Controllable Movement of the Azobenzene Linked Crown Ether Conjugated Liposome

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Abstract: Drug delivery systems have been developed to reduce the side toxicity of drugs by localizing them in the site of action. But it depends on the circulation of the blood and it doesn't have the function of locomotive mechanism of itself for searching for the region of disease. However, this problem could be solved by nanobot which have the locomotive function. So, we mimic the movement of cell that can move in a human body. In this paper, to polymerize the encapsulated actin within the liposome, electroporation technique is employed. In order to optimize polymerization and depolymerization of the liposome, we compare the time of polymerization and depolymerization by concentration of crown ether. we synthesis the liposome which contain azobenzene Linked crown Ether conjugated Actin protein. Azobenze linked crown ether holds the K+ ion by exposure of UV light and this disturbs the actin polymerization. In result, UV light could control the liposome growth. Finally, we could develop the liposome robot and control the growth and degeneration of the liposome by external stimuli such as UV light. The merit of the controlling by UV light doesn't need to inject proteins which induce polymerization and depolymerization of actin protein.

Keywords: Giant Liposome, Actin, Electroporation, Crown ether, Azobenzene

1. INTRODUCTION

Liposomes encapsulating actin or actin along with actin regulatory proteins have been widely applied as a simplified model of eukaryotic cells. This biomimetic system has been used to study complex cellular processes such as cellular morphogenesis, locomotion, and surface protrusioni [1-8]. Giant liposomes were first used as a simplified biophysical model to study the morphogenesis of the cell membrane. In particular, Cortese et al [2]. encapsulated actin filaments inside the aqueous core of liposomes and demonstrated that the initially spherical liposomes can be forced into asymmetric, irregular shapes. They also observed that the magnitude of liposome deformation was dependent on the length of encapsulated actin filaments. Miyata and Hotani[3] expanded on this previous study and observed that, by polymerizing actin filaments inside liposomes, actin-containing liposomes could be transformed from an originally spherical geometry into a dumbbell or disklike geometry.

To polymerize the encapsulated actin within the liposome, two techniques are commonly employed. The first method induces G-actin polymerization into filamentous actin (F-actin) by increasing the temperature of G-actin liposome dispersions [3]. The initially spherically shaped liposomes deformed into both dumbbell or disk geometries. The second method creates transient pores in the liposome membrane by electroporation [7]. This permits KCl in the bulk solution to diffuse into the aqueous core of the liposome through the transient pores, which polymerizes the G-actin. In this case thin protrusive invaginations consisting of actin filaments encased in a lipid bilayer extended from the surface of the initially spherical liposome surface.

In this paper, to polymerize and depolymerize the encapsulated actin within the liposome, Crown ether is linked wiht liposome and electroporation technique is employed to introduce KCL. Electroporation creates transient pores in the liposome membrane. This permits KCl in the bulk solution to diffuse into the aqueous core of the liposome through the transient pores, which polymerizes the G-actin. In this case, thin protrusive invaginations consisting of actin filaments encased in a lipid bilayer extended from the surface of the initially spherical liposome surface. The polymerization forces cause significant deformations of the liposome. Morphological changes of liposome caused by actin assembly without crosslinking protein. With this method, we could initiate at a desired instance actin polymerization in a selected liposome and we could continuously observe liposome shape change.

Crown ethers contain intramolecular cavities, capable to bind K+ ion. By introducing an azo group the function of crown ethers can be controlled by an on-off light switch. the inner cavity of the crown ether opens and it now has a greater affinity for K^+. Simply turning a light source on or off can lead to the selective removal of ions from a solution [9].

In order to opimize polymerization and depolymerization of the liposome, we compare the time of polymerization and depolymerization by concentration of crown ether. We first presented that crown ether can be used to depolymerize the actin within liposomes encapsulating the cytoskeletal polymer actin. we synthesis the liposome which contain azobenzene Linked crown Ether conjugated Actin protein. This liposome could be controlled by exposure of UV light. The liposome was growth by actin polymerization and degeneration by actin depolymerization. Azobenze linked crown ether holds the K+ ion by exposure of UV light and this disturbs the actin polymerization. In result, UV light could control the liposome growth.

Previous studies have only been presented the polymerization of liposome. In contrast, we realized not only the polymerization but also depolymerization of liposme. Thus, our study can provide the most similar model system with biological cells to study cytoskeletal organization or understand the mechanism of cell locomotion.

2. MATERIALS AND METHODS

2.1 Materials

Purified actin from rabbit skeletal muscle was obtained from Fluka. Dimyristoylphosphatidylcholine and cardiolipin (from bovine heart, Na salt) were purchased from Avanti Polar-Lipids. All other chemicals were of analytical grade. A sample of internal solution (G buffer composition: 2 mM tris(hydroxymethyl)aminomethane (pH 8.0)/0.2 mM $CaCl_2$/0.2

mM ATP/0.2 mM NaN_3/5 mM 2-mercaptoethano/0.4mg/ml BSA, 100mM sucrose) containing of G-actin was then added to the dried lipid films at 4°C. the liposome suspensions were diluted 100-fold with external solution (F buffer composition: 2 mM tris(hydroxymethyl)aminomethane (pH 8.0)/0.2 mM $CaCl_2$/1 mM ATP/0.2 mM NaN_3/0.2mg/ml BSA/50mM 5 mM /2-mercaptoethanol/glucose/30mM KCl) to prevent actin from polymerizing outside liposomes, and actin polymerization inside liposomes was the initiated by electroporation.

2.2 Preparation of Actin-Containing Liposomes

Actin-containing liposomes were prepared by swelling at 4°C of the dried lipid film (prepared from the mixture of 150 µg dimyristoyl phosphatidylcholine and 150 µg cardiolipin) in 50 µl internal solution containing actin (110 µM). After the swelling, 1 µl of the concentrated liposome solution was mixed with 100 µl external solution in an observation chamber that fabricated by Microelectro mechanical systems (MEMS) process (100 µm thick) stuck onto a 18 × 18 mm cover glass which had an adequate hole in the regular square without adhesive. The concentration of actin outside the liposomes was 1/100 of the initial concentration, and the polymerization of actin outside the liposomes should be negligible.

2.3 Synthesis Method of Azobenzene Linked Crown Ether

Concentrated sulfuric acid 5.52g (56.3mmol) was added in 4.47g (70.9mmol) in a 250-ml round-bottomed flask in portions with shaking. The mixture kept cool during the addition by immersing the flask in cold water. N, N`-Dibenzyl-1, 4, 10, 13-tetraoxa-7, 16-diazacyclooctadecan 0.8g (1.8mmol) was added into the mixture in portions with shaking the flask well. When all starting material has been added and heated in a sand bath maintained at 60℃ for 40-45 minutes. The contents of the flask was poured into about 500ml cold water in a beaker, stired the mixture well. And the mixture was transferred to the separatory funnel, 300ml methylene chloride was poured to the mixture and shaked it vigorously. The solvent was removed under reduced pressure. The crude product was chromatographed on a silica gel using methylene chloride and methanol [10]. Water (3.33ml) was added portionwise to a vigorously stirred mixture of dinitro derivative (0.4g, 0.75mmol), stannous chloride dehydrate (0.73g, 3.25mmol) , potassium hydroxide(1.3g, 23mmol) and acetone(4.5ml). Boiling began within few minutes. Boiling continued for 3 hours. Toluene (30ml) was added to the cooled reaction mixture and organic red colored layer was separated. The organic layer was washed with water and the solvent was removed under reduced pressure. The crude product was chromatographed on a silica gel using methylene chloride and methanol [11].

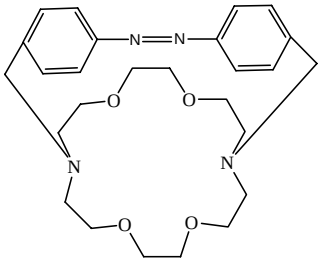


Fig. 1 molecular structure of Azobenzene Linked Crown Ethere. Crown ethers contain intramolecular cavities, capable to bind K+ ion. By introducing an azo group the function of crown ethers can be controlled by an on-off light switch.

2.4 Preparation of Crown Ether or Azobenzene Linked Crown Ether Linked Liposome

Crown ether or Azobenzene linked Crown ether dissolved in a chloroform/methanol solution, 98:2 (vol/vol) as added to the mixture of 150 µg dimyristoylphosphatidyl choline (PC) and 150 µg cardiolipin (CL). The organic solvent was evaporated under a flow of nitrogen gas. The resultant dried crown ether/PC/LC film was rehydrated in 50 µl internal solution of 110 mM G-actin dissolved in G-buffer for liposomes containing actin at 4°C. The lipid films immediately started swelling to form liposomes, and swelling was facilitated by agitating the test tubes occasionally by hand. After 30 minutes, 10 µM liposome suspensions were used immediately after their preparation. in an observation chamber that fabricated by Microelectromechanical systems (MEMS) process stuck onto a 18 × 18 mm cover glass which had an adequate hole in the regular square without adhesive.

2.5 Design and microfabrication of electroporation microchips

The electroporation (EP) microchip was fabricated using MEMS microfabrication techniques, which has the advantages of the use of a very low applied voltage, a simple power supply and a much simpler liposome preparation process. The EP microchips were designed in two components: the well-defined cavity for liposome accommodation and testing; and the electrodes for providing electroporation electric power. The cavity region was designed to match with the electrode position and provide a precise liposome elongation area, $10\times10mm^2$. Fig. 2b shows schematic drawings of the EP microchip with three-dimensional (3D) and top views [12].

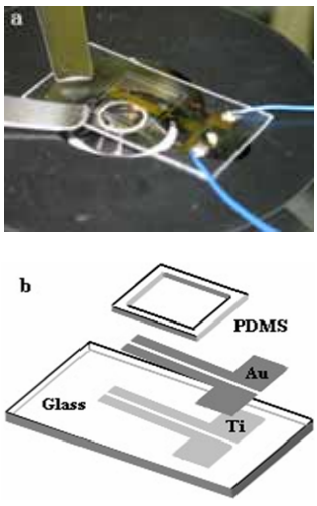


Fig. 2 (a) Photo image, (b) expanded view

2.6 Electroporation of Liposomes

Generally, the liposome membrane does not show permeation for metal cation like K ion, but the application of electric fields results in membrane permeation for K ion. The center-to-center distance of the electrodes was 75 µm. In each experiment a liposome having a diameter of 9-15 µm was selected by phase contrast microscopy and was placed in the middle of the electrode pair. A synthesized functional generator (Model 9302, Protek, Korea), was used as a signal

source to produce 10kHz sine wave alternating electric fields. Typically the voltage used was up to 10 V peak to peak. The duration of electric field application was controlled by a laboratory-built computer-controlled timer system, and was usually 250 ms. The voltage was applied between two electrodes 75 μm apart. The sample chamber contained 10 μl of sample solution. The temperature of the sample before application of the voltage was controlled to be 4 °C. and then was applied to the selected liposomes. The liposome was then immediately loaded between two electrodes in chamber. KCl entered the liposomes within 1 sec after the application of the electric pulse.

2.7 Observation of Liposomes

Observation of the actin-containing liposomes was carried out on an inverted microscope (IX81, Olympus, Japan) equipped with a 400×. A color video camera (XC003, Sony, Japan) was used to acquire phase contrast images. For presentation of the process of liposome deformation, appropriate images were intermittently captured. When the protrusion growth was analyzed, the images were captured every 30s after the application of the electric pulse. To quantify the development of protrusions, distance was measured between the tip of the protrusion and a reference point arbitrarily fixed in the liposome, as indicated with the symbol * in Fig. 4. From the video sequence, we judged the appearance of protrusions by eye, and the time of the start of the elongation was determined accordingly. Change in the distance between the reference point and the tip was plotted and was presented as a time course of the protrusive elongation.

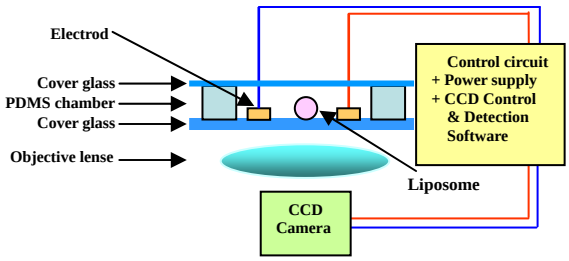


Fig. 3 Schematic setup of the detection system. A liposome was placed in the middle of a pair of gold electrodes.

3. RESULTS

3.1 Morphological changes of liposomes caused by actin polymerization

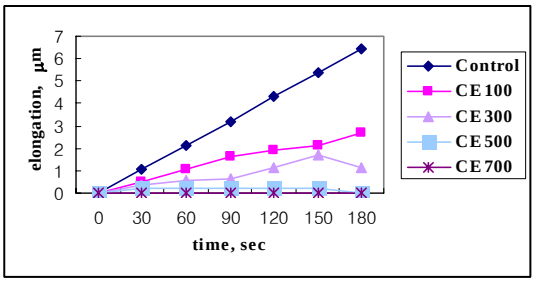
We observed the shape change of the liposomes containing actin. The applied voltage was 10 Vp-p (sine wave, 10 kHz) with a pulse duration of 250 ms for one pulse. When the encapsulated actin polymerized into F-actin, liposomes transformed into disk shapes or flat spoon shapes [13]. The F-actin which had polymerized in liposomes spontaneously aligned along the periphery of transformed liposomes to form bundles [13]. These liposomes are fairly rigid, as judged by the low fluctuation in their shapes. Their membranes were quiescent, and bending motions were restrained. Monomeric actin self-assembles into actin filaments, which in turn could further self-assemble into actin bundles. Because the size of the liposome is much smaller than the persistence length of the actin filaments, the liposome membrane restricts actin filament elongation. Due to the higher density of actin filaments in larger liposomes, the interplay between actin filament elongation and liposome confinement will stretch and elongate the liposomes and force actin filaments to bend and accumulate into bundles against the inner liposome membrane [14]. The protrusions possessed a variety of sizes and shapes. Some liposomes developed several protrusions in various directions [7]. Correlation between the direction of the protrusive growth and that of the electric field was not clear, because rotation of liposomes could have occurred immediately after the pulse application. Despite their large axial ratio, the protrusions were considerably rigid: fluctuation of the shape, such as bending, was small compared with that of long sausage-like liposomes having similar dimensions. These rigid characteristics were quite similar to that of the dumbbell-shaped liposomes containing polymerized actin [3].

Individual protrusions grew at relatively uniform rate of an average of 2.14 μm/min. The shape change of the liposomes as shown in Fig. 4. Morphological changes of liposomes caused by actin. Polymerized actin bundled and then aligned along their periphery.

Fig. 4 Phase-contrast micrographs of the liposomes containing actin observed after the electroporation. Transformed liposomes obtained by the polymerization of encapsulated G-actin into F-actin. Number in each frame represents the time in sec elapsed after the pulse application. The symbol * indicates the reference point used to determine the rate of protrusive formation. The scale bars represent 10 μm.

3.2 Morphological changes of liposomes caused by actin depolymerization with crown ether

We investigated the effect of crown ether on morphological changes of actin-containing liposomes. We tested various crown ether contents (0, 100, 300, 500 and 700 μg) to produce actin-containing giant liposomes. Among them, 500 μg crown ether was found to be the most suitable for preparing morphological changes of giant actin-containing liposomes caused by actin depolymerization with crown ether and polymerization without crown ether (Fig. 5). At 0 or 100 μg crown ether linked liposome, the actin-containing liposomes transformed into a protrusive shape, whereas, At 500 or 700 μg crown ether linked liposome, the actin-containing liposomes retained a spherical shape. At 300 μg crown ether linked liposome, the actin-containing liposomes transformed into a protrusive shape. But, As time goes by, caused by actin depolymerization with crown ether (fig. 7). The frequency of transformed liposomes and lengths of projections decreased with increasing concentration of crown ether (fig 5).



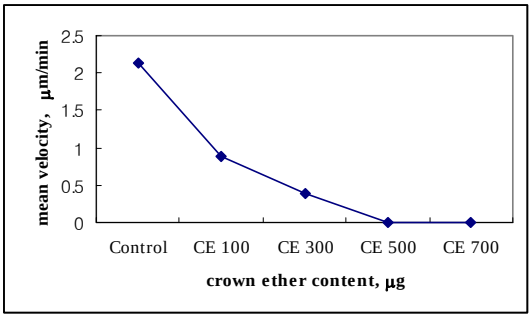


Fig. 5 (*Upper*) Time course of the protrusion elongation. Morphological changes of liposomes caused by actin depolymerization with crown ether (CE). tested various crown ether contents (0, 100, 300, 500 and 700 μg). (*Lower*) Mean velocity (μg /min) of liposome elongation.

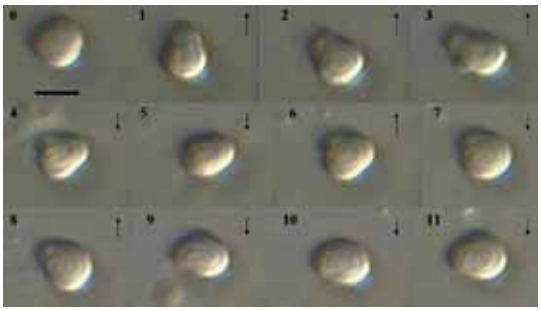


Fig. 6 Morphological changes of liposomes caused by actin depolymerization with crown ether 300 μg. Number in each frame represents the time in minute elapsed after the pulse application. the upper direction arrow express elongation of liposome. the lower direction arrow express contraction of liposome. The scale bars represent 10 μm.

3.2 Morphological changes of liposomes caused by actin depolymerization with azobenzene linked crown ether on UV

The horseshoe-shaped liposome is shown in Figure 7. the Morphological changes of liposomes caused by actin polymerization and depolymerization with azobenzene linked crown ether (500 μg) on the switch on/off time. Each frame represents captured image every thirty seconds. Liposome size increases with the increase in UV off time. But liposome size decreases with the increase in UV on time. The lower part shows the morphological changes of liposomes caused by actin polymerization without ACE. Liposome size increases with the increase in OFF time. There was almost no effect on UV. This shape transition at higher G-actin concentrations could be due to the self-assembly of G-actin inside the liposomes into actin filaments.

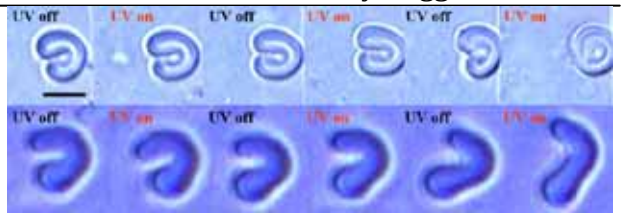


Fig. 7 Each frame represents captured image every thirty seconds. The duration time of UV on/off kept for 30 seconds. The upper part shows the morphological changes of liposomes caused by actin polymerization and depolymerization with azobenzene linked crown ether (ACE) 500 μg. The lower part shows the morphological changes of liposomes caused by actin polymerization without ACE. There was almost no effect on UV. The scale bars represent 10 μm.

4. CONCLUSIONS

Many groups have studied the morphological changes of giant liposomes (5-20 mm in diameter) during the internal polymerization of G-actin into actin filaments. Hotani observed the transformation of originally spherical actin-containing liposomes into either dumbbell or disklike shapes. Hackl et al. and Boulbitch et al. observed the presence of a thin actin shell or cortex contiguous to the periphery of the inner liposome bilayer [5, 15]. Our study has shown that the morphological changes of actin-containing liposomes is dependent on the concentration of crown ether and the azobenzene linked crown ether. The observed liposome shapes were a result of a balance between the lipid membrane tension and the elastic stress of the actin bundles or filaments. With the confinement afforded by the liposome closed structure, which is much smaller than the persistence length of actin filaments, actin filaments could either bend and nestle beneath the inner leaflet of the liposome or stretch and deform the liposome, which is dependent on the interplay between the actin filament stiffness and the differing extents of confinement [14]. Dumbbell- and horseshoe-shaped actin assemblies were observed at different crown ether and azobenezene linked crown ether concentration. Previous studies have only been presented the polymerization of liposome. In contrast, we realized not only the polymerization but also depolymerization of liposme. Thus, our study can provide the most similar model system with biological cells to study cytoskeletal organization or understand the mechanism of cell locomotion. In addition, the use of these actin-containing liposomes as drug-delivery vehicles could increase the mechanical stability of these novel carriers versus plain liposomes, thereby increasing their circulatory half-life in the systemic circulation [14].

REFERENCES

[1] M. Barmann, J. Kas, H. Kurzmeier and E. Sackmann, *The structure and conformation of amphiphilic membranes*, Springer-Verlag, Berlin, pp. 137-143, 1992.

[2] J.D. Cortese, B. Schwab, C. Frieden and E.L. Elson, "Actin polymerization induces a shape change in actin-containing vesicles," *Proc. Natl. Acad. Sci. U.S.A,.*, Vol. *86*, pp. 5773-5777, 1989.

[3] H. Miyata and H. Hotani, "Morphological changes in liposomes caused by polymerization of encapsulated actin and spontaneous formation of actin bundles," *Proc. Natl. Acad. Sci. U.S.A.*, Vol. *89*, pp. 11547-11551, 1992.

[4] M. Honda, K. Takiguchi, S. Ishikawa and H. Hotani, "Morphogenesis of liposomes encapsulating actin depends on the type of actin-crosslinking," *J. Mol. Biol.*, Vol. *287*, pp. 293-300, 1999.

[5] A. Boulbitch, R. Simson, D. A. Simson, R. Merkel, W. Hackl, M. Barmann and E. Sackmann, "Shape instability of a biomembrane driven by a local softening of the underlying actin cortex ," *Phys. Rev. E*, Vol. *62*, pp. 3974-3985, 2000.

[6] H. Miyata and K. Kinosita, "Transformation of actin-encapsulating liposomes induced by cytochalasin D," *Biophys. J.*, Vol. *67*, pp. 922-928, 1994.

[7] H. Miyata, S. Nishiyama, K. Akashi, and K. J. Kinosita, "Protrusive growth from giant liposomes driven by actin polymerization," *Proc Natl Acad Sci U S A*, Vol. 96, No. 5, pp. 2048-53, 1999.

[8] H. Hotani, F. Nomura and Y. Suzuki, "Giant liposomes: from membrane dynamics to cell morphogenesis," *Curr. Opin. Colloid Interface Sci.*, Vol. *4*, pp. 358-368, 1999.

[9] S. Shinkai, H. Kinda, M. Ishihara and 0. Manabe, "Photoresponsive Crown Ethers," *Journal of Polymer Science.*, Vol. 21, pp. 3525-3539, 1982.

[10] B. S. Furniss, A. J. Hannasford, W. G. Smith and A. R. Tatchell, *Vogel's Textbook of Practical Organic Chemistry Fifth edition*, Woolwich Polytechnic, London, p. 854-855, 1992.

[11] E. Luboch, J. F. Biernat, Y. A. Simonov and A.A. Dvorkin, "Synthesis and electrode properties of 16-membered azo- and azoxycrown ethers. Structure of tribenzo-16-azocrown-6," *Tetrahedron*, Vol. 54 , pp. 4977-4990, 1998.

[12] Y. C. Lin and M. Y. Huang, "Electropporation microchips for in vitro gene transfection," *J. Micromech. Microeng.* Vol. 11, pp. 542-547, 2001.

[13] M. Honda, K. Takiguchi, S. Ishikawa and H. Hotani, "Morphogenesis of liposomes encapsulating actin depends on the type of actin-crosslinking," *J Mol Biol.* Vol. 287, No. 2, pp.293-300, 1999.

[14] Shuliang Li and Andre F. Palmer, "Structure of small actin-containing Liposomes Probed by atomic force microscopy," *Lanmuir*, Vol. 20, pp. 7917-7925, 2004.

[15] W. Hackl, M. Barman and E. Sackmann, "Shape Changes of Self-Assembled Actin Bilayer Composite Membranes," *Phys. Rev. Lett.*, Vol. 80, pp. 1786-1789. 1998