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The Single GUV Method for Probing Biomembrane Structure and Function

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Giant liposomes or giant unilamellar vesicles (GUVs) of lipid membranes, with diameter greater than 10 µm have a great advantage over smaller liposomes, e.g., large or small unilamellar vesicles, LUVs or SUVs in the investigation of biomembranes. Studies of single GUVs (the ‘single GUV method’) yield especially useful information on their structure and physical properties as a function of time and spatial coordinates. Here we show three examples of studies using the single GUV method: analysis of shape changes of GUVs; membrane fusion and vesicle fission; and use of a GUV as a microscopic container. [DOI: 10.1380/ejssnt.2005.218]

Keywords: Biophysics; Bioimaging; Self-assembly; Thermodynamic properties; Lanthanides; Membrane Interface; Giant Unilamellar Vesicle (GUV); Membrane Fusion and Fission

I. INTRODUCTION

Giant liposomes or giant unilamellar vesicles (GUVs) of lipid membranes, with diameters greater than 10 µm have a great advantage over smaller liposomes such as large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) in investigating the physical properties of, and structural changes in, liposomes. So far almost all studies of liposomes have been carried out on small liposomes such as LUVs and SUVs, or on unilamellar vesicles (MLVs) using light scattering, fluorescence spectroscopy, ESR, and X-ray scattering [1–3]. In these studies average values of physical parameters of liposomes have been obtained from large numbers of liposomes. In contrast, studies of GUVs afford information on the structure and physical properties of a single GUV as a function of time and spatial coordinates. It is expected that much new information will be obtained on the structure and function of biomembranes and lipid membranes from these kinds of studies of a single GUV (i.e., the single GUV method). In this short review based on our recent works, we show several examples of this method.

II. ANALYSIS OF SHAPE CHANGES INDUCED IN GUVS BY THE INTERACTION OF VARIOUS SUBSTANCES WITH THE GUV MEMBRANE INTERFACE

Interactions of proteins with lipid membranes play important roles in the static and dynamic structures of biomembranes and in their functions. It is reported that many water-soluble proteins can be reversibly bound to lipid membrane regions in biomembranes and that their binding depends on their aqueous phase concentration, conformation, and local net charge. The electrostatic interaction between a cluster of basic amino acid residues of proteins and negatively charged lipid membranes, and also the hydrophobic interaction between the hydrophobic core of lipid membranes and fatty acids covalently linked proteins are especially well understood [4, 5]. However, the interactions of various substances with the membrane interface of electrically neutral lipids such as phosphatidylcholine (PC), and their effects on the structure of lipid membranes are not well understood. The lipid membrane interface is composed of hydrophilic segments (so-called head groups), hydrophobic hydrocarbon chains, and water molecules because of large thermal motions of the membranes such as undulation and protrusion. Therefore, there exist many kinds of intermolecular interactions between the membrane interface and various substances, and as a result, the membrane interface has molecular recognition capabilities for different substances [6]. The analysis of shape changes in GUVs induced by substances is a highly sensitive method to study the interaction between such substances and the membrane interface [7, 8]. We discuss two examples.

Lanthanide ions such as La³⁺ and Gd³⁺ are well-known to exert large effects on the function of membrane proteins such as mechanosensitive ion channels and voltage-gated sodium channels, and also on the structure of phospholipid membranes. However, the effects of the interaction of lanthanides with membrane interfaces on the stability of membrane structure, and on the function of membrane proteins are not well understood. We first investigated the effects of La³⁺ and Gd³⁺ on the shape of GUVs of dioleoylphosphatidylcholine (DOPC-GUVs) by phase-contrast microscopy [7]. The addition of 10-100 µM La³⁺ through a 10-µm diameter micropipette near a DOPC-GUV triggered several kinds of shape changes. When 10 µM La³⁺ was added to the neighborhood of a discocyte (Fig. 1-(1)), its shape changed via an intermediate structure (Fig.1-(2)) to a stomatocyte (Fig.1-(3)), after

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FIG. 1: Reversible shape change of DOPC-GUV induced by the addition of 10 µM La\textsuperscript{3+} at 20°C. During the addition of La\textsuperscript{3+}, a shape transformation occurred from discocyte via stomatocyte to the inside budded form. The times after starting injection of La\textsuperscript{3+} solution through the micropipette were (1) 0 s, (2) 9 s, (3) 10 s, and (4) 12 s for these pictures. After the addition of La\textsuperscript{3+} was stopped, the shape change was reversed. The times after stopping injection of La\textsuperscript{3+} solution through the micropipette were (5) 7 s, (6) 12 s, (7) 22 s, and (8) 33 s for the pictures. The bar in the picture corresponds to 20 µm. This figure is reprinted from Ref. [7] with permission from Elsevier Inc.

which the stomatocyte invagination became unstable and a small vesicle budded into the inside of the GUV (Fig. 1-(4)). When the La\textsuperscript{3+} concentration near the GUV was decreased, shape changes were induced as shown in Fig. 1 ((5)-(8)), culminating with the return of the GUV’s shape to its original form. This result showed that the shape change induced by La\textsuperscript{3+} was reversible, indicating that no vesicle fission occurred. Other shape changes were also observed. The addition of 10 µM La\textsuperscript{3+} changed a GUV consisting of two spheres connected by a neck (Fig. 2a-(1)) into a dumbbell (Fig. 2a-(2)), and then into a prolate shape (Fig. 2a-(3)). When we added 10 µM La\textsuperscript{3+} near GUVs made up of three or more spherical vesicles connected by a narrow tube (the so-called ‘pearls on a string’ shape) (Figs. 2b-(1), 2c-(1)), the shape changed into a cylinder or a tube (Figs. 2b-(3), 2c-(3)). All these shape changes were reversible. 10-100 µM Gd\textsuperscript{3+} also induced the same shape changes in DOPC-GUVs, which were reversible.

It is of interest to know what kinds of effects of La\textsuperscript{3+} (or Gd\textsuperscript{3+}) on PC membranes induced such shape changes in GUVs. The shapes of GUVs of lipid membranes are determined by minimizing the elastic energy ($W_{el}$) of the closed GUV membrane. The area-difference-elasticity (ADE) model (i.e., the generalized bilayer-couple model) can reasonably explain shape changes in a GUV [9, 10]. In this model, the area of each monolayer is not fixed at the equilibrium value, allowing the monolayer membrane to stretch elastically to increase the nonlocal elastic energy of the membranes. Thus, $W_{el}$ can be expressed as a sum of the membrane bending energy ($W_b$) and the energy of
relative monolayer stretching ($W_r$) as follows:

$$W_{el} = W_b + W_r = \frac{\kappa_c}{2} \int (C_1 + C_2 - C_0)^2 dA + \frac{\kappa_r}{2Ah^2} (\Delta A - \Delta A_0)^2,$$

where $\kappa_c$ is the local bending modulus of the membrane; $C_1$ and $C_2$ are two principal curvatures of the monolayer membrane; $C_0$ is the spontaneous curvature of the bilayer membrane; the integration is over the whole area of the neutral surface; $\kappa_r$ is the nonlocal bending modulus of the membrane, $\kappa_r = K_a h^2 / 2$ ($K_a$ is the area expansivity modulus); $\Delta A$ is the area difference between the two monolayers in the bilayer membrane; and $\Delta A_0 (= A^0_0 - A^0_0)$ is the area difference between the two monolayers in the bilayer membrane under non-stretched conditions. In the ADE model, the shape of a GUV is determined by minimization of the membrane elastic energy $W_{el}$ for a given area $A$, a given volume $V$, and also a given area difference $\Delta A_0$ between the two monolayers under relaxed conditions. An analysis based on the ADE model showed that, under constant volume conditions of the GUV, the following shape changes occurred with a decrease in $\Delta A_0$: (1) discocyte $\rightarrow$ stomatocyte, (2) two spheres connected by a narrow neck $\rightarrow$ dumbell (i.e., symmetric prolate shape) $\rightarrow$ prolate shape, and (3) pearls on a string $\rightarrow$ cylinder (or tube) [9, 10].

These shape changes predicted by the ADE model are the same as those induced by La$^{3+}$ or Gd$^{3+}$ in DOPC-GUVs, and therefore, the mechanism of these shape changes can be reasonably explained based on this model, as follows. Only the area of the external monolayer membrane of the DOPC-GUV, $A^e_{ex}$, decreases due to its interaction with La$^{3+}$ (or Gd$^{3+}$), and as a result, $\Delta A_0 (= A^e_{ex} - A^o_{ex})$ decreases with an increase in La$^{3+}$ concentration, which is the main factor in these shape changes.

The main physical factor responsible for the decrease in area of the DOPC membrane induced by La$^{3+}$ (or Gd$^{3+}$) will now be considered. We have observed that the chain-melting phase transition temperature, $T_m$, of PC and phosphatidylethanolamine (PE) membranes increased with an increase in La$^{3+}$ concentration [7, 11]. At equilibrium, three kinds of lateral pressures in the membrane have to be balanced:

$$\Pi_{head} + \Pi_{chain} = \gamma,$$

where $\Pi_{head}$ is the repulsive steric interaction between the head groups in the phospholipid membrane, $\Pi_{chain}$ is the repulsive chain pressure, and $\gamma$ is the attractive interfacial pressure due to hydrophobic interactions between the hydrocarbon chains and water at the membrane interface. Experimental results showing an increase in $T_m$ indicated that the lateral compression pressure of the membrane at the hydrophobic core, $\gamma - \Pi_{head}$, increased with an increase in La$^{3+}$ (or Gd$^{3+}$) concentration. This caused the decrease in the area of the DOPC membrane, supporting the above analysis of shape changes in GUVs. The mechanism of the La$^{3+}$-induced increase in lateral compression pressure is as follows: a PC headgroup has one positive charge ($N^+$) and a negative charge at the phosphate group, resulting in a large electric dipole moment of 20 Debye. In the absence of La$^{3+}$, the lateral component of the dipole moment of PC headgroups orient randomly in the membrane due to thermal motions. When La$^{3+}$ binds with the phosphate group of the PC headgroup, it causes the lateral polarization of dipole moments of its neighboring PC molecules, because the electrostatic interaction of surface charge (La$^{3+}$) with neighboring dipoles of PC headgroups tends to orient the dipoles in a certain direction. This lateral polarization can increase the lateral compression pressure of the membrane [12, 13].

As mentioned above, a membrane interface composed of three components has the ability to engage in molecular recognition. The interaction of the membrane interface with diverse substances has a profound effect on the structure and physical properties of the membrane interface, which often governs large changes in the structure and phase stability of lipid membranes and biomembranes [14]. It is not easy to predict molecular recognition in membrane interfaces due to their complicated structure, and therefore, it is important to obtain information on these systems experimentally.

In the case of amino acid residues, an interfacial hydrophobicity scale (i.e., free energies of transfer of amino acid residues from the membrane interface to water, $\Delta G_{tr}$) for PC membranes has been constructed [15]. These data show that aromatic amino acid residues such as Trp (W) and Phe (F) have high interfacial hydrophobicity ($\Delta G_{tr}$ of Trp and Phe are 1.85 kcal/mol and 1.13 kcal/mol, respectively), indicating strong partitioning of these residues in the lipid membrane interface. Using this interfacial hydrophobicity data, we have synthesized a de novo designed peptide, WLFLKKK (peptide-1), having positive charges (KKK and an amino group of the N-terminus) on both its sides and a segment (WLFL) capable of being partitioned into the lipid membrane interface of electrically neutral lipids (Fig. 3). We have investigated the effect of peptide-1 on the stability of electrically neutral lipid membranes in the liquid-crystalline (L$n_{I0}$) phase [8] and in the cubic phase [16].

The effect of peptide-1 on the shape of PC-GUVs was investigated initially. The addition of 5 µM peptide-1 through a micropipette near a DOPC-GUV induced several kinds of shape changes. For example, a prolate shape changed to a dumbbell, and then to two spheres connected...
FIG. 4: Shape change of 60%DPPC/40%chol-GUV induced by the addition of 0.2 µM lyso-PC (16:0) at 20°C. Change of prolate shape to pear shape, and subsequently to two unequal-sized spheres connected by a narrow neck. The times after starting injection of 0.2 µM lyso-PC solution through the micropipette were (1) 0 s, (2) 26 s, (3) 30 s, and (4) 31 s. After stopping the addition of lyso-PC, the shape change was reversed. The times after stopping injection of lyso-PC were (5) 0 s, (6) 180 s, (7) 190 s, and (8) 195 s. The bar in the picture corresponds to 10 µm. This figure is reprinted from Ref. [20] with permission from the American Chemical Society.

The effects of peptide-1 on the stability of the cubic phase of monoolein (MO) membranes were also investigated. Lipid membranes in cubic phases have a non-bilayer structure with connections in three-dimensional (3-D) space, and its membrane structure has a cubic symmetry. Regular 3-D structures of biomembranes similar to cubic phases have been observed in various cells by transmission electron microscopy. These have been postulated to play several important biological roles such as membrane fusion, control of functions of membrane proteins, and ultrastructural organization inside cells. One family of cubic phases, which includes the Q\textsubscript{224} phase (space group Pn3m, Schwartz’s D surface), Q\textsubscript{229} phase (Im3m, P surface) and Q\textsubscript{230} phase (Ia3d, G surface), has an infinite periodic minimal surface (IPMS) consisting of bicontinuous regions of water and hydrocarbon [17–19]. We have found that, as the concentration of peptide-1 increased, a phase transition from Q\textsubscript{224} to Q\textsubscript{229} occurred, and that, at higher peptide-1 concentrations, MO/peptide-1 membranes were in the L\textsubscript{α} phase [16]. Salts in solution inhibited these phase transitions, indicating that electrostatic interactions with adsorbed peptide-1 in the membrane interface induced these phase transitions. These results showed that peptide-1 can be partitioned into the MO membrane interface, and the electrostatic interactions due to surface charges of the adsorbed peptides play an important role in these phase transitions.

III. ANALYSIS OF MEMBRANE FUSION AND VESICLE FISSION USING THE SINGLE GUV METHOD

Membrane fusion and vesicle fission are very important processes of biomembranes in cells, but their mechanisms are unclear and controversial. Recently we succeeded for the first time in revealing details of membrane fusion and vesicle fission processes using the single GUV method [20, 21]. We first examine an example of vesicle fission [20].

Binary mixture membranes of cholesterol and saturated PC such as dipalmitoyl-PC (DPPC) or sphingomyelin (SM) are in the liquid-ordered (L\textsubscript{o}) phase, which has intermediate properties between those of a liquid-crystalline (L\textsubscript{α}) phase and a gel phase. In the L\textsubscript{o} phase, acyl chains of PC have high orientational order, but the lateral diffusion coefficient of lipids in the membrane is relatively high [22]. In cells, rafts or microdomains in plasma membranes are thought to play important roles in signal transduction and cell migration [23].

We investigated the effects of low concentrations (much lower than its critical micelle concentration, CMC) of lyso-PC (16:0) with a long acyl chain (CMC: 7 µM) on GUVs of 60 mol% DPPC/40 mol% cholesterol mem-
FIG. 5: Shape change of 60%DPPC/40%chol-GUV induced by the addition of 1 µM lyso-PC (16:0) at 20°C. (a) Change of prolate shape to pear shape, and subsequently to two unequal-sized spheres connected by a narrow neck. The times after starting injection of 1 µM lyso-PC solution through the micropipette were (1) 0 s, (2) 17 s, (3) 18 s, and (4) 40 s. After stopping the addition of lyso-PC, the distance between the two spherical vesicles increased with time. The times after stopping injection of lyso-PC was (5) 240 s. (b) The cylinder changed to pearls on a string. The times after starting injection of 1 µM lyso-PC solution through the micropipette were (1) 0 s, (2) 21 s, (3) 25 s, and (4) 45 s. After stopping the addition of lyso-PC, the distance between spherical vesicles again increased with time. The time after stopping injection of lyso-PC was (5) 270 s. (c) The tube changed to pearls on a string. The times after starting injection of 1 µM lyso-PC solution through the micropipette were (1) 0 s, (2) 22 s, and (3) 37 s. After stopping the addition of lyso-PC, the distance between spherical vesicles increased with time. The time after stopping injection of lyso-PC was (4) 180 s. The bar in the picture corresponds to 10 µm. This figure is reprinted from Ref. [20] with permission from the American Chemical Society.

brane in the lo phase (i.e., 60%DPPC/40%chol-GUVs) [20]. Figure 4 shows several kinds of shape changes of a 60%DPPC/40%chol-GUV in the lo phase induced by addition of 0.2 µM lyso-PC through a 10-µm diameter micropipette near the GUV. In Fig. 4, at first (in the absence of lyso-PC), the GUV had a prolate shape (Fig. 4-(1)). After the addition of lyso-PC, the shape change to a pear shape (Fig. 4-(2)) and then became a pair of unequal-sized spheres connected by a narrow neck (Fig. 4-(3)). Finally the diameter of the neck became very small (we define this shape as vesiculation; see also the following analysis of shapes), as shown in Fig. 4-(4). In order to determine the reversibility of the shape change, the addition of lyso-PC was stopped after the shape change of the GUV was complete, after which we continued to observe the GUV’s shape. Figures 4-(5) to 4-(8) show the course of the shape change of the GUV over time, after stopping the addition of lyso-PC. The two connected spheres first changed back to the pear shape (Fig. 4-(7)), and then to the prolate shape (Fig.4-(8)). After its addition was stopped, lyso-PC diffused away from the vicinity of the GUV into the bulk solution, lowering its concentration near the GUV and thereby decreasing the partition of lyso-PC into the membrane (i.e., lyso-PC molecules in the outer monolayer of the GUV move into the aqueous solution). This result indicated that the lyso-PC-induced shape change of 60%DPPC/40%chol-GUVs was reversible, and that no vesicle fission occurred. The shape change predicted by the ADE model (with an increase in $\Delta A_0$) is identical to the lyso-PC-induced shape changes of 60%DPPC/40%chol-GUVs shown in Fig. 4. Therefore, this analysis shows that lyso-PC entered the external monolayer of GUVs from the surrounding aqueous solution, increasing $\Delta A_0$, and also that ‘flip-flop’ (exchange of lyso-PC between external monolayer and internal monolayer in one bilayer) of lyso-PC in the lo phase membrane is very slow, i.e., there is almost no flip-flop within 1 min (which is the time scale of the shape changes). This is the
FIG. 6: (a) Association of DOPC-GUV induced by 100 µM La³⁺. The times after starting injection of La³⁺ solution through the micropipette were (1) 0 s, (2) 2 s, and (3) 4 s. The scale is the same as in (b). (b) Membrane fusion of 30% DPOPE/70%DOPC-GUV induced by 100 µM La³⁺. The times after starting injection of La³⁺ solution through the micropipette were (1) 0 s, (2) 2 s, (3) 4 s, and (4) 6 s. The bar in the pictures corresponds to 20 µm. This figure is reprinted from Ref. [21] with permission from the American Chemical Society.

TABLE I: Threshold concentrations of lyso-PC induced shape changes and membrane fission of 60%DPPC/40%chol-GUVs. This table is reprinted from Ref. [20] with permission from the American Chemical Society.

| Lyso-PC (C10:0) | 5 × 10⁵ | 1 × 10⁷ | 7 × 10⁸ |
| Lyso-PC (C12:0) | 2 × 10¹⁰ | 4 × 10¹⁰ | 7 × 10¹⁰ |
| Lyso-PC (C14:0) | 9 × 10⁻¹ | 8 | 7 × 10⁴ |
| Lyso-PC (C16:0) | 7 × 10⁻² | 5 × 10⁻¹ | 7 |

main reason for the lyso-PC-induced shape changes of the GUVs. Moreover, this analysis indicates that substances with a long hydrocarbon chain such as lyso-PC can enter into the lo phase membrane and also into rafts in cell membranes.

Higher concentrations (1 µM; less than its CMC) of lyso-PC induced the same shape changes in 60%DPPC/40%cholesterol-GUVs in the lo phase as did lower concentrations (Fig. 5), in the initial stage: prolate shape (Fig. 5a-(1)) → pear shape (Fig. 5a-(2)) → two unequal spheres connected by a narrow neck (Fig. 5a-(3)), but in the final stage two spherical vesicles were separated (Fig. 5a-(4)). In order to determine the reversibility of this change, the addition of lyso-PC was stopped after the shape change was complete, after which we continued to observe the shape of the GUV. The distance between the two spherical vesicles increased with time (Fig. 5a-(5)), indicating that 1 µM-lyso-PC induced vesicle fission in these GUVs. Vesicle fission also occurred in other GUV shapes such as a cylinder and a tube (Figs. 5b, c).

In the ADE model [9], further increase in ΔA₀ induces vesiculation where the neck diameter goes to zero at a critical value of ΔA₀. However, above the critical value of ΔA₀, the ADE model does not give us reliable predictions, and also cannot explain vesicle fission, since this involves topological changes in the membrane. To elucidate the effect of acyl chain length of lyso-PC on shape changes and vesicle fission induced by this substance, we investigated the effects of low concentrations of three other kinds of lyso-PC with different acyl chain lengths (lyso-PC (14:0), lyso-PC (12:0), and lyso-PC (10:0)) on 60%DPPC/40%cholesterol-GUVs. All three lyso-PCs induced the same shape changes and vesicle fission observed when using lyso-PC (16:0), as described above. In Table 1, we summarize the threshold concentrations of lyso-PC needed to induce the shape changes observed in these GUVs (i.e., prolate → two spheres connected by a narrow neck) and concentrations needed to induce vesicle fission. These threshold values were defined as lyso-PC concentrations at which shape changes (or vesicle fission) occurred in 50% of the examined GUVs (i.e., prolate → two spheres connected by a narrow neck) and concentrations needed to induce vesicle fission. These threshold values were defined as lyso-PC concentrations at which shape changes (or vesicle fission) occurred in 50% of the examined GUVs. Both threshold concentrations increased greatly with a decrease in chain length (i.e., a decrease in the number of carbons in the hydrocarbon chain), and both were much lower than the CMC of each lyso-PC. Thermodynamic analysis of this result indicated that the shape change and vesicle fission of GUVs with lo phase membranes occurred at the threshold concentrations of lyso-PC in the membrane [20].
have proposed a mechanism for the lyso-PC induced vesicle fission of GUVs [20] on the basis of these results.

Having examined vesicle fission, we now turn our attention to membrane fusion [21]. In section 2, we described the effect of La$^{3+}$ on the shape of DOPC-GUVs. Here, we describe the effect of La$^{3+}$ on the interaction between two GUVs composed of a mixture of DOPC and dipalmitoyl-PE (DPOPE) (i.e., DPOPE/DOPC-GUVs). A GUV was first adsorbed on the surface of a micropipette, and brought into contact with another GUV. When 100 µM La$^{3+}$ solution was added from a micropipette into the vicinity of the two DOPC-GUVs, association between the two GUVs occurred (Fig. 6a), but not membrane fusion. Interaction of La$^{3+}$ with membranes of the GUVs suppressed (or eliminated) undulation of the membranes, causing the association of GUVs. Figure 6b shows the shape change of two spherical 30 mol%-DPOPE/70 mol%-DOPC-GUV (i.e., 30%DPOPE/70%DOPC-GUV) in 2% (w/v) PEG 6K aqueous solution induced by addition of 100 µM La$^{3+}$ solution. During the addition of La$^{3+}$ in this case, the two GUVs became associated with each other (Fig. 6b-(1)), followed by a gradual increase in their mutual contact area (Fig. 6b-(2), (3)). Further addition of La$^{3+}$...
induced membrane fusion between these GUVs to produce a larger spherical GUV (Fig. 6b-(4)). Membrane fusion occurred at ≥ 30 μM La$^{3+}$. The efficiency of membrane fusion depended on the concentration of DPOPE in the DPOPE/DOPC membranes: at ≤ 10 mol% DPOPE, there was no fusion; at 20 mol% DPOPE, 50% of associated GUVs fused; at ≥ 30 mol%, 100% of associated GUVs fused.

To elucidate the mechanism of La$^{3+}$-induced membrane fusion, we investigated the process of fusion in depth. Figure 7 shows, in detail, the steps in 100 μM La$^{3+}$-induced membrane fusion of 30%DPOPE/70%DOPC-GUVs. In Fig. 7a, at 1/30 s, two GUVs were strongly associated with each other, and a partition membrane composed of two bilayers of each GUV had formed between the GUVs. At 2/30 s, the partition membrane had suddenly broken at one end (i.e., disconnected from the GUV membranes). Subsequently, the cross sectional length of the partition membrane gradually decreased, finally forming a small structure that was difficult to define. In some cases of membrane fusion, after the sudden rupture of the partition membrane, it curled up to form a smaller spherical vesicle (Fig. 7b). The area of the partition was almost equal to the decrease in the total surface area at membrane fusion. This was the first study to reveal the detailed process of membrane fusion of biomembranes [21].

On the basis of these results, we propose a mechanism of La$^{3+}$-induced membrane fusion of GUVs, the ‘partition breakage’ model (Fig. 8) [21]. After association of two GUVs, addition of La$^{3+}$ near the GUVs increases the density of La$^{3+}$ bound in the outer monolayer membrane facing the buffer, increasing the lateral compression pressure of the monolayer membrane (Fig. 8-(1)). This induces fusion between the external monolayer membranes at the edge of the partition membrane (Fig. 8-(2)), if the membrane contains a high concentration of DPOPE, because such a membrane favors formation of a surface with high negative curvature. As a result, chain packing at the edge of the partition membrane (i.e., at the interstitial hydrocarbon region indicated by a green triangle in Fig. 8-(2)) is destabilized, causing breakage of the membrane at one site on the edge (Fig. 8-(3)). The area of this breakage site then gradually expands from the site of the first breakage, eventually causing the partition membrane to separate from the GUVs, and membrane fusion of the two GUVs is simultaneously completed (Fig. 8-(4)).

The two examples in this section are the first successful application of the ‘single GUV method’ to the study of biomembrane dynamics such as vesicle fission and mem...
brane fusion. However, we acknowledge that these are in the rudimentary stages, and that the method needs further development to elucidate the mechanisms of various kinds of vesicle fission, and membrane fusion of biomembranes.

IV. SINGLE GUVS AS CHEMICAL REACTION VESSELS WITH PICO-LITER INTERNAL VOLUME AND ARTIFICIAL CELLS

It is difficult to make PC-GUVs in aqueous solutions containing high concentrations of salts such as NaCl, which has prevented their more extensive use. We therefore developed a new method to prepare PC-GUVs in aqueous solution containing high concentrations (up to 2.0 M) of salts [24]. Hydrophilic polymers attached to the surface of lipid membranes by including a small amount of PEG-grafted phospholipid (Fig. 9a) in the membrane increase the repulsive forces between the membranes, which makes it possible to form GUWs in high ionic strength solutions (Fig. 9b). This method (the PEG-lipid method) is useful in many cases where GUWs cannot be formed easily (e.g., in buffers with low ionic strength, or when using PC with saturated hydrocarbons, such as DPPC) [20, 26]. Using this method, we could grow a micron-sized (ca. 10-50 µm) protein single crystal inside a GUV (Fig. 10). Preparation of protein crystals by the batch method requires a high concentration of salts and proteins in aqueous solution, and therefore the conventional method of preparation of GUWs is not suitable under these conditions. For our first study on the use of GUWs in protein crystallization, we used hen egg-white lysozyme as the protein. Immediately after the lysozyme and NaCl in 0.1 M sodium acetate buffers (pH 4.6) were mixed at 37°C [final concentrations were 8.0%(w/v) lysozyme and 3.5%(w/v) NaCl], the mixture was transferred onto a pre-hydrated lipid film in a vessel, and incubated at 37°C for 1 to 3 hrs. This solution was then diluted 20-fold into the same buffer containing no protein, and incubated at 15-20°C. After less than 30 min, we found a small (~10 µm) tetragonal crystal within a GUV, and observed it as it grew for another 30 min (Fig. 10). These results demonstrated that this method is a promising tool for the preparation of 'artificial cells' under various conditions.

Lipid membranes as biomaterials have an advantage over other materials such as metals, mica, SiO2, gold-coated substrates, and polymer films, because there is no nonspecific adsorption of proteins on lipid membranes [25]. However, GUWs of lipid membranes in the Lα phase have some technical drawbacks; one is that they are easily disrupted by detergents or surfactants, and the other is that hydrolysis of phospholipids prevents long-term usage of these GUWs in water. Recently, we have found that in the presence of high concentrations of Triton X-100 (a strong, non-ionic detergent used to solubilize lipid membranes), the structures of GUWs and LUVs of DPPC/chol and sphingomyelin(SM)/chol membranes in the Lα phase were very stable, allowing no leakage of fluorescent probes from the vesicles [26]. We have also investigated the effect of cholesterol on membranes of an ether-linked dialkylphospholipid, viz., dihexadecyl-PC (DHPC), which can be used in water for long periods due to its resistance to hydrolysis, and found that GUWs and LUVs of DHPC/chol membranes formed the Lα phase and that they were stable against Triton X-100 [26]. These data indicate that GUWs and LUVs of the Lα phase membranes are very valuable for a variety of practical uses.

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