Morphological and Topological Transformation of Membrane Vesicles

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Abstract. Liposomes are micro-compartments made of lipid bilayer membranes with characteristics quite similar to those of biological membranes. To form artificial cell-like structures, we generated liposomes that contained subunit proteins of cytoskeletons: tubulin or actin. Spherical liposomes were transformed into bipolar or cell-like shapes by mechanical forces generated by the polymerization of encapsulated subunits of microtubules. Disk- or dumbbell-shaped liposomes were developed by the polymerization of encapsulated actin. Dynamic processes of morphological transformations of liposomes were visualized by high intensity dark-field light microscopy.

Topological changes, such as fusion and division of membrane vesicles, play an essential role in cellular activities. To investigate the mechanism of these processes, we visualized in real time the liposomes undergoing topological transformation. A variety of novel topological transformations were found, including the opening-up of liposomes and the direct expulsion of inner vesicles.

Key words: actin, cytoskeleton, liposome, membrane vesicle, microtubule, optical microscopy, phospholipid, surfactant, topology

1. Introduction

Phospholipids assemble into a bilayer membrane spontaneously in aqueous solution, and necessarily form liposomes that are closed membrane vesicles. Liposomes have been well studied as simplified models of biological membranes, and are now used as such in a number of applications from pharmacology to bioengineering, for example as carriers of DNA vectors or anti-cancer drugs for internal delivery.

Cellular organelles, such as the nucleus, the Golgi apparatus and the endoplasmic reticulum, are all compartmentalized by lipid bilayer membranes and show characteristic shapes depending on their specific functions. In order to study the mechanism that determines vesicular shape, we investigated the transformation processes of liposomes. Electron microscopy is not suitable for visualizing the three-dimensional morphology of liposomes, because the liposomes often become

Figure 1. Transformation pathways of liposomes. The biconcave form transformed via one of the possible pathway as shown by the arrows.

deformed during the preparation of dried specimens. Thus, the dynamic characteristics of liposomes were visualized using dark-field light microscopy and their images were recorded using a highly sensitive SIT video camera.

Living cells themselves also have specific shapes depending on their physiological roles. That morphology is thought to be determined and maintained by cytoskeletal networks of microtubules or actin filaments. Therefore, we generated liposomes containing subunit proteins of those cytoskeletons to study their roles in the morphogenesis of the cells.

The morphologies of cellular organelles are quite changeable in response to physiological conditions. In particular, topological changes such as the fusion and the division of membranes play essential roles in cellular activities, for example, in endocytosis or in exocytosis that occurs during vesicle trafficking within cells. It has been thought that such topological transformations are made possible through interactions with proteins. However, direct observation of liposomes reveals that the lipid bilayer itself possesses the ability to undergo topological transformation.

2. Morphological Transformation of Liposomes

2.1. TRANSFORMATION PATHWAYS

Many years ago, we reported a systematic study on liposomes undergoing transformation (1). Liposomes were found to transform sequentially in a well-defined manner through one of several transformation pathways. A circular biconcave form was the initial shape in all pathways and it then transformed into a stable thin flexible filament or small spheres via a variety of regularly shaped vesicles which possessed geometrical symmetry (Figure 1). The driving force for those transformations was found to be osmotic pressure that decreases the liposome volume thereby altering the surface to volume ratio.

Figure 2. Schematic representation of liposome transformation caused by microtubule growth.

Based on the mechanical principle of the least bending energy of membranes, we investigated the stability and shape transformation of liposomes. We evaluated the bending energy within constraints of the constant surface area and volume. We also assumed that any shape transformations due to volume change would occur without altering the total surface area of the liposome [1]. The bending energy stored in the lipid bilayer was calculated numerically for each liposome shape formulated by the modified Cassini equation. By computer simulations, we showed that there are energetically stable liposome shapes that possess intrinsic geometrical symmetry [2]. We found that by reducing the volume, the stable shape of a liposome changed from a circular biconcave shape to elliptical, triangular, square or other polygonal shapes.

Based on the theory of mechanics, Pamplona and Calladine analyzed these transformations in more detail [3, 4]. They used a finite-deformation theory of axisymmetric, quasi-static thin shells to theoretically analyze bifurcations and changes of liposome shape under decreasing volume. The main structural response in a lipid bilayer is basically agreed to be its elastic resistance to bending of a two-dimensional liquid.

2.2. TRANSFORMATION CAUSED BY MICROTUBULE GROWTH

Cell morphology is thought to be determined and maintained by cytoskeletal networks of microtubules (MTs) and actin filaments (F-actins). To study the role of MTs on the morphogenesis of cells, we made model liposomes encapsulating tubulin [5, 6]. The encapsulated tubulin was polymerized into MTs by raising the temperature. By monitoring individual liposomes, it was found that the elongation of MTs by polymerization of tubulin caused shape changes of the liposomes, indicating that the self-assembly process itself can generate a mechanical force strong

Figure 3. Bipolar liposomes formed by microtubule growth viewed by dark-field microscopy. Bar = 10 μm .

Figure 4. Length-force relations obtained by computer simulation and corresponding liposome shapes according to the pressure difference $p = 0$ (a) or $p = -40$ (b).

enough to deform membranes. When tubulin polymerized into MTs, spherical liposomes were transformed into rugby-ball shapes due to the mechanical force generated by the MT polymerization. Tubular projections of the membranes then grew from both ends of the rugby-ball shaped liposomes, and the liposomes were eventually transformed into characteristic shapes consisting of a central ellipsoid and two straight tubes (Figure 2, 3). This morphology was designated as a bipolar shape.

2.3. THEORETICAL ANALYSIS OF TRANSFORMATIONS CAUSED BY MICROTUBULE ASSEMBLY

We investigated the mechanical aspects of the shape transformation of liposomes caused by MT growth. We calculated the liposome shape using a mathematical model based on the notion of the minimum bending energy of the liposome membrane [7]. The force generated by the MT assembly is incorporated in the model by considering the local force balance to the membrane. Numerical analysis yields a series of shapes that are similar to those observed experimentally (Figure 4). To develop the bipolar shape, the inside positive pressure was found to be indispensable in this theoretical model. The estimated pressure required to develop the bipolar shape can be easily generated from osmotic pressure due to an increase in the concentration of encapsulated tubulin because the transformation causes a decrease in the liposome volume. The force-transformation relationship thus obtained predicts the existence of a critical force required for the formation of tubular projections (Figure 4). Recently, this critical force has been measured experimentally by applying tension to polystyrene beads inside liposomes using laser tweezers (in preparation).

2.4. FORMATION OF CELL-LIKE SHAPED LIPOSOMES

We have developed a method to make multi-polar liposomes whose morphologies are similar to those of living cells. The key steps to obtain such multi-polar liposomes are as follows: (i) make giant-sized liposomes that are prepared slowly at low temperature, (ii) encapsulate large amounts of tubulin into the liposomes using high concentrations of tubulin in the preparation, (iii) assemble MTs in liposomes attached to the surface of a glass slide, and (iv) warm the liposome specimen to room temperature on the microscope stage. Many MTs grow in each liposome so that almost all liposomes that are attached to the glass surface began to transform. Unlike the case of bipolar liposomes, several membrane projections were formed on each of these liposomes [5].

The projections are different from each other in shape and direction of growth, and they are distributed randomly on each liposome. As a result, giant liposomes are transformed into a variety of multipolar liposomes similar to cultured living cells (Figure 5).

Why do free-floating liposomes transform into bipolar liposomes, and why do liposomes attached to a glass surface transform into multipolar liposomes? In the former case, elongating MTs inside the liposome can change their directions freely so that they can easily align along the long axis of the liposome. In the latter case however, MTs may interact with the flat surface of the liposome membrane attached to the glass surface. As a result, individual MTs cannot alter their direction so that each MT elongates in its own direction.

Figure 5. A multipolar liposome similar to living cells viewed by dark-field microscopy.

2.5. TRANSFORMATION CAUSED BY GROWTH OF F-ACTIN

To study the role of F-actin (another cytoskeletal filament) in the morphogenesis of cells, an experimental strategy similar to that for MTs was employed. Spherical liposomes that encapsulated G-actin (a subunit of F-actin) were prepared by swelling a dried lipid film in a solution of G-actin at 5 °C. Polymerization of the encapsulated G-actin into F-actin was achieved by raising the temperature, and subsequent shape changes were observed [8]. After actin polymerization, two shapes of liposome developed: dumbbell and disk shapes. Polarization microscopy showed that F-actins formed thick bundles in the liposomes and that these filaments were contiguous to the periphery of the liposomes. Localization of F-actin in the liposomes was confirmed by fluorescent labeling. Dumbbell- and disk-shaped liposomes were rigid and kept their shapes as long as the F-actins were stabilized, indicating that the actin bundles serve as a mechanical support for the liposome shapes.

The development of protrusions in living cells is indispensable to the process of cell motility. Membrane protrusions have been suggested to occur as a result of actin polymerization. In our work however, liposomes transformed only into dumbbell or disk shapes, and never formed protrusions.

It is well established that many kinds of actin binding proteins play regulatory roles in the organization of F-actin networks in living cells. To study such organization roles, we characterized the transformation of liposomes encapsulating G-actin with its crosslinking proteins, fascin, α -actinin or filamin [9]. As the temperature increased, the encapsulated G-actin polymerized into F-actins and formed bundles or gels, depending on the type of crosslinking protein that was

Figure 6. Liposomes caused by actin assembly with actin-crosslinking protein. Transformed liposomes with fascin (A), fascin in the presence of 5 mM MgCl_2 , (B), α -actinin (C) or filamin (D).

co-encapsulated, which caused various morphological changes of liposomes (Figure 6). The differences in morphology seen in these transformed liposomes indicate that the actin-crosslinking proteins actually determine liposome shape by organizing their specific actin networks. Morphological analysis reveals that the manner of crosslinking, i.e. the distance and the angular flexibility between adjacent crosslinked F-actins, is essential for morphogenesis.

3. Topological Transformation of Liposomes

3.1. DIRECT OBSERVATION OF THE MEMBRANE FUSION PROCESS

Membrane fusion plays an essential role in cellular activities such as exocytosis, vesicle transport among cell organelles and release of neurotransmitters at synapses. To investigate the mechanism of membrane fusion, we used liposomes as a model of biological vesicles, and monitored the fusion process in real time using optical dark-field microscopy [10]. To induce membrane fusion, we used the following peptides, i) HA (influenza hemagglutinin) peptide, a 20 residue peptide derived from the amino-terminal end of the HA2 subunit; ii) synthesized analogue peptides of HA, a negatively charged analogue (E5) or a positively charged analogue (K5); and iii) bee venom peptide: melittin.

We succeeded in visualizing the membrane fusion process induced by melittin or by a mixture of E5 and K5 at neutral pH. In addition to the fusion phenomenon, various behaviors of membrane vesicles, including aggregation, shrinkage and opening-up of liposomes, were observed depending on the buffer conditions. However, HA or E5 alone did not induce membrane fusion at acidic pH, which had been previously reported to be the optimal condition for membrane fusion. Such fusion

Figure 7. Fusion process of multi-lamellar liposomes caused by amphiphilic peptide.

occurred only in a narrow range of conditions involving the peptide concentrations, the ionic strength and the lipid composition of the membrane.

When unilamellar liposomes composed of phosphatidylcholine and phosphatidyl glycerol were mixed with E5 and K5 peptides, they transformed into multilamellar liposomes prior to the fusion. Their average diameters decreased by 50% and the light scattering intensity of each liposome increased remarkably. Only the multilamellar liposomes fused with each other (Figure 7), and the fusion was completed within several seconds, just as occurred with unilamellar liposomes. This result gives an insight into the mechanism of formation of multi-lamellar cell organelles such as lysosomes.

Opening-up of liposomes

Recently, we found that band 4.1 family proteins make extremely stable holes in liposomes. When talin, a member of the band 4.1 family, was added to a liposome solution, liposomes opened stable holes and were subsequently transformed into cup-shaped liposomes [11]. The holes became larger with increasing talin concentration, and finally, at sufficiently high concentrations of talin, the cup-shaped liposomes were transformed into a lipid bilayer sheet. These morphological changes could be reversed by protein dilution, i.e. the sheets could be transformed back into closed spherical liposomes (Figure 8). We demonstrated that talin is localized mainly along the membrane verges, presumably to avoid exposure of its hydrophobic portion at the edge of the lipid bilayer. This was the first demonstration that a lipid bilayer can stably maintain a free verge in aqueous solution, and refutes the established dogma that all lipid bilayer membranes inevitably form closed vesicles.

This opening-up topological change was analyzed by a statistical theory [12], and was found to be due to the adsorption of talin to the verges of cup-shaped liposomes. The adsorption isotherm of talin between an aqueous solution and a liposome membrane was analyzed by taking into account the bending energy of the membrane. The equilibrium was determined by the balance of the energy gain obtained by the adsorption of talin to the periphery of the liposome and the change of the bending energy of the membrane due to the shape change.

Figure 8. Opening-up of a liposome caused by an increase in talin concentration (A–H) and reverse transformation caused by dilution of talin (I–L). Bar = 5 μm .

Figure 9. Expulsion of inner liposomes caused by detergent. Bar = 5 μm .

It is well known that cellular organelles, including the nucleus, the Golgi apparatus, and the endoplasmic reticulum, convert into small membrane vesicles when cells enter metaphase during mitosis. Further, those fragmented vesicles reverse into native organelles after mitosis. Such fragmentation and reassembling processes could be regulated by unknown proteins possessing activities similar to those of talin.

3.2. CYCLIC SHRINKAGE OF LIPOSOMES AND DIRECT EXPULSION OF INNER VESICLES

Membrane fusion and lysis would seem to be opposite phenomena. Actually however, they are closely related behaviors, because the membranes must be lysed once in the vicinity of the fusion point to undergo the membrane fusion. Hence, we studied the effect of membrane-destabilizing reagents, such as surfactants, on liposome topology.

When Triton X-100, an electrically neutral detergent, was added to a liposome solution, the spherical liposomes became smaller in size while maintaining their spherical shape and membrane thickness. During this process, the shrinking liposomes exhibited two states alternatively, i.e. quaking and tense states. Liposomal quakes represent transitions from the tense state to the transient state and are characterized by vigorous fluctuations in their spherical shape. Liposomes quaked individually and intermittently, and decreased their size step by step [13].

Some liposomes could be observed to encapsulate a few smaller liposomes within them. When Triton X-100 was added, the outer liposomes shrank, and surprisingly, their encapsulated liposomes escaped when the outer liposomes vigorously fluctuated in shape [13]. Since the outer liposomes prepared by our method are unilamellar, this is conclusive evidence that Triton X-100 transiently generates holes in liposomes and thereby excludes inner vesicles directly.

Liposomes were able to discharge water from their interiors, as well as encapsulated vesicles, only during the intermittent quaking by perforation of their lipid bilayers. Liposomes vigorously fluctuate their membranes within each quaking state due to membrane perforation and also due to the sudden decrease in internal pressure which results from the discharge of the water within. Recently, these processes have been investigated using 2-D Fourier analysis of video images.

4. Conclusions

Direct observation of liposomes by optical microscopy shows that membrane vesicles possess the ability to transform into a variety of cell organelle-like shapes in response to alterations in the surface-to-volume ratio caused by osmotic pressure. The characteristic morphologies of liposomes are also determined by the growth of encapsulated cytoskeletal filaments such as MTs and F-actins, indicating that the polymerization of proteins can generate sufficient force to deform liposome membranes. Theoretical studies can interpret quite well the mechanism of liposome transformation that are caused by osmotic pressure and/or by the growth of cytoskeletons. The mechanism of topological changes of vesicles, such as fusion, division and poration, have been investigated by observing liposome behavior in the presence of surfactants or amphipathic peptides. Of particular interest is talin, an F-actin binding protein, which opens stable holes in liposomes by binding the exposed edge of the lipid bilayer. This reversible opening may be related to cell

organelle membrane metabolism, and may be used as a technique to encapsulate huge structures into liposomes.

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