundles formed with methylcellulose in the presence of Mg^{2+} in bulk solution were added with ATP, they transformed into the asters. The same kind of aster-like assembly of F-actin can also be found *in vivo* [37, 38].

Figure 3D shows that, after the addition of α-hemolysin to supply ATP into inside of the liposomes, which are containing actoHMM bundles that have formed with methylcellulose, the same aster-like assemblies tend to appear in some liposome (less than 20% of liposomes). Note that, even the encapsulated actoHMM bundles transform to the asters, all liposome observed does not show any transformation.

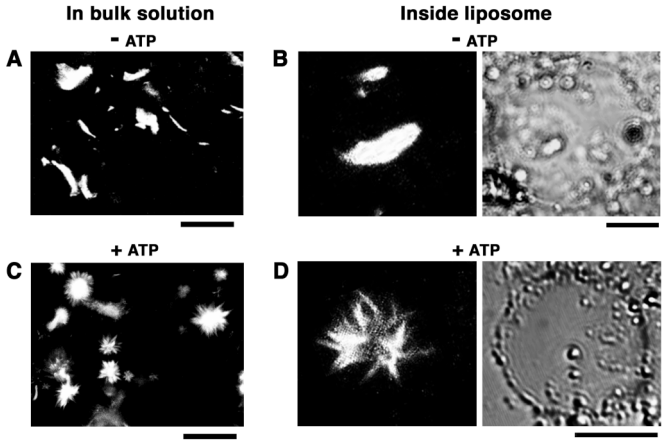

Figure 3. Confocal microscopy images of the actoHMM bundles that have formed with methylcellulose without (A and B) or with (C and D) the ATP supply. The concentrations of F-actin and HMM are 10 and 20 μM, respectively. (A and C) Fluorescence microscopy images of actoHMM bundles in bulk solution (Buffer A). Note that the actoHMM bundles are observed in the bulk solution (10 μl Buffer A) after the pipetting in the same way as to form W/O droplets containing bundles (A). After that, an ATP solution (5 μl Buffer A, that was containing final 10 mM ATP) was added, and then the sample was observed about 30 min later (C). The transformation of actoHMM bundles in bulk solutions is not effected by the addition of α-hemolysin. (B and D) Each shows a representative giant liposome encapsulating actoHMM bundles that have formed with methylcellulose obtained by the spontaneous transfer method (left: fluorescence, right: transmission). The lipid composition was DOPC/DPPC/Cholesterol. The small spherical objects situated on the surfaces of liposomes in the transmission images are attributed to oil droplets in the water phase. Bundles without (B) or with (D) the ATP supply by the addition of α-hemolysin are shown. After the ATP supply, aster-like assemblies could be observed (C and D). Bars indicate 100 (A and C) or 20 μm (B and D).

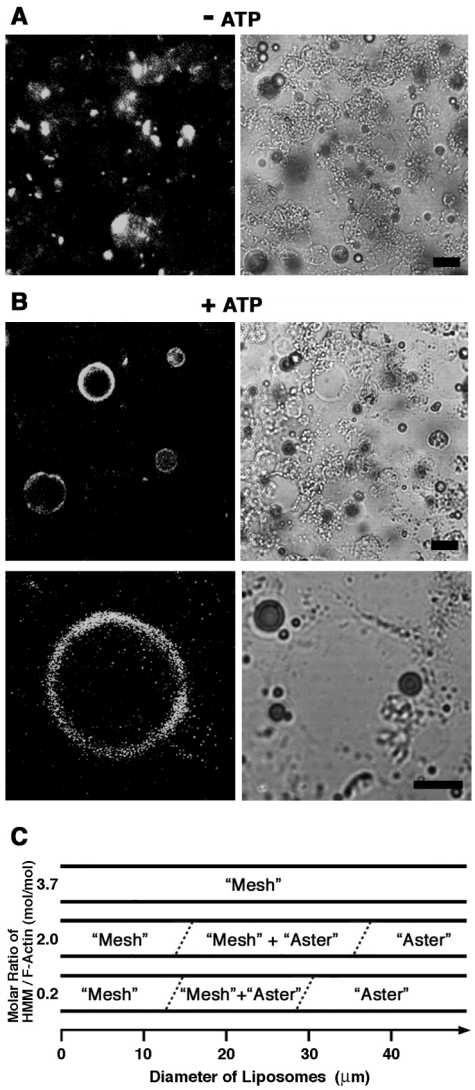

Figure 4. (A and B) Confocal microscopy images of giant liposomes made from DOPC/DPPC/Cholesterol by the spontaneous transfer method (left: fluorescence, right: transmission). The liposomes are encapsulating actoHMM bundles that are made with methylcellulose. The concentrations of encapsulated F-actin and HMM are 10 and 37 μM, respectively. Fluorescence images (left column) show the distribution of F-actin. Bundles without (A) and with (B) the ATP supply are shown. The bottom panel of (B) shows an enlarged fluorescence image of a representative liposome, which the encapsulated F-actins redistributed to the periphery of inner surface of the liposome after the ATP supply. Bars = 20 μm. The small spherical objects situated on the surfaces of liposomes in the transmission images are attributed to oil droplets in the water phase. (C) Diagram of the changes in the encapsulated actin bundles depending on the liposomal size and on the concentration of co-encapsulated HMM. Concentration of F-actin is 10 μM. In all liposome where the encapsulated actin bundles showed changes after the ATP supply, the actin bundles either situated around the inner periphery of the liposomes and formed a cortex ("Mesh") or transformed into the aster-like assemblies ("Aster"). For each HMM concentration, more than 30 liposomes were observed and counted.

As shown in Figure 4, interestingly the encapsulated actoHMM bundles redistribute to the periphery of inner surface of the liposome in almost all cases (about 90%), even though almost actoHMM bundles transform to the asters in the bulk solution (please see Figure 3C): when the concentrations of F-actin and HMM are 10 and 37 μM, respectively, all bundles transform to aster; when those are 10 and 20 μM, 89% of bundles transform to aster; when those are 10 and 2 μM, 44% of bundles transform to aster. Whereas, inside giant liposomes, no aster is formed in the condition of combination of 10 μM of F-actin and 37 μM of HMM as long as we have observed. Incidentally, when the concentrations of the encapsulated F-actin and HMM are 10 and 20 μM, aster is found in 9.7% liposome; when those are 10 and 2 μM, aster is found in 14% liposome. In other cases, the encapsulated F-actins situate around the inner periphery of the liposomes.

To investigate the reason of redistribution of the encapsulated actin bundles, we measured diameter of liposomes. Figure 4C shows that diameter of liposomes where the encapsulated actin bundles transformed to the aster have larger size as compared with those where F-actins situated around the inner periphery of the liposomes. In addition, the diameter of the former liposomes is comparably longer than the length of actin bundles formed (data not shown).

As control, three cases, without both of α-hemolysin and ATP, or only one or the other of them is added to the bottom aqueous solution, are tested. In either case, any change of the encapsulated F-actin or actoHMM bundle is not observed.

4. CONCLUSION

In this study, adopting α-hemolysin, we successfully supply ATP inside the giant liposomes encapsulating actoHMM, which are obtained by the spontaneous transfer method.

Accompanied by the supply of ATP, self-organized actin network-like structures are formed from the encapsulated actoHMM gradually dispersed into individual F-actins. Such disassembly of actoHMM networks is attributable to the dissociation of F-actins they have been crosslinked by strong binding between F-actins and double-headed HMM, because both ATP, the fuel for HMM, and α-hemolysin, membrane-channel for introducing ATP into liposomes, are indispensable for the observed F-actin redistribution.

In the presence of methylcellulose, F-actins form bundles even without HMM [35]. By the supply of ATP, actoHMM bundles, that have been formed with methylcellulose and subsequently encapsulated into liposomes, changed shape and/or distribution in a characteristic manner. Also in this case, since both ATP and α-hemolysin are indispensable, the aster formation is attributable to the active sliding between F-actins and HMM. These results indicate that the supplying ATP into liposomes utilize α-hemolysin does just what we expect in this study.

Recently we have reported that, by exposure to HMM and Mg-ATP, actin bundles formed with a depletion reagent such as methylcellulose are transformed into aster-like assemblies, that is result of the active sliding motion between F-actins and HMM [30, 31]. Inside the giant liposomes, asters formed from actoHMM reproducibly tend to appear after the supply of ATP. However, the formation of aster is rarely observed in the liposomes. Rather, in almost cases, F-actins situate around the periphery of inner surface of the liposome after the supply of ATP. Such a remarkable difference would be resulted from the effect of encapsulation into a closed cell-sized space made from lipid membrane.

5. ACKNOWLEDGMENT

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