Cutting-Edge Studies Using Artificial Membranes

Morphological Control of Microtubule-Encapsulating Giant Vesicles by Changing Hydrostatic Pressure

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For the development of artificial cell-like machinery, liposomes encapsulating cytoskeletons have drawn much recent attention. However, there has been no report showing isothermally reversible morphological changes of liposomes containing cytoskeletons. We succeeded in reversibly changing the shape of cell-sized giant vesicles by controlling the polymerization/depolymerization state of cytoskeletal microtubules that were encapsulated in the vesicles using pressure changes. The result indicates that it is possible to manipulate artificial cell models composed of molecules such as lipids and proteins. The findings obtained in this study will be helpful in clarifying the details of cooperation between cytoskeletal dynamics and morphogenesis of biological membranes and in improving the design and construction of further advanced artificial cell-like machinery, such as drug-delivery systems. In addition, the experimental system used in this study can be applied to research to elucidate the adaptive strategy of living organisms to external stimuli and extreme conditions such as osmotic stress and high-pressure environments like the deep sea.

Key words artificial cell model; vesicle; giant liposome; microtubule; hydrostatic pressure

1. INTRODUCTION

Currently, as a part of robotics evolution, research on a methodology for creating advanced, complex macromolecular machinery through step-by-step expansion of function to develop the first generation of molecular robots is addressing the challenge of utilizing cell-sized giant liposomes encapsulating the reconstruction systems of biomacromolecules.1) Molecular robots are small robots with dimensions ranging from nanometers to micrometers made from molecules, e.g., nucleic acids like DNA origami, proteins, and/or other polymers. Molecular devices are the components that make up molecular robots. Molecular robots are expected to function as core technologies for advanced drug-delivery systems (DDS) in the field of pharmaceutical and medical engineering.

Cytoskeletons and molecular motors are biomacromolecules responsible for the movements and morphogenesis of living organisms. Therefore, their major components, i.e., actin and myosin or microtubule (MT) and kinesin/dynein, have attracted attention in a wide range of research fields from basic science studies to application studies in medicine and engineering as elements of molecular robots. Trials on creating highly efficient nanometer-sized molecular devices made from such macromolecules have been actively conducted. In particular, hybrid devices combining micro-fabricated semiconductor materials and biomacromolecules are progressing to the prototype stage.11) However, the molecular robots developed so far cannot move autonomously as cells do. Only a few examples of the construction of a molecular device that acts like a robot have been reported, and all of them operated under the on/off switching mode and were limited to a simple form that can perform a series of movements only once. Considering their applications, for example, in DDS, those molecular devices are still within the conventional category. In order to develop a molecular robot that can generate reversible shape changes and repeatable movements like living cells, it is insufficient to follow a top-down analytical approach alone, and therefore a bottom-up reconstructed approach based on demonstration and verification is essential.2,3)

2. RESULTS AND DISCUSSION

2.1. Bottom-Up Approach Using MTs-Encapsulating Cell-Sized Giant Liposomes

In our research, we employ tubulin as the biomacromolecule that is encapsulated and reconstructed in cell-sized giant liposomes.31) Tubulin forms MTs, representative cytoskeletal filaments, by polymerization. It is known that the dynamics of MTs, including the kinetics of
their polymerization/depolymerization reactions, are controllable by both native and artificial stimuli. An example of the former is MT-associating protein (MAP). A variety of MAPs are expressed in cells and involved in the regulation of MT function. Examples of the latter are physicochemical stimuli, such as solution salinity, temperature, and hydrostatic pressure, as mentioned below.

4) Toxins and drugs such as paclitaxel or nocodazole are also known to act as effective controls of MT dynamics.

When a lipid bilayer where phospholipids assemble in aqueous solution is closed spontaneously, it is called a vesicle or liposome. Liposomes have been used for many experimental studies as the most simplified model of biological membranes. They have attracted attention as useful materials in applications including the manufacture of cosmetics and pharmaceuticals. The liposomes used in our experimental studies have diameters ranging from a few to tens of microns and are called giant liposomes. Their size is comparable to that of living cells and has the advantage of allowing direct observation with various optical microscopes.

MT-encapsulating cell-sized giant liposomes were prepared and then observed under a microscope at the desired high pressure at 25°C. At ambient pressure (0.1 MPa), many liposomes formed tubular protrusions due to tubulin polymerization within them at 25°C. The majority maintained a bipolar shape with a central sphere and two tubular protrusions aligned in a straight line.

The high-pressure microscope developed by Nishiyama is optimized both for the best image formation and for stability under hydrostatic pressure up to 150 MPa. This exceeds the hydrostatic pressure of the deepest ocean site on the earth (the Mariana Trench, about 11000 m). Using the high-pressure microscope, the shortening of a single paclitaxel-stabilized MT in bulk solution resulting from depolymerization induced under high pressure was analyzed.

2.2. Reversibility of Liposome Deformation Induced by Changes in Pressure

We succeeded in repeatedly deforming cell-sized giant liposomes by repeating the polymerization reaction from tubulin to MTs and the reverse depolymerization reaction from MTs to tubulin inside the liposomes (Fig. 1). This is one of the critical steps in constructing a micrometer-sized robot from biomacromolecules and establishing a methodology for achieving robot locomotion.

At ambient pressure (0.1 MPa) and 25°C, the majority of MT-encapsulating cell-sized giant liposomes maintained a bipolar shape, as mentioned above. When high pressure (60 MPa) was applied, the protrusions shrank within several tens of seconds. This process was repeatedly inducible, and after the pressure was released, the protrusions regenerated within several minutes (Fig. 1a). It should be noted that, when the liposomes encapsulate no tubulin, or when tubulin encapsulated into the liposomes has not yet polymerized to MTs, these liposomes maintain the same spherical shape as when they were first prepared.

When the application and release of pressure were repeated over a long period, the shrinkage/elongation cycle of the reversible protrusions of the MT-encapsulating liposomes (concentrations of tubulin and guanosine 5’-triphosphate (GTP) of
the encapsulated solution were 68 and 200 µm, respectively) could be induced for approximately three repetitions (Fig. 1b). The consumption of GTP is thought to be the limiting factor on the number of times that liposome deformation can be repeated, because GTP is indispensable for the activity of tubulin and the polymerization and depolymerization process of MTs is closely coupled with GTP hydrolysis.\(^9,10\) To overcome this restriction, a system for maintaining the concentrations of guanine nucleotides such as a GTP regeneration system will be required.

Most of the effect of the application of high pressure consisted of shortening of the bipolar protrusions, followed by reelongation after pressure release. As in other cases, bending of the bipolar shape with increased pressure was observed (Fig. 1c). As a result of the bending, the bipolar-shaped liposomes became spherical. Upon the return to ambient pressure, the liposomes redeformed to a bipolar shape. The response to the pressure change in the case of bending deformation was faster than in the case of the protrusion shortening and reelongation. This might be due not only to shortening and reelongation of the encapsulated MTs but also to the breaking up of bundles of MTs as a result of depolymerization and rearrangement on a straight line as a result of MT annealing. This case, however, is rare, and the conditions that cause bending instead of shortening remain unclear. Various factors that can affect the results, e.g., the conditions of solution, lipid composition of the membrane, or effects of MAPs on the behaviors of MTs, should be investigated.

2.3. Rate of Shape Changes  The rate of shortening of the long axis of bipolar-shaped MT-encapsulating giant liposomes induced under high-pressure conditions is dependent on the strength of pressure applied (Fig. 2a). The logarithm of the shortening rate is linearly proportional to the pressure. This tendency is the same as the relationship previously obtained for single paclitaxel-stabilized MTs in bulk solution.\(^10\) However, the extrapolated shortening rate of the long axis of bipolar-shaped liposomes (0.009 µm/s at 0.1 MPa) was slow, at approximately several tenths of the directly observed shortening rates of an individual MT that was without any modification and thus showing the dynamic instability in bulk solution.\(^11-13\) This is thought to be due to the effect of many MTs being reconstituted inside the confined space of a micrometer-sized membrane vesicle.\(^31\

The pressure-induced changes in the morphology of the liposomes were also studied using time-lapse microscopy. The end-to-end distance between the tips of the membrane protrusions, i.e., the long-axis length of the bipolar shape, of the same MT-encapsulating giant liposome at specific intervals was measured (Fig. 2b). When the pressure was increased from 0.1 to 60 MPa at 0s, the protrusions started to shorten from both ends. The shortening rate at 60 MPa was 0.9 µm/s. After the release of pressure, the two protrusions reappeared and elongated. The reelongation velocity was 0.03 µm/s at ambient pressure. The deformation speeds of the MT-encapsulating liposomes, i.e., the elongation and shortening rates of the protrusions, rather than those of the individual MTs with or without paclitaxel treatment measured in bulk solution are on the same order as the rates of morphological changes or migrations shown by living cells. This is also thought to be due to the effect of many MTs being confined in a micrometer-sized membrane vesicle.\(^31\)

2.4. Tubulin and MTs at High Pressure  A single MT comprises 13 protofilaments, each of which consists of a head-to-tail arrangement of tubulins (tubulin is a heterodimer consisting of tubulin-α and tubulin-β, and its length is 8 nm). MT length is regulated by both polymerization and depolymerization reactions at the ends of the protofilaments. In a steady state, the length reaches a constant, at which the depolymerization rate is equal to the product of the polymerization rate and concentration of tubulin in solution. This relation is characterized by the critical concentration of tubulin in solution. It was reported that the critical concentration increases with pressure, meaning that the application of pressure works to weaken the intermolecular interactions between tubulin heterodimers. It is expected that similar results will be obtained in our current experimental system.

Next, we estimated the critical concentration of tubulin in MT-encapsulating giant liposomes at high pressure. It is assumed that the tubulins are free or polymerized in MTs (the length is the same as the long-axis length of the liposome), without any short segments. The applied pressure works to release tubulin from both ends of the MTs, but does not sever

![Fig. 2. Rate of Morphological Changes in Cell-Sized Liposomes](image-url)

(a) Semi-logarithmic plot of the shortening rate of the distance between the tips of the protrusions, i.e., the long-axis length of the bipolar shape. The points plotted correspond to the results obtained from individual liposomes (n=22). (b) Time course of changes in the end-to-end distance between tips of protrusions of the liposome. Data were obtained from the image shown in Fig. 1(a). The inset shows the results during the entire period. The period when the pressure was 60 MPa is indicated by a gray background; at other periods, the pressure was 0.1 MPa.
MTs or increase the number of MTs in giant liposomes. Under ambient pressure conditions, we set the critical concentration of tubulins at 5 µM. When the pressure was increased from 0.1 to 60 MPa, the long-axis length of the liposome rapidly decreased from 28 ± 12 µm, and then reached to 13 ± 6 µm (Figs. 1a, 2b). This length change corresponds to the addition of 34 ± 6 µM of free tubulins in the liposome. The critical concentration in this liposome (Figs. 1a, 2b) was calculated to be 39 ± 12 µm at 60 MPa. Therefore, the average value was 33 ± 12 µM at 40–60 MPa (n=7), consistent with the results in bulk solution.4)

The number of MTs in a giant liposome also could be estimated. A single MT consisting of 13 protofilaments contains 1.6×10^6 tubulins per 1 µm. The number of tubulins in the liposome (Fig. 1a) was calculated to be 1.4×10^6 in a volume of 36 µm^3, and the number of MTs was 31. In this experimental system, a single giant liposome with projections has 22±12 MTs (n=7). These results are consistent with those in previous reports.5)

### 2.5. Progress in Molecular Robots and Development of an Artificial Motile Cell Model

Many research groups have tried to induce folding of nucleic acids into various mesoscopic-sized structures in bulk solution using DNA origami or to regulate liposome morphology utilizing state changes in biopolymers and/or artificial macromolecules encapsulated in the liposomes.6) However, the former corresponded to the stage prior to putting the “engine” in the “machine,” and the latter was a one-time deformation, falling well short of the intended movement that can be achieved only by repeating deformations. So far, “reversibly repetitive” deformation has been possible only in principle, with no research actually demonstrating the desired results.

Therefore, the results obtained in our study were a very important step in the research process to establish a methodology for constructing a motile molecular robot. Cells can change their morphology and become motile by repeating shape deformation. By utilizing the outcomes of this study, a molecular robot that mimics living cells could be constructed. In addition to the biological question of what is life, creating a molecular robot capable of movement through multiple repeated deformations will open new frontiers in bioengineering technology. Our attempts to construct a molecular robot should be seen as pioneering efforts in this area.

We were able to prepare many liposomes encapsulating target proteins at high concentrations comparable to the intracellular environment using a new preparation method and then analyzed their deformation processes by direct observation. In addition, using a high-pressure microscope, we could manipulate the morphology of the liposomes by changing the pressure, which is a familiar physical parameter. The results of these methodologies will be important in developing our research and extending the scope of application.

In particular, the experimental system used in these studies has the advantage of allowing observation of samples in real time with an optical microscope while changing pressure and/or temperature. Therefore, it can be applied to elucidate the types of strategy adopted while living organisms adaptively evolved in response to external stimuli such as osmotic stress or extreme high-pressure environments like the deep sea.

### 2.6. Interface between Lipid Bilayer Membranes and Ends of MTs Undergoing Shortening or Elongation

Elongation/shortening of the cytoskeletal filament, such as MTs or actin filaments, occurs by polymerization/depolymerization of polymerization units at both ends of the filaments. The polymerization unit is a tubulin dimer in the case of MTs, and monomeric G-actin in the case of actin filaments. Therefore, assuming that cytoskeletal filaments, MTs, or actin filaments encapsulated in liposomes deform the membrane by direct pushing or being pushed from the inside due to elongation/shortening, an important question is how tubulin dimers or G-actins polymerize or depolymerize at the ends of filaments that are interacting with the inner surface of the membrane. In the current hypothesis, the gap formed between the ends of the filaments and the membrane surface resulting from thermal fluctuations, thermal bending of the filaments, and thermal vibration of the membrane, is thought to provide a site for polymerization/depolymerization. However, the actual mechanism is still unknown. In particular, since the ends of MTs under elongation/shortening are considered unique, complicated structures such as tapered or coiled tips based on cryo-electron microscopic observation of MTs in bulk solution, this is a serious concern in the case of MT-encapsulating liposomes.7)

In this study, using changes in hydrostatic pressure, repeated elongation/shortening of the membrane protrusions of bipolar-shaped MT-encapsulating giant liposomes was achieved successfully. A previous study reported that shortening of MTs under high pressure occurs as a result of depolymerization at both ends, and that severing in the middle of MTs only rarely occurs.8) These results indicate that tens or hundreds of MTs bundled within membrane protrusions of bipolar-shaped liposomes can be polymerized/depolymerized without hindrance even when the ends of MTs and the membrane are in contact. It was shown that sufficient membrane deformation directly caused by the elongation/shortening of MTs could occur even in living cells. On the other hand, it is important to investigate whether the ends of MTs that are undergoing polymerization/depolymerization within cells actually take the tapered or coiled structures described above.

Whether similar changes could occur in actin, which functions as a dynamic cytoskeleton for various cell functions, remains unclear. Utilizing the experimental system employed here, studies investigating whether reversible movements or specific morphological changes in cell-sized liposomes could be induced even in the case of actin are in progress.9,10)

### 2.7. Utilization of Giant Liposomes as Reactors and Carriers

When encapsulating genes, drugs, or other bioactive factors within carriers such as vesicles or liposomes and transporting them inside the bodies of living organisms, the following problems arise: 1) robustness of the carrier, which must not collapse easily and leak the encapsulated cargo in sites other than the target; 2) stability of cargo; and 3) targeting, i.e., how to equip the carrier with sensing and/or chemotaxis functions.

In relation to the first problem, not much attention has been paid to how membrane vesicles are affected by substances to be delivered once the carrier stably encapsulates them. As a special case, this study revealed the morphological changes that occur in giant vesicles due to assembly/disassembly of the internal contents. During the repeated deformation of the giant vesicles, the surface area of the membrane was constant while the internal volume changed, although the confinement...
of tubulin and MTs within the lipid bilayer was maintained, even though water moved across the membrane. This means that the current giant vesicle model holds a potential clue for solving the first problem.

Furthermore, this study also confirmed that the activity of tubulin and MT, i.e., the ability to polymerize/depolymerize, is maintained even after the encapsulation in giant vesicles. In relation to the second problem, we need to determine whether encapsulation in vesicles or liposomes causes alterations in the properties of substances encapsulated due to interaction with the lipid membrane, the confined conditions, or depletion effects. Our results indicate that encapsulated bioactive factors, which were proteins in this case, maintain their functions if the conditions are appropriate. As a result of these advantages, it was possible to calculate the critical concentration of tubulin and the number of MTs in liposomes, as discussed in Section 2.4, and thus the critical concentration at high pressure could be determined.

Repetitive deformation of MT-encapsulating giant vesicles caused by changing temperature or pressure demonstrated in this study indicates that it is possible to make vesicles and liposomes respond to external stimuli by using the properties of the encapsulated substance, although the stimulus employed here was not biochemical but physical. This will be helpful in solving the third problem as well as in functionalization of lipid membranes of vesicles/liposomes.

Following those approaches to the problems, a trial of enhancing the reaction of bioactive factors confined in a giant vesicle at high concentration under external stimuli, including light irradiation, is now in progress. By constructing an artificial motile cell model, it is believed that a cell-sized carrier capable of morphological change and/or locomotion can be developed in the future.

It is already possible to produce highly sensitive markers with low biotoxicity by encapsulating a dye or contrast agent in giant vesicles. Toyota et al. reported a high-sensitivity magnetic resonance imaging (MRI) contrast agent that densely encapsulates iron oxide nanoparticles in giant vesicles, and a vesicle aggregate-type marker that allows visual recognition of injection sites with both X-ray computed tomography and a near-IR fluorescent camera. Combining these with endoscopy and laparoscopy enables accurate laparoscopic surgery.

When a carrier consists of multilayered structure and/or integrated assembly containing multiple spaces that are compartmentalized, simultaneous parallelization or hierarchization of the reactions of encapsulated bioactive factors is possible. This feature enables linkage of the response to external stimuli with the secretion of the encapsulated substance. Utilizing multilayered liposomes, a system capable of repeatedly releasing the necessary amount of drug (e.g., insulin) only when necessary was developed. Collaboration among researchers attempting to confer sense and intelligence on carriers and allow carriers to deform or move, as focused on in this review, will evolve much smarter transporters for DDS. For that reason, we note that it is important for giant vesicle preparation methods to provide good encapsulation efficiency and methods for obtaining clusters of giant vesicles or giant vesicles composed of multilayered or integrated structures must be developed.

3. CONCLUSION

We succeeded in repeatedly changing the shape of cell-sized giant vesicles by iteratively manipulating the state of the protein polymer encapsulated within. The results demonstrated that the reversible, repetitive deformation necessary for freely moving micrometer-sized robots made of molecules could be achieved using familiar molecules such as proteins and phospholipids. The importance of cooperation between biology and engineering in studying biological materials is emphasized, and the effectiveness of a new method for preparing giant liposomes encapsulating biological materials was demonstrated.

4. EXPERIMENTAL

Tubulin was prepared from porcine brain by three cycles of polymerization and depolymerization, as described previously. Tubulin solutions were stored at −80°C until use, and GTP was added appropriately.

Cell-sized giant liposomes used in this study were prepared by transferring water-in-oil (W/O) emulsions formed in oil in which phospholipids were dissolved through the interface between the oil and aqueous phases using centrifugal force. The W/O emulsions were prepared from a solution containing the target materials, e.g., proteins (tubulin or MTs in this study) or DNA, to be encapsulated in liposomes. In this method, the reproducibility of the number and size of giant liposomes prepared and the uniformity and upper limit of concentration of target materials encapsulated are markedly greater than the efficiency of the gentle hydration method employed previously.

In this study, a tubulin solution containing 6.8 mg/mL (about 68 µM) of tubulin in buffer (80 mM piperoxane-1,4-bis(2-ethanesulfonic acid) (PIPES)–HCl, pH 6.8, 1 mM MgCl2, 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N′,N″,N‴-tetra acetic acid (EGTA), and 200 µM GTP) was used. Before encapsulation, the tubulin solution was kept on ice for more than 30 min to depolymerize the tubulin.

MT-encapsulating cell-sized giant liposomes were prepared from W/O emulsions of a tubulin solution as above and then observed under the desired high pressure with high-pressure microscope at 25°C. A high-pressure microscope was constructed to acquire high-resolution microscopic images regardless of the pressure applied. The details of the system were described previously. Briefly, the high-pressure chamber was connected to a separator, pressure gauge, and high-pressure pump. The separator conferred the advantage of reducing the total dead volume of the buffer solution in the pressure line. Hydrostatic pressure was applied to the pressure line using a hand pump. The inside of the Teflon cap was filled with buffer solution and connected to the chamber. The water pressure was transduced to the buffer solution by deformation of a thin Teflon cap in the separator, and the pressure was then transmitted to the chamber. We changed the internal pressure by several dozen MPa within a few second without any overshooting. Pressure was also released nearly instantaneously by opening a valve. The hydrostatic pressure in the pressure line was measured using a pressure gauge. These pressure devices were combined with an inverted microscope on a vibration-free table. All microscopic images were stored on a computer.
and then analyzed offline using Image J computer software (http://imagej.nih.gov/ij/).

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