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Rapid report

A new method for the preparation of giant liposomes in high salt concentrations and growth of protein microcrystals in them

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Abstract

We have developed a new method for the preparation of giant liposomes in aqueous solution containing high salt concentrations (up to 2.0 M). Hydrophilic polymers attached to the surface of lipid membranes by including a small amount of poly(ethylene glycol)-grafted phospholipid in the membrane increase the repulsive force between the membranes, which makes it possible to form giant liposomes at high ionic strength. Using this method, we could grow micron-sized (10–50 μm) protein crystals in a giant liposome. These results demonstrate that this method is a promising tool for the preparation of 'artificial cells' under various conditions.

Keywords: Giant liposome; Artificial cell; Microcrystal of protein; Intermembrane repulsive force; Poly(ethylene glycol)

Giant liposomes of phospholipids such as phosphatidylcholine (PC), whose diameters are more than 10 μm, can be formed in water in the absence of salts or at low ionic strength. They have been used for physical and biological investigations such as shape change of liposomes [1–4], elastic properties of lipid membranes [5], interaction of cytoskeleton proteins with membranes [6,7], and dynamic structures of biomembranes [8,9]. However, in an aqueous solution containing high concentrations of salts such as NaCl, it was difficult to make giant liposomes of PC, which hindered their more extensive use. Here we report a new method for the preparation of giant liposomes in aqueous solution containing high salt concentrations (up to 2.0 M). In this method, hydrophilic polymers are attached to the surface of lipid membranes by including a small amount of poly(ethylene glycol) (PEG)-grafted phospholipid such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly-(ethylene glycol) 2000] (PEG2K-DOPE) (Fig. 1a) in the PC membrane. Since this new method enables us to prepare giant liposomes in various kinds of aqueous solution, it may be a promising tool to make an 'artificial cell' composed of many kinds of proteins and DNA, and also for a chemical reaction vessel with a small-confined volume. To demonstrate this possibility, we have successfully tried to make a protein crystal in a giant liposome prepared by this method. Part of this research was presented at the 36th Annual Meeting of the Biophysical Society of Japan [10].

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and PEG2K-DOPE were purchased from Avanti Po-

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lar Lipids (Alabaster, AL, USA). 2-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Molecular Probes (Eugene, OR, USA). Hen egg white lysozyme was purchased from Seikagaku Kogyo (Tokyo, Japan).

PEG-lipid/DOPC giant liposomes were prepared as follows. 100 μl of 1 mM phospholipids (a mixture of DOPC and PEG2K-DOPE) in chloroform in a small glass vessel were dried by a stream of nitrogen gas at room temperature. Then, the residual solvent was completely removed by a rotary vacuum pump for more than 10 h. A small amount of water (2 μl) was added to this vessel, and incubated at 45°C for a few minutes (prehydration). To prepare a giant liposome without proteins, 200 μl of 0.1 M sucrose aqueous solution containing various concentrations of NaCl were added to the prehydrated lipid film in the vessel, and incubated at 37°C or other temperatures for 0.5–3 h. When we observed the giant liposome with a phase-contrast microscope, 20 μl giant liposome solution (0.1 M sucrose solution containing several NaCl concentrations; internal solution) were diluted into 300 μl of 0.1 M glucose aqueous solution whose NaCl concentration was equal to that in the internal solution (external solution) in order to increase the contrast of the membrane. To prepare a giant liposome in protein solution, immediately after a lysozyme solution and a NaCl solution in 0.1 M sodium acetate buffer (pH 4.6) were mixed (final concentration: 7.0–8.0% (w/v) lysozyme and 3.5–5.0% (w/v) (i.e. 0.60–0.86 M) NaCl), the mixture was transferred to the prehydrated lipid film in the vessel, and incubated at 37°C for 1–3 h. Then, this solution was diluted 20 times by the same buffer containing no proteins, and then incubated at 15–25°C in the incubator. To observe the protein crystals in the giant liposome, a Hoffman modulation-contrast

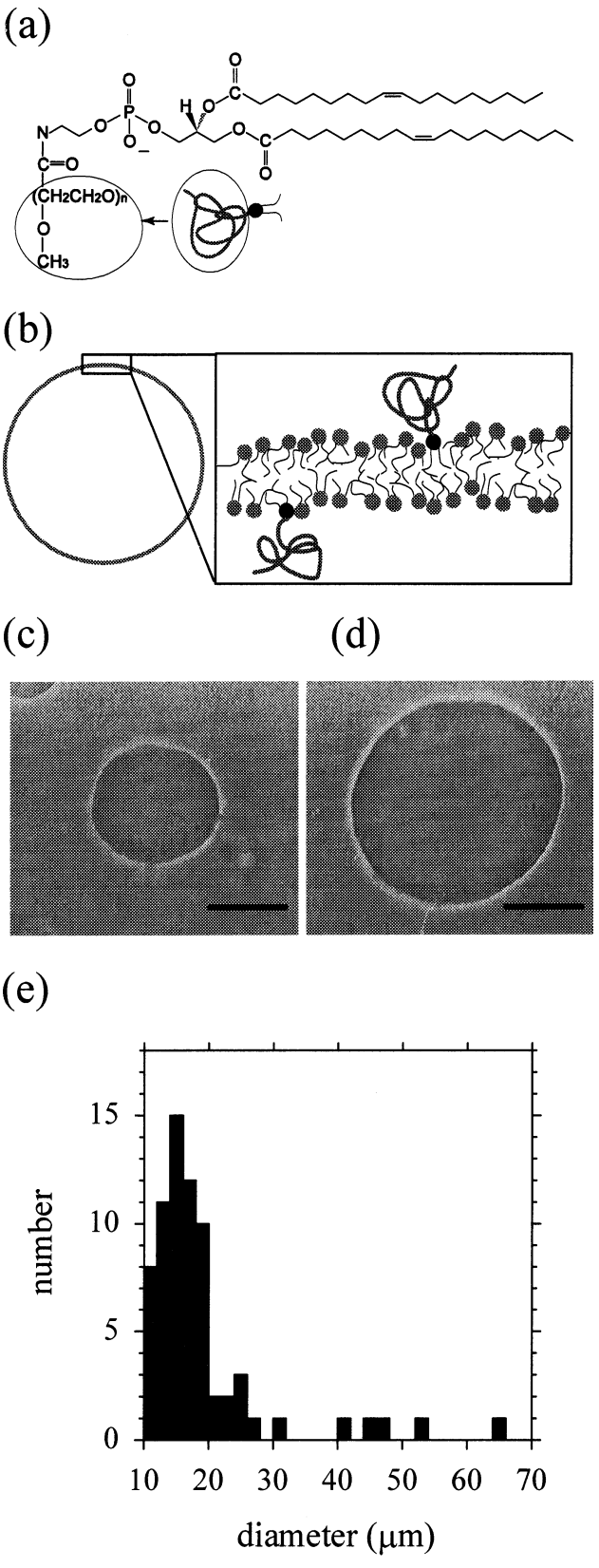

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Fig. 1. (a) Chemical structure of PEG2K-DOPE (PEG-lipid). (b) Schematic model of PEG-lipid/DOPC giant liposome. (c,d) Phase-contrast images of a 0.2% PEG-lipid/DOPC giant liposome formed in 150 mM NaCl in 0.1 M sucrose aqueous solution (c) and a 2.0% PEG-lipid/DOPC giant liposome formed in 500 mM NaCl in 0.1 M sucrose aqueous solution (d). The scale bar is 20 μm. (e) Size distribution of a population of 0.2% PEG-lipid/DOPC giant liposomes prepared in 150 mM NaCl.

microscope was used instead of a phase-contrast microscope, because the former is suitable to observe thick samples. For the observation with the Hoffman microscope, we used no sucrose and glucose in the solution.

The diluted solutions containing the giant liposomes were then transferred to a handmade microchamber (1 cm × 1 cm wide and 3 mm high, internal volume ~0.3 ml) on a slide glass using a silicone-rubber spacer and a coverslip. We observed giant liposomes under an inverted fluorescence microscope (IX-70, Olympus, Tokyo, Japan) with phase-contrast optics or with Hoffman modulation-contrast optics. The phase-contrast images and Hoffman modulation-contrast images of the giant liposomes were recorded through a CCD camera (DXC-108, Sony, Tokyo, Japan) on a video recorder. We measured the isothermal area compressibility modulus, and manipulated the movement of the giant liposome containing the lysozyme crystal by the micropipet aspiration technique [11]. A glass micropipet (10 μm inner diameter) was pulled from 1.0 mm glass tubing (G-1, Narishige, Tokyo, Japan) to a needle point by a puller (PP-83, Narishige), and then broken by quick fracture to the desired tip diameter. The micropipet filled with the external solution of the giant liposome was held by a micromanipulator (MMW-23, Narishige), enabling to control the position of the tip of the micropipet. We controlled the aspiration pressure by changing the height of the vertical column of water in the U-shaped glass tube to which the micropipet was hydraulically connected.

We could produce many giant liposomes of DOPC in aqueous solution containing high concentrations (up to 2.0 M) of salts such as NaCl or $CaCl_2$ by including a small amount (0.2–5 mol%) of PEG2K-DOPE (PEG-lipid) in the DOPC membrane. In most cases, these liposomes were spherical. Fig. 1c and d show phase-contrast images of the DOPC giant liposome containing 0.2 mol% PEG2K-DOPE (0.2% PEG-lipid/DOPC giant liposome) prepared in 150 mM NaCl (Fig. 1c) and that of the 2% PEG-lipid/DOPC giant liposome prepared in 0.5 M NaCl in water (Fig. 1d), respectively. To evaluate the size distribution of these giant liposomes, histograms of their diameter were made. Fig. 1e shows a histogram obtained by the analysis of a population of the 0.2% PEG-lipid/DOPC giant liposomes prepared in 150 mM NaCl (Fig. 1c). It shows that most giant liposomes prepared under these conditions have diameters of 10–30 μm, but some have diameters of more than 50 μm. We obtained similar results from other histograms of various kinds of giant liposomes. The efficiency of the formation of the giant liposome was higher when incubated at a higher temperature during the preparation. However, incubation at 4°C also produced many giant liposomes.

An undulation motion of the membranes of these PEG-lipid/DOPC giant liposomes in the presence of high concentrations of NaCl was observed, which was much larger than that of DOPC giant liposomes in water. For the observation of these giant liposomes using a fluorescence microscope, a small percentage (0.5 mol%) of long-chain fluorescent phospholipid, NBD-PC, was mixed with the unlabeled phospholipid. The intensities of fluorescence from the giant liposomes were much lower than those from multilamellar vesicles. To confirm that these vesicles are unilamellar vesicles, we also measured the isothermal area compressibility modulus, K, by the micropipet aspiration technique [11]. For 0.2% PEG-lipid/DOPC giant liposome in 150 mM NaCl at 20°C, the value of K was 105 ± 49 mN/m. The undulation of the membrane makes the evaluated value of K smaller than the real K, but it corresponds to a unilamellar vesicle. Both the results of the fluorescence microscope and the isothermal area compressibility modulus indicate that most of PEG-lipid/DOPC giant liposomes are unilamellar vesicles or at least oligolamellar vesicles composed of two or three layered membranes. At the measurement of K of the PEG-lipid/DOPC giant liposomes containing more than 1.5 mol% PEG-lipid, a budding or a fission of small vesicles from the giant liposome occurred at the inside of the micropipet at a high suction pressure, which makes it difficult to measure K (manuscript in preparation, Yamashita et al.).

What kind of factor is important for the formation of the PEG-lipid/DOPC giant liposome in aqueous solution containing high salt concentrations? After preincubation of dry lipid film in a vessel with a small amount of water, many bilayer membranes are stacked to form a multilamellar sheet-like membrane at the bottom of the vessel. To form a giant liposome, a sheet of the bilayer membrane has to come off the multilamellar membranes into the aque-

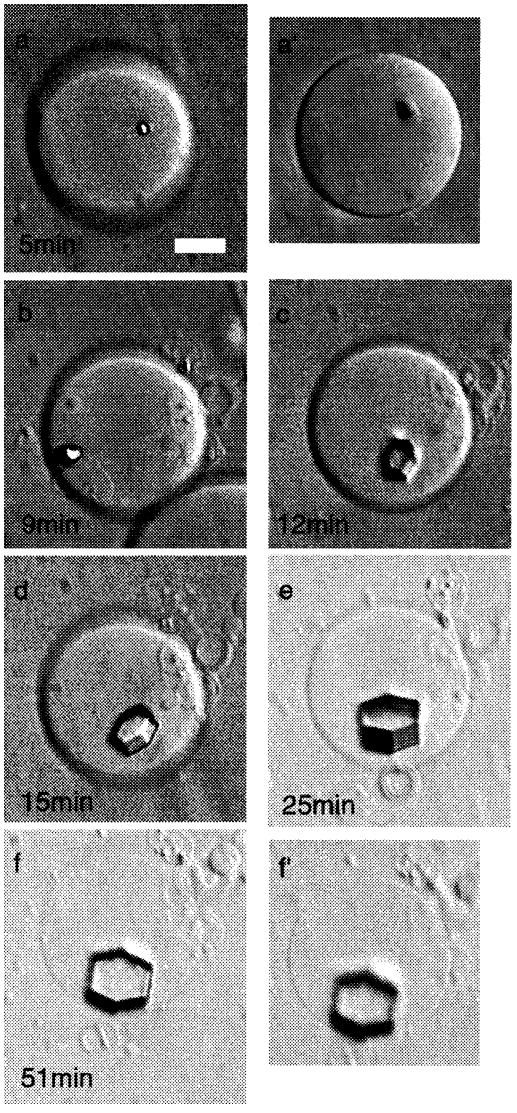

Fig. 2. Hoffman modulation-contrast images for the growth of a tetragonal microcrystal of lysozyme in a 2.0% PEG-lipid/DOPC giant liposome at 19°C. The final concentrations in the giant liposome were 8% (w/v) lysozyme and 3.5% (w/v) (i.e. 0.60 M) NaCl in 0.1 M sodium acetate buffer (pH 4.6). The giant liposomes were prepared by incubation at 37°C for 1 h. (a,a′) 5 min incubation at 19°C after dilution, (b) 9 min incubation, (c) 12 min incubation, (d) 15 min incubation, (e) 25 min incubation, and (f,f′) 51 min incubation. The focus of the microscope was on the crystal in a–f, and on the giant liposome in a′ and f′. The scale bar is 20 μm. Between 5 min and 9 min incubation, the giant liposome was moved and manipulated with a micropipet to confirm that the protein crystal exists inside the giant liposome.

ous solution. We can reasonably consider that a large repulsive force between the neighboring membranes favors this step. Fig. 1b shows a schematic model of the PEG-lipid/DOPC giant liposomes. Hydrophilic polymer chains of PEG exist in the interface of these membranes, which produce a large repulsive force between adjacent membranes due to the steric-overlapping force [12–14]. We also observed that the undulation motion of the membranes of these giant liposomes containing the PEG-lipid was much larger than that of the DOPC giant liposome in water. It is well established that the intermembrane repulsive force in the multilamellar membrane increases with an increase in the undulation motion of the membrane [15,16]. Therefore, we can conclude that the large repulsive force between the membranes due to the existence of the hydrophilic polymer chains is one of the main reasons for the success of the formation of giant liposomes at high ionic strength.

Since this new method enables us to prepare giant liposomes in various kinds of aqueous solution, it is a promising tool to make an 'artificial cell' composed of many kinds of proteins and DNA, and also for a chemical reaction vessel with a small-confined volume [17]. To demonstrate this possibility, we tried to make a protein crystal in a PEG-lipid/DOPC giant liposome. Preparation of protein crystals with the batch method requires high concentrations of salts and proteins in aqueous solution [18]; therefore the conventional method for the preparation of giant liposomes is not suitable for this purpose. As a protein, we used hen egg white lysozyme (MW = 14 307), because the mechanism of its crystal growth has been thoroughly investigated [19,20]. Immediately after a lysozyme solution and a NaCl solution in 0.1 M sodium acetate buffer (pH 4.6) were mixed at 37°C (final concentrations were 8.0% (w/v) lysozyme and 3.5% (w/v) (i.e. 0.60 M) NaCl), the mixture was transferred to the prehydrated lipid film in the vessel, and incubated at 37°C for 1–3 h. Then, this solution was diluted 20 times into the same buffer containing no protein, and then incubated at 15–20°C. After less than 30 min incubation, we found a small-size ($\sim$10 μm) tetragonal crystal in a giant liposome, and then observed that it grew to a larger-size tetragonal crystal

in the giant liposome for another 30 min (Fig. 2). For example, after 20 min incubation of the giant liposome solution at 15°C, we found 12 giant liposomes containing one large-size (20–40 μm) tetragonal crystal among 232 giant liposomes whose diameters were larger than 30 μm (i.e. 5% giant liposomes had a microcrystal). On the other hand, when we prepared giant liposomes in 7.0% (w/v) lysozyme and 4.0–5.0% (w/v) (i.e. 0.68–0.86 M) NaCl by incubation at 37°C for 3 h, and then the solution diluted by the same buffer was incubated at 20–24°C, we found needle-like crystals [21,22] and orthorhombic crystals [20] in giant liposomes after more than 10 h incubation. In most cases, we observed that the needle-like crystals grew to larger-size ones in the giant liposome (Fig. 3). This needle-like crystal is considered as a monoclinic or triclinic form [21,22]. The growth of the needle-like crystals induced a shape change of the giant liposome (Fig. 3b,c), and in most cases broke the liposome (Fig. 3d). This result clearly indicates that filaments or rods made of proteins can induce a shape change of the giant liposome, which supports the actin bundle-induced shape transformation of giant liposome [7]. The efficiency of the production of the needle-like and orthorhombic crystals in the giant liposomes was estimated as follows. After 14 h incubation of the giant liposomes containing 7.0% lysozyme and 5.0% NaCl at 22°C, we found 87 large-size (more than 20 μm) needle-like and orthorhombic crystals in solutions which contained 640 giant liposomes (more than 30 μm diameter) at the beginning of the incubation at 22°C. In control experi-

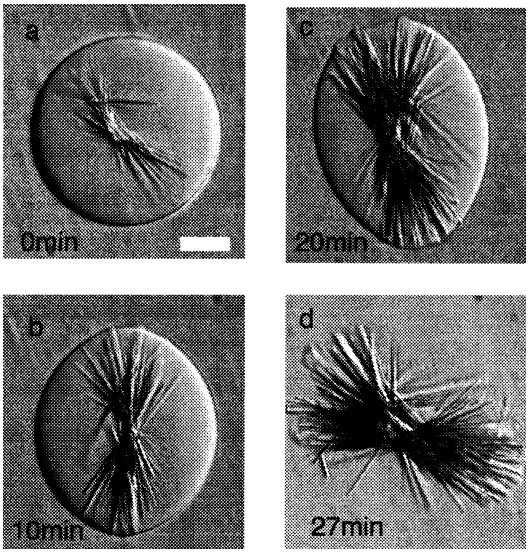

Fig. 3. Hoffman modulation-contrast images for the growth of needle-like microcrystals of lysozyme in a 2.0% PEG-lipid/DOPC giant liposome at 24°C. The giant liposomes were prepared by incubation at 37°C for 3 h. The final concentrations in the giant liposome were 7.0% (w/v) lysozyme and 4.0% (w/v) (i.e. 0.68 M) NaCl in 0.1 M sodium acetate buffer (pH 4.6). (a) Needle-like crystals were found in a giant liposome after 11 h and 17 min incubation at 24°C. To follow the crystal growth in the figure, we define the time of panel a as the start time (i.e. 0 min). (b) After 10 min from the start time. (c) After 20 min. (d) After 27 min. The scale bar is 20 μm.

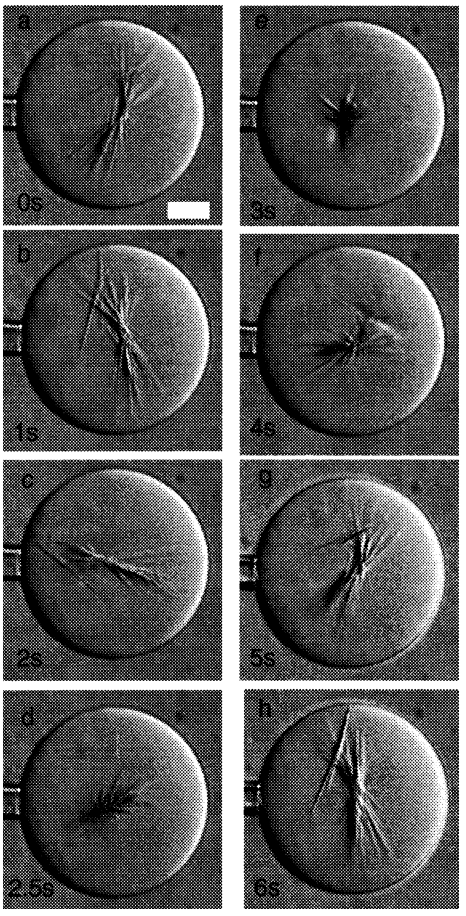

Fig. 4. Rotation of lysozyme crystals in a giant liposome. The giant liposome containing needle-like microcrystals of lysozyme was moved by aspiration through the micropipet, and then fixed at the tip of the micropipet. Then, the needle-like crystals rotated inside the giant liposome while the liposome did not rotate due to the fix at the micropipet by weak aspiration. (a) 0 s; (b) 1 s; (c) 2 s; (d) 2.5 s; (e) 3 s; (f) 4 s; (g) 5 s; (h) 6 s. The microcrystals in the giant liposome were observed with a Hoffman modulation-contrast microscope. The scale bar is 20 μm.

ments in the absence of lipid membranes, i.e. the giant liposomes, under the same condition, no needle-like and orthorhombic crystals were produced after 14 h incubation at 22°C. Therefore, this result shows that 14% giant liposomes produced these protein crystals in them.

To confirm that the protein crystal existed inside the giant liposome, we manipulated the liposome using the micropipet aspiration technique. When the giant liposome containing lysozyme crystals was moved by the flow of water injected or aspirated through the micropipet, the crystal was moved together with the liposome (data not shown). Fig. 4 shows that the needle-like crystals rotated in several directions inside the giant liposome while the liposome did not rotate due to the fix at the micropipet by weak aspiration. These results clearly indicate that the crystal did not attach to the membrane surface outside the giant liposome, but existed inside the liposome.

The results in this report clearly indicate that the PEG-lipid/DOPC giant liposome is a promising vessel for making micron-sized (10–50 μm) protein crystals (i.e. microcrystals). Recently, the large progress in protein crystallography using third-generation synchrotron sources enabled us to determine the 3-D structure of proteins by using microcrystals [23,24]. The giant liposome has a picoliter internal volume (e.g. 4.2 pl for a 20 μm diameter liposome), and thus the volume of the protein solution for preparing crystals is small. Therefore, further development of the giant liposome method to prepare microcrystals of proteins may be a promising methodology. Moreover, the success of the formation of micron-sized protein crystals in a giant liposome strongly supports the conclusion that most of the PEG-lipid/DOPC giant liposomes are unilamellar or at least oligolamellar vesicles, and also that this new method can be used to construct 'artificial cells' under various conditions.

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