Real-World Modeling of Artificial Motile Cell

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Abstract:
Using a spontaneous transfer method, 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 successfully constructed in the presence of 5 mM $MgCl_2$ and 50 mM KCl. The encapsulated actoHMM formed self-organized actin network-like structures, and non-spherical liposomes were obtained in a reproducible manner. However, actoHMM requires ATP, the fuel of molecular motors, to generate force and motility. Here we tried to introduce ATP to actoHMM inside of the constructed liposomes through protein pores inserted in their membranes. By the ATP supply, the network structures of F-actin that have formed inside the liposomes showed redistributions, which are attributable to the sliding between F-actin and HMM. 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.

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, an artificial cell model that consists of cytoskeletal proteins and giant liposomes 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^+). However, to the best of our knowledge, no one had succeeded in constructing giant liposomes that contain 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]. Note that 200 μM is comparable to the actin concentration expressed in living cells [27, 28], 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 heavy meromyosin (HMM) and F-actin (actoHMM) into giant liposomes [25]. 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 [29, 30]. Noted here that, the motor domain of myosins that is required and sufficient for generating actin-sliding movement is termed “head”. 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 how to control 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 tried to adopt α-hemolysin, a bacterial membrane pore-forming toxin, which can spontaneously penetrate in lipid bilayer membrane, assembly heptamer and form large channel larger than 2 nm in diameter [22, 26, 31, 32], to introduce ATP into the system.

2. METHODS

2.1 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 [29, 30]. 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 (R-415, Molecular Probes, Eugene, OR, USA) was added to the actin or actoHMM solution (the molecular ratio against actin monomer was approximately 1/40). Alpha-hemolysin was purchased from Sigma (St. Louis, MO, USA).

2.2 Preparation and observation of liposomes

Dioleoylphosphatidylcholine (DOPC) and phosphatidylcholine isolated from native source (EggPC) were purchased from Sigma or Avanti Polar Lipids (Alabaster, AL, USA). The preparation and observation of liposomes were performed as previously reported [23, 25]. 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 DOPC or 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 DOPC or EggPC) that had been placed above an aqueous phase (10 μl, buffer B: pH 7.5 with 50 mM imidazol-HCl, 10 mM $MgCl_2$, 100 mM KCl and 20 mM DTT with 10 mM ATP). Buffer B was used as the bottom aqueous phase to regulate the osmolarity. 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. 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 10 μg/ml) solved in buffer B (2 μl, without ATP) was added to the bottom aqueous phase using a narrow pipette tip (Catalogue No. 010, Quality Scientific Plastics, Kansas City, KS, USA). 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.

3. RESULTS AND DISCUSSION

3.1 Construction of actoHMM-containing giant liposomes by the spontaneous transfer

Figure 1 shows giant liposomes encapsulating F-actin (50 μM) and HMM (5.0 μM) in the presence of $MgCl_2$. The small spherical objects situated around the liposomes or on their surfaces in the transmission images are attributed to oil droplets at the oil/water interface [23]. Inside of those liposomes, F-actin that is co-encapsulated with HMM assembles into large bundles or networks (Figure 1A) [25]. We have confirmed the appearance of similar assemblies of F-actin in aqueous solution in control experiments.

On the other hand, in the case of the liposomes that are encapsulating the same concentration of F-actin only, F-actins inside the liposomes exist in a homogeneous manner (Figure 1B) [25].

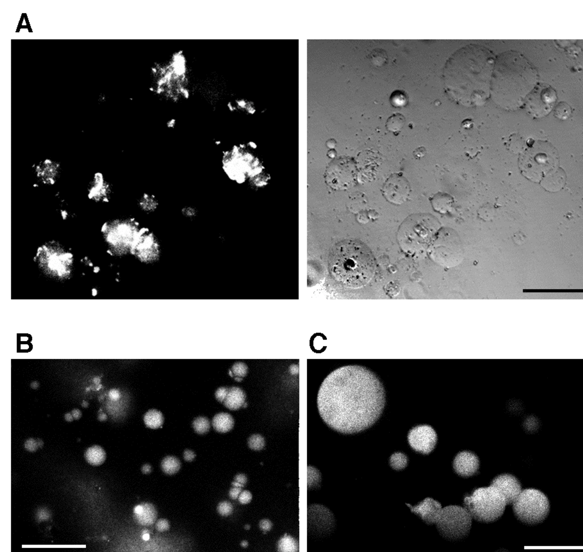

Figure 1. (A) Confocal microscopy images of actoHMM-encapsulating giant liposomes made from DOPC by the spontaneous transfer method (left: fluorescence, right: transmission). The concentrations of encapsulated F-actin and HMM are 50 and 5.0 μM, respectively. Transmission images indicate the squeezed out oil-droplets outside the liposome. (B and C) Confocal microscopy images of giant liposomes encapsulating F-actin only (B) or actoS-1 (C) obtained by the spontaneous transfer (fluorescence images). The concentrations of encapsulated F-actin and S-1 are 50 μM and 60 μM, respectively. Fluorescence images show the distribution of rhodamine-phalloidin-labeled F-actin. Under these conditions, most liposomes are spherical and no protrusions develop. Bars = 100 μm.

Moreover as shown in Figure 1C, giant liposomes co-encapsulating the same concentration of F-actin and S-1, instead of HMM, did not show non-uniform distribution of F-actin, even under conditions that excess molar of S-1 was co-encapsulated [25].

Myosins are classified into two groups according to their head-structure, *i.e.* double- or single-headed myosins. Myosin belonging to the double-headed type, such as myosin-II, and a double-headed derivative of myosin-II, such as HMM, which frequently has been studied as a representative double-headed myosin [29, 30]. On the other hand, S-1 has often been studied as a representative of simple single-headed myosin. S-1 has only one actin-binding motor domain and is unable to crosslink F-actins. Thus, these indicate that the crosslinking F-actins by double-headed HMM is a motive force for organizing the actin bundles and networks.

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

In the absence of ATP, the strong binding takes place between each myosin head and F-actin (rigor state). Once Mg-ATP is supplied, myosin head escapes from the rigor state and starts sliding motion along F-actin. Therefore, when Mg-ATP is presence, actin bundles resulted from the crosslinking of F-actins by double-headed HMM should be weakened.

Figures 2 and 3 show actoHMM-encapsulating giant liposomes. When the concentration of encapsulated F-actin is 50 μM and that of co-encapsulated HMM is 5.0 (Figure 2) or 7.5 μM (Figure 3), robust actin bundles and networks are efficiently observed inside of the liposomes before ATP supply [25]. By adding α-hemolysin, as ATP in the outside solution was introduced into the internal of liposomes, actin bundles and networks gradually dispersed. Finally, F-actins inside the liposomes redistributed in uniformly.

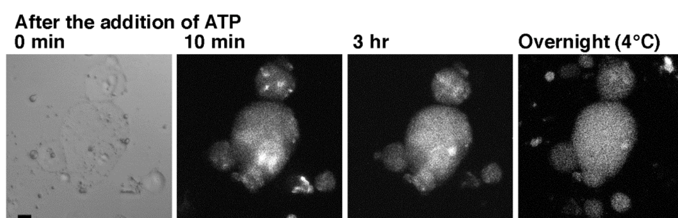

Figure 2. Confocal microscopy images of actoHMM-encapsulating giant liposomes made from DOPC by the spontaneous transfer method (left panel: transmission, other panels: fluorescence, they were observed at the same microscopic field). The concentrations of encapsulated F-actin and HMM are 50 and 5.0 μM, respectively. Fluorescence images show disassembly process of F-actin bundles that have formed with HMM. Time after the addition of α-hemolysin is indicated in top of each panel. 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. Bars = 10 μm.

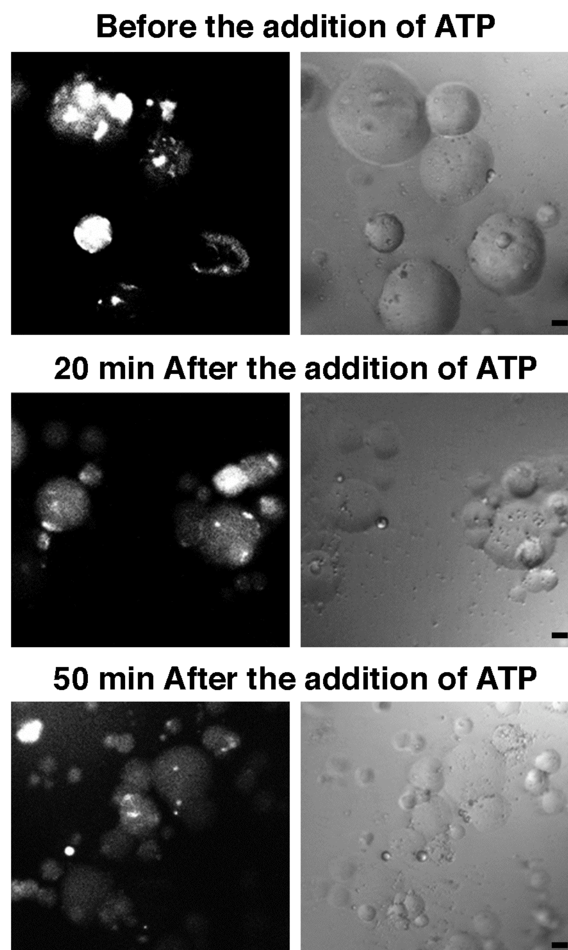

Figure 3. Confocal microscopy images of actoHMM-encapsulating giant liposomes made from DOPC by the spontaneous transfer method (left: fluorescence, right: transmission). The concentrations of encapsulated F-actin and HMM are 50 and 7.5 μM, respectively. Fluorescence images (left column) show the distribution of F-actin. Time after the addition of α-hemolysin is indicated in top of each panel. Bars = 10 μm.

3.3 The effect of ATP supply on the actoHMM networks that have formed with methyl-cellulose inside of the liposomes

When actoHMM is mixed with inert high polymer, such as methyl-cellulose, a large number of F-actins closely align in parallel with overlaps to form a long and thick bundles with several tens of micrometers long [33]. When they were exposed to Mg-ATP, they contracted to about one-third the initial length, while becoming thicker [29, 30]. 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.

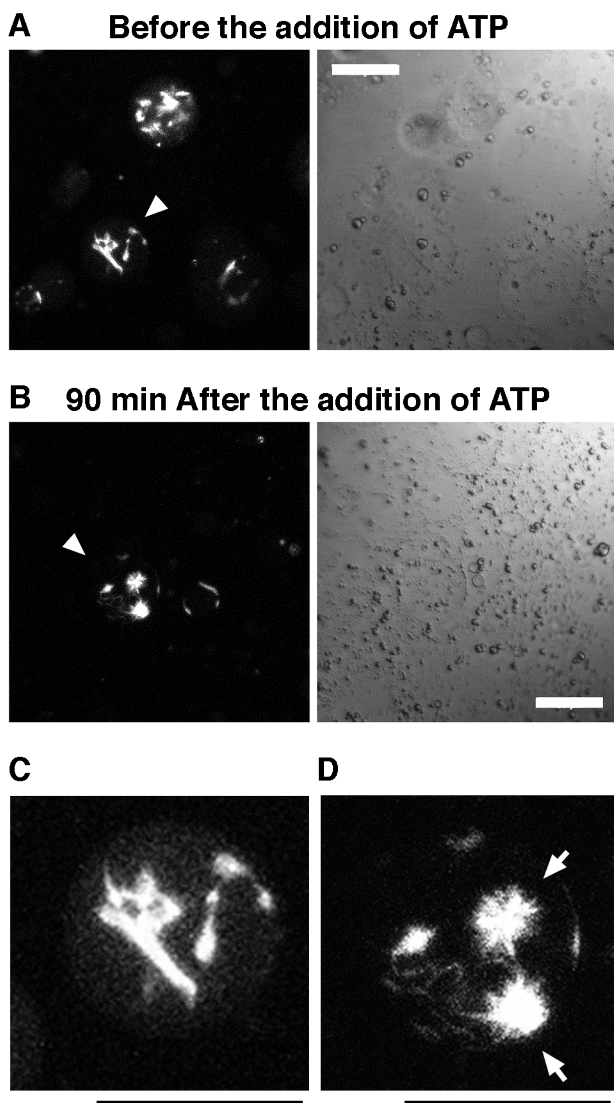

Figure 4. (A and B) Confocal microscopy images of actoHMM-encapsulating giant liposomes made from DOPC by the spontaneous transfer method (left: fluorescence, right: transmission). The concentrations of encapsulated F-actin and HMM are 20 and 15 μM, respectively. The concentration of methyl-cellulose is 0.3%. Fluorescence images (left column) show the distribution of F-actin. Images of before (A) and 90 minutes after (B) the addition of α-hemolysin are shown. (C and D) Enlarged fluorescence images of liposomes indicated by arrowheads in A and B, respectively. Arrows in D indicate the aster-like assemblies of F-actins, which are formed after the contraction of actin bundles [29, 30]. Bars = 50 μm.

Figure 4 shows that, after the addition of α-hemolysin to supply ATP into inside of the liposomes that have contained actoHMM with methyl-cellulose, the same aster-like assemblies tend to appear in some liposome.

4. CONCLUSION

In this study, adopting α-hemolysin, we successfully supply ATP to actoHMM that is encapsulated inside the giant liposomes, which are obtained by the spontaneous transfer method. As a result, self-organized actin network-like structures formed from the encapsulated actoHMM gradually dispersed into individual F-actins after addition of ATP. That disassembly of actoHMM networks is attributable to the dissociation of F-actins they have been crosslinked by strong binding between F-actin and HMM. In the presence of methyl-cellulose, F-actins form bundles even without HMM [33]. Previously we have reported that such actin bundles are transformed into an aster-like structure by motor activity of HMM [29, 30]. Also inside the giant liposomes, in the presence of methyl-cellulose, asters formed from actoHMM tend to appear after addition of ATP.

However, any movement or shape change of the giant liposomes yet could not be observed. In our system, the following subjects should be dissolved. i) Possibly there is difference in the optimum conditions for generating force or motion between the internal the liposomes and bulk solutions. Thus we have to more consider the experimental conditions, for an example, combination of concentrations of actoHMM. ii) The amount of ATP that is introduced into each liposome through the protein pore should be evaluated precisely. Systems adopting luciferine-luciferase reaction or using fluorescence labeled probes may be useful to determine the quantity. iii) In living cells, a number of actin-associating proteins are involved in their morphology and motility. Many of such proteins localize within or beneath the cellular membranes. Therefore other factors, as a candidate, protein crosslinker between F-actin and membrane, may be required for the liposomal morphogenesis or movement.

This is the first step for developing the motile artificial cell model for developing cell-sized giant liposomes containing cytoskeletons and molecular motors, and to realize spontaneous motion by mimicking living cells.

5. ACKNOWLEDGMENT

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REFERENCES

[1] Schroeder, T. E. 1973. Actin in dividing cells: contractile ring filaments bind heavy meromyosin. *Proc. Natl. Acad. Sci. USA* **70,** 1688-1692.

[2] Albrecht-Buehler, G. & Lancaster, R. M. 1976. A quantitative description of the extension and retraction of surface protrusions in spreading 3T3 mouse fibroblasts. *J. Cell Biol.* **71,** 370-382.

[3] Taylor, D. L. & Condeelis, J. S. 1979. Cytoplasmic structure and contractility in amoeboid cells. In *Int. Rev. Cytol. Vol. 56,* pp. 57-144, Academic Press, New York.

[4] Byers, H. R. & Fujikawa, K. 1982. Stress fibers in cells in situ: immunofluorescence visualization with antiactin, antimyosin, and anti-a-actinin. *J. Cell Biol.* **93,** 804-811.

[5] Mitchison, T. J. & Kirschner, M. 1988. Cytoskeletal dynamics and nerve growth. *Neuron* **1,** 761-772.

[6] Mitchison, T. J. & Cramer, L. P. 1996. Actin-based cell motility and cell locomotion. *Cell* **84,** 371-379.

[7] Hotani, H., Inaba, T., Nomura, F., Takeda, S., Takiguchi, K., Itoh, T. J., Umeda, T. & Ishijima, A. 2003. Mechanical analyses of morphological and topological transformation of liposomes. *Biosystems* **71,** 93-100.

[8] Rodriguez, O. C., Schaefer, A. W., Mandato, C. A., Forscher, P., Bement, W. M. & Waterman-Storer, C. M. 2003. Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat. Cell Biol.* **5,** 599-609.

[9] Bormann, M., Kos, J., Kurzmeier, H. & Sackmann, E. 1992. In *The Structure and Conformation of Amphiphilic Membranes*, (Lipowsky, R., Richter, D., and Kremer, K., eds), Springer, Heidelberg.

[10] Cortese, J. D., Schwab, B., III, Frieden, C. & Elson, E. L. 1989. Actin polymerization induces a shape change in actin-containing vesicles. *Proc. Natl. Acad. Sci. USA* **86,** 5773-5777.

[11] Fygenson, D. K., Marko, J. F. & Libchaber, A. 1997. Mechanics of microtubule-based membrane extension. *Phys. Rev. Lett.* **79,** 4497-4500.

[12] Häckl, W., Bärmann, M. & Sackmann, E. 1998. Shape changes of self-assembled actin bilayer composite membranes. *Phys. Rev. Lett.* **80,** 1786-1789.

[13] Limozin, L., Bärmann, M. & Sackmann, E. 2003. On the organization of self-assembled actin networks in giant vesicles. *Eur. Phys. J. E* **10,** 319-330.

[14] Limozin, L., Roth, A. & Sackmann, E. 2005. Microviscoelastic moduli of biomimetic cell envelopes. *Phys. Rev. Lett.* **95,** Art. No. 178101.

[15] Limozin, L. & Sackmann, E. 2002. Polymorphism of cross-linked actin networks in giant vesicles. *Phys. Rev. Lett.* **89,** Art. No. 168103.

[16] Miyata, H. & Hotani, H. 1992. Morphological changes in liposomes caused by polymerization of encapsulated actin and spontaneous formation of actin bundles. *Proc. Natl. Acad. Sci. USA* **89,** 11547-11551.

[17] Miyata, H., Nishiyama, S., Akashi, K. & Kinosita, K. Jr. 1999. Protrusive growth from giant liposomes driven by actin polymerization. *Proc. Natl. Acad. Sci. USA* **96,** 2048-2053.

[18] Bangham, A. D. 1995. Surrogate cells or Trojan horses. The discovery of liposomes. *BioEssays* **17,** 1081-1088.

[19] Lasic, D. D. 1995. Applications of liposomes. In *Handbook of Biological Physics*, **1a,** *Structure and Dynamics of Membranes*, (Lipowsky, R., and Sackmann, E., eds), p. 491-519, Elsevier, Amsterdam.

[20] Pautot, S., Frisken, B. J. & Weitz, D. A. 2003. Engineering asymmetric vesicles. *Proc. Natl. Acad. Sci. USA.* **100,** 10718-10721.

[21] Pautot, S., Frisken, B. J. & Weitz, D. A. 2003. Production of unilamellar vesicles using an inverted emulsion. *Langmuir* **19,** 2870-2879.

[22] Noireaux, V. & Libchaber, A. 2004. A vesicle bioreactor as a step toward an artificial cell assembly. *Proc. Natl. Acad. Sci. USA* **101,** 17669-17674.

[23] Yamada, A., Yamanaka, Y., Hamada, T., Hase, M., Yoshikawa, K. & Baigl, D. 2006. Spontaneous transfer of phospholipid-coated oil-in-oil and water-in-oil micro-droplets through an oil/water interface. *Langmuir* **22,** 9824-9828.

[24] Hamada, T., Miura, Y., Komatsu, Y., Kishimoto, Y., Vestergaard, M. & Takagi, M. 2008. Construction of asymmetric cell-sized lipid vesicles from lipid-coated water-in-oil microdroplets. *J. Phys. Chem. B* **112,** 14678–14681.

[25] Takiguchi, K., Yamada, A., Negishi, M., Tanaka-Takiguchi, Y. & Yoshikawa, K. 2008. Entrapping desired amounts of actin filaments and molecular motor proteins in giant liposomes. *Langmuir* **24,** 11323-11326.

[26] Pontani, L.-L., van der Gucht, J., Salbreux, G., Heuvingh, J., Joanny, J.-F. & Sykes, C. 2009. Reconstitution of an actin cortex inside a liposome. *Biophys. J.* **96,** 192-198.

[27] Janson, L. W., Kolega, J. & Taylor, D. L. 1991. Modulation of contraction by gelation/solation in a reconstituted motile model. *J. Cell Biol.* **114,** 1005-1015.

[28] Pollard, T. D., Blanchoin, L. & Mullins, R. D. 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29,** 545-576.

[29] Takiguchi, K. 1991. Heavy meromyosin induces sliding movements between antiparallel actin filaments. *J. Biochem.* **109,** 520-527.

[30] Tanaka-Takiguchi, Y., Kakei, T., Tanimura, A., Takagi, A., Honda, M., Hotani, H. & Takiguchi, K. 2004. The elongation and contraction of actin bundles are induced by double-headed myosins in a motor concentration-dependent manner. *J. Mol. Biol.* **341,** 467-476.

[31] Gibrat, G., Pastoriza-Gallego, M., Thiebot, B., Breton, M.-F., Auvray, L. & Pelta, J. 2008. Polyelectrolyte entry and transport through an asymmetric α-hemolysin channel. *J. Phys. Chem. B* **112**, 14687-14691.
[32] Langzhou, S. L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H. & Gouaux, J. E. 1996. Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859-1866.
[33] Suzuki, A., Yamazaki, M., Ito, T. 1989. Osmoelastic coupling in biological structures: formation of parallel bundles of actin filaments in a crystalline-like structure caused by osmotic stress. *Biochemistry* **28,** 6513-6518.