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Kinetic Study on Giant Vesicle Formation with Electroformation Method

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Giant vesicles (GVs) composed of zwitterionic phospholipids were prepared by the electroformation method. The growth behavior of GVs was quantitatively analyzed as a first-order kinetics of the radius of GVs to obtain the apparent growth rate constant k_{Gr}. On the basis of the dependence of the k_{Gr} value on the preparation temperature and the lipid composition of GVs, the membrane fluidity of vesicle membranes was found to be dominant in the growth behavior of GVs. The comparison of the k_{Gr} values with the membrane fluidity for GVs suggested the validity of the model that the swelling of the lipid membrane could induce the growth of GVs.

Introduction

Giant vesicles (GVs) are cell-size liposomes and are known to be advantageous to the visualization of physiological phenomena.[1−6] Some preparation methods of GVs have been presented, such as electroformation,[7−9] the hydration method,[10−13] chemically induced vesiculation using a cell,[14,15] membrane perturbation effect by the protein molecule,[16] and emulsification using a microchannel.[17] However, its formation mechanism has not been clarified in details. This is because the vesicle formation highly depends on the preparation conditions.

The electroformation method has widely been utilized among the possible preparation methods because the immobilization of GVs on Pt electrodes is advantageous to the direct microinjection of biomaterials.[1−4] This method successfully permits the preparation of GVs mainly composed of phosphatidylcholine, negatively charged lipids, phosphatidylethanolamine, and cholesterol (Chol). However, the mechanism of GV formation has remained unclear. Montes et al. reported the fusion-mediated GV formation mechanism.[10] On the other hand, Dimitrov and Angelova reported the electro-swelling mechanism based on (i) the electro-osmotic effect, (ii) the osmotic pressure, (iii) the membrane elasticity, (iv) hydration for membrane separation, and (v) other factors including van der Waals forces.[7,8] Almost all cases reported were performed with distilled water to neglect the effect of osmotic pressure. Therefore, it is important to consider the electro-osmotic effect as a main factor for GV formation.

The electro-osmotic effect depends on the voltage and frequency of the applied alternating current (AC) electric field.[2,8] The preparation condition has been qualitatively optimized to be about 1.5 V and 10 Hz of AC electric field.[2] The theoretical model based on the electro-osmotic effect as a main factor explains neither the optimal preparation condition nor the initial stage of GV formation.[7] Therefore, the presented model that the lipid bilayer is swelled to induce GV growth has involved ambiguous points in relation to the initial stage of GV formation.

The main goal in this study is to investigate the kinetics and mechanism of GV formation. First, the kinetic analysis of GV growth was studied by using first-order kinetics. The electroformation method was used to observe the behavior of GV formation under heating conditions. The quantitative parameter obtained from the analysis of the first-order kinetics was compared with the dynamic property of the lipid membrane (membrane fluidity). From the obtained results, we discussed the mechanism of GV formation involving its initial stage.

Results and Discussion

Analysis Procedure on the Growth Behavior of GVs. In the first series of experiments, we investigated the quantitative analysis method of GV formation. Figure 1 shows the image of the growth process of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) GVs under an AC electric field (1.5 V, 10 Hz) at 25 °C. A GV with a mushroom-type shape was observed. In order to quantitatively analyze the growth behavior of GVs, the diameter of GVs parallel to the electrode ($D = 2R_v$), as the representative diameter, was measured according to the method of Bucher et al.[2] Also, we performed the electroformation of GVs in a lipid concentration of 0.01−0.1 mg/mL to avoid vesicle−vesicle interaction/fusion or morphological change.

Non-Gaussian distribution was observed in the GV diameter, consistent with a previous report.[18] On the other hand, it has

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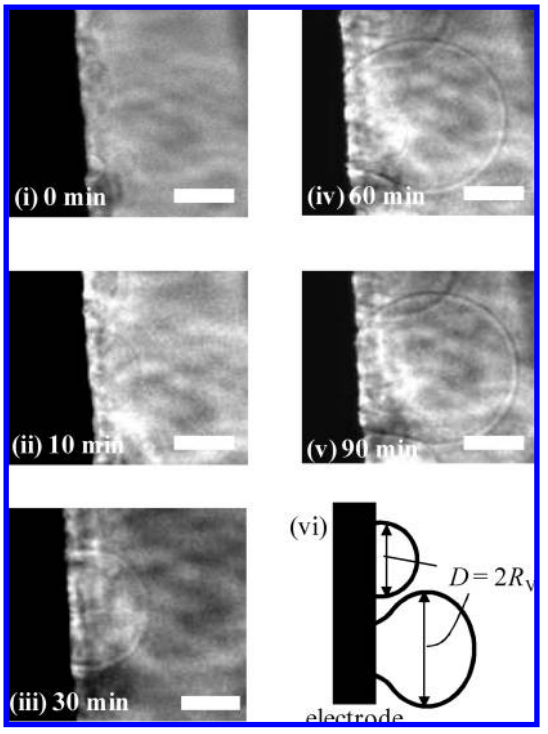

Figure 1. Light micrographs of the time-course of GV formation. An AC electric field of 1.5 V (10 Hz) was applied to the chamber at 25 °C. The amount of POPC lipids was about 13 μmol. Images are (i) 0 min, (ii) 10 min, (iii) 30 min (iv) 60 min, and (v) 90 min. (v) The schematic illustration of a vesicle with diameter D ($= 2R_V$v). The length of bar represents 20 μm. The time course of these images were corresponding to vesicle 1 in Figure 2(a).

been reported that the diameter distribution of GVs obtained with a hydration method was monodispersion.[19] Therefore, the diameter distribution of GVs depends on the preparation method. To clarify whether the diameter distribution is related to the kinetics of the GV formation, the time-courses of three vesicles with different final diameters (14.2, 34.0, and 50.1 μm) were compared with one another (Figure 2a). It is thought that the apparent kinetics of three vesicles could be different from one another because of the initial rate of GV diameter. However, the plots of $R_v(t)/R_{v,max}$ against the time resulted in an identical curve, as shown in Figure 2b. Here, $R_v(t)$ and $R_{v,max}$ are the radius of the vesicle at time t and the maximal one, respectively. In order to clarify the GV growth mechanism, the effects on the growth rate were investigated, as shown in Figure 2a. The results show the validity of the variation of their diameters based on the first-order kinetics, as expressed in the following equation:

$$R_v(t) = R_{v,max}(1 - \exp(-k_{Gr}t)) \quad (1)$$

where k_{Gr} is the apparent growth rate constant and reflects the growth kinetics of GV. The growth rate constant for GVs with various diameters, k_{Gr}, were analyzed with eq 1 (Figure 2b,c). The histogram of k_{Gr} values obtained from the treatment of Figure 2a−c showed a single peak (Figure 2d), which indicates the validity of analyzing the growth behavior of GV on the basis of one kinetic process. The average value of k_{Gr} was estimated to be 0.062 ± 0.014 min^{-1}. It is, therefore, concluded that the apparent growth rate constant of GVs can be analyzed by using eq 1, independent of the maximal diameter of GVs.

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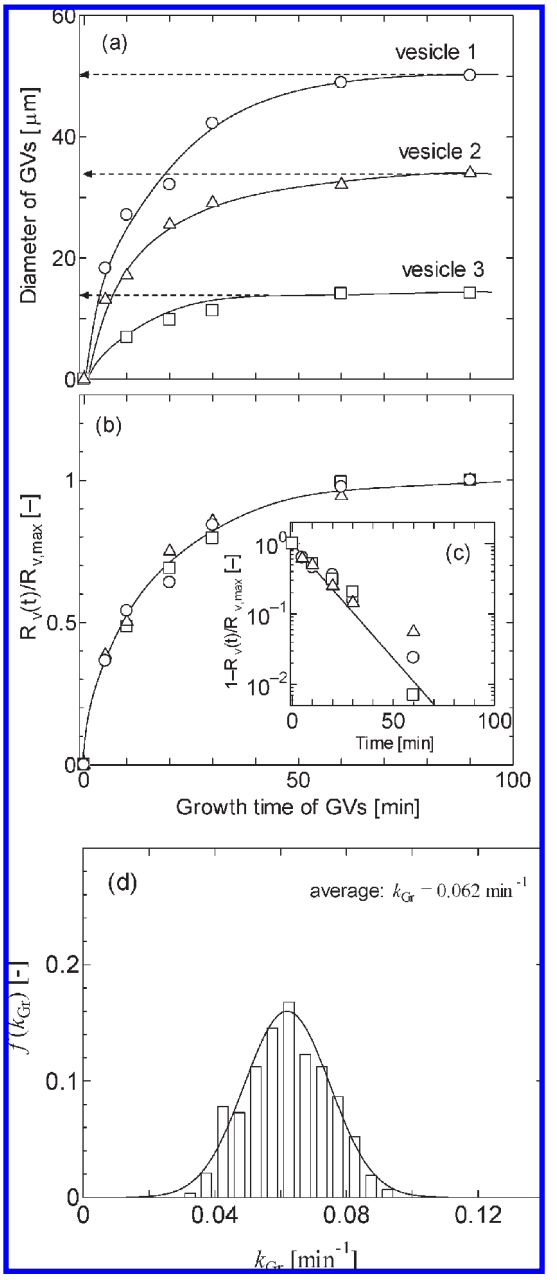

Figure 2. The analysis procedure on the growth process of GVs formed on electrodes. (a) Time-course of their diameters. Three GVs with different diameters (14.2, 34.0, 50.1 μm) were selected. (b) Time-course of their normalized radii ($R_v(t)/R_{v,max}$). Here, $R_{v,max}$ was tentatively determined and shown as a dotted line on each hyperbolic curve. R_v at 90 min was used as $R_{v,max}$. (c) The inset shows the semilogarithmical plots of the normalized radii: $1 - R_v(t)/R_{v,max}$ versus the growth time in each vesicle. Linear least-squares fit was performed for each vesicle. (d) Histogram of the k_{Gr} value obtained from more than 500 GVs. Solid curve shows the Gaussian distribution function.

In the following section, the optimal preparation condition was investigated using the k_{Gr} value as an index of GV electroformation.

Electric Field-Dependent GV Formation. There is the possibility that the optimal k_{Gr} value depends on (i) the operational condition such as the voltage and the frequency of the applied AC field and (ii) the membrane property. Here, the first factor was investigated.

The influence of the frequency of the applied AC electric field on the k_{Gr} value was first investigated. All the GV formation was

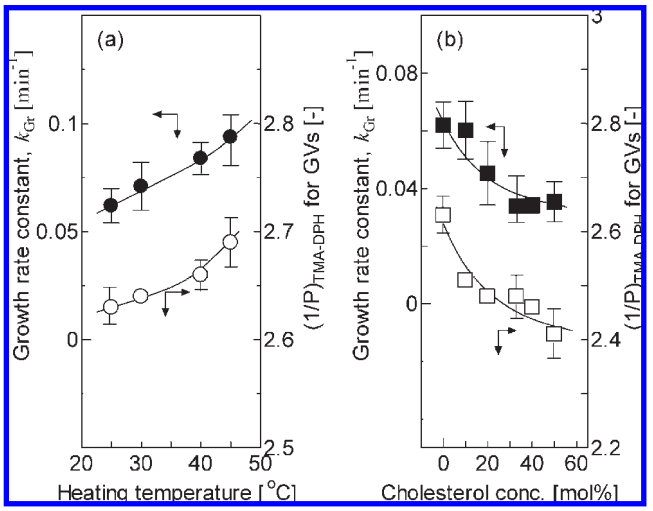

Figure 3. (a) The temperature dependency of the k_{Gr} value and membrane fluidity for POPC GVs and (b) the dependency of the cholesterol concentration against the k_{Gr} value and membrane fluidity for POPC/Chol GVs. Data for membrane fluidity were obtained from three to five independent measurements.

observed below 30 Hz, consistent with the previous findings. The k_{Gr} value showed a maximal value at around 10 Hz. The GV formation was strongly inhibited in the higher frequency range (above 100 Hz). The influence of the voltage was then investigated. In the lower voltage (0.2 V, 10 Hz), the k_{Gr} value was 0.02 min^{-1} ($< 0.062 \pm 0.014$ min^{-1} at 1.5 V, 10 Hz). The higher voltage condition above 1.5 V resulted in the generation of bubbles by electrodegradation around platinum electrodes, although the k_{Gr} value increased, which led to a decrease in the number density of the formed GVs.

In conclusion, it is considered that 1.5 V and 10 Hz of the AC electric field was the optimal condition in our experiments.

Effect of Temperature against GV Formation. The effect of membrane property was then investigated by using the k_{Gr} value as an index. It is reported that the GV formation was related to the permeability of the solvent.[7−9] The permeability of the vesicle membrane can be enhanced by heating conditions since the permeability is controlled by the membrane fluidity.[20] Hence, the temperature change is expected to enhance the growth behavior of GVs. The growth kinetics of GVs was investigated together with its membrane fluidity under various heating conditions (25−45 °C).

Almost all vesicles prepared in pure water under higher temperature range were not spherical shapes, although drastic deformations to nonspherical vesicles such as *stomatocyte*, *dumbbell*, or *budding* in the previous literature[6,21] were not observed in our experiments. To avoid any misunderstanding, such unspherical vesicles were not used to analyze the growth rate. The experimental results on the formation in the case of heating conditions are summarized in Figure 3a. The obtained k_{Gr} and the corresponding membrane fluidity, $(1/P)_{TMA-DPH}$ (TMA-DPH = 1-(4-trimethyl-aminophenyl)-6-diphenyl-1,3,5-hexatriene), were plotted against the heating temperature (Figure 3a). Both parameters increased with elevating temperature. It is, therefore, obvious that physicochemical properties of vesicle membranes such as membrane fluidity are related to the growth behavior of GVs.

Table 1. Summary of Properties of GVs Made from Various Phospholipids and Observations for GV Formation (AC Electric Field: 1.5 V and 10 Hz)

lipid	T_m[a] [°C]	GV formation at 25 °C[b]	$(1/P)_{TMA-DPH}$ [-]	k_{Gr}[c] [min^{-1}]	ΔE [kJ/mol]	K_a [N/m]
DOPC	−18.3	yes	2.66	0.078 ± 0.007	13.8	0.122
POPC	−2.5	yes	2.63	0.062 ± 0.014	16.1	0.135
DMPC	23.6	yes	2.31	0.021 ± 0.005	44.9	0.145
DPPC	41.3	no	n.d.[d]	0	n.d.	0.198
DSPC	54.5	no	n.d.	0	n.d.	n.d.

[a] T_m means the gel-to-liquid crystalline transition temperature. These values were referred to in the previous literature.[22,23] [b] All experiments were carried out at 25 °C. The GV formation was confirmed 2 h later from the application of the electric field. [c] All the data are the mean values for various GVs at 25 °C. [d] n.d.: not determined.

Effect of Lipid Composition against GV Formation. The growth rate constant for various neutral phospholipids was investigated in the same manner as the treatment in Figure 2. In Table 1, the obtained k_{Gr} values for respective phospholipids were summarized together with their physicochemical properties such as phase transition temperatures (T_m) and membrane fluidities ($(1/P)_{TMA-DPH}$) for GVs. The formation of GVs for each phospholipid was observed at 25 °C. In the case of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), POPC, 1,2-dimirystoyl-*sn*-glycero-3-phosphocholine (DMPC), (T_m < 25 °C), their GV formation could be observed, while no GV formation was observed in the case of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (T_m > 25 °C). From the temperature dependency of the k_{Gr} value, the activation energy was estimated according to Arrhenius's law. The resulting ΔE values for growths of POPC, DOPC, and DMPC GVs were summarized in Table 1. The larger the ΔE value, the higher the phase transition temperature. The ΔE value represents the energy necessary for the swelling of the lipid membrane and its growth to GVs by overcoming the viscous resistance generated by the curvature elasticity of the lipid membrane. Therefore, in the case of DPPC and DSPC (no GV formation), one can predict that the ΔE values for these lipid systems should be considerably higher than those for POPC, DOPC, and DMPC GVs.

Effect of Cholesterol against GV Formation. It is well-known that cholesterol has the capability to increase the stiffness of membranes of GVs,[24−26] in other words, to decrease membranes fluidity.[27] Although the phospholipid/cholesterol binary system shows the liquid-ordered (l_o) phase, liquid-disordered (l_d) phase, and mixture $l_d + l_o$ phase,[28] it has been reported that the permeability to solvent is proportional to the membrane fluidity.[20] The effect of cholesterol on the growth behavior of GVs was then examined.

The GV formation of a POPC/Chol binary system (0−50 mol % of cholesterol) was analyzed at 25 °C according to the treatment in Figure 2 (Figure 3b). The cholesterol addition favors the formation of GVs with smaller diameter (data not

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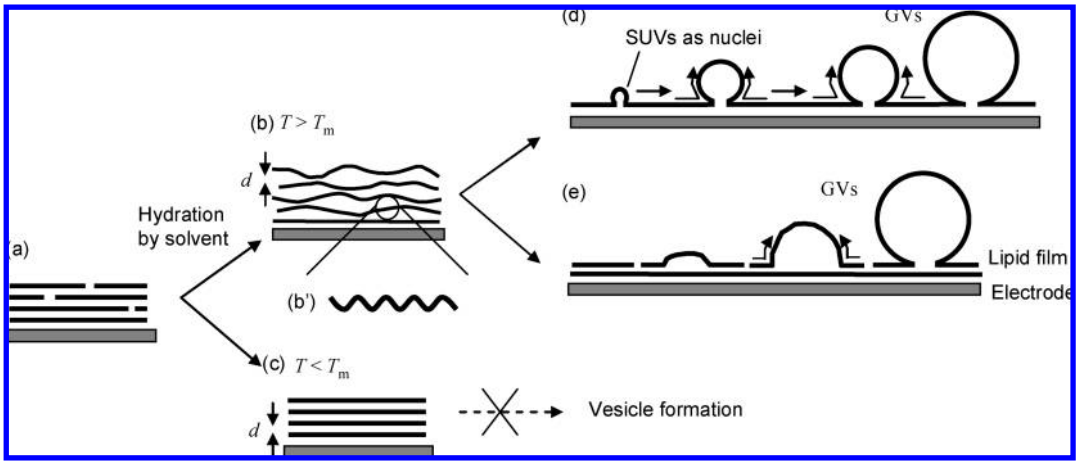

Figure 4. Schematic illustrations of the possible mechanism of GV formation by the electroformation method: (a) multilayer stack of membranes before its hydration by solvent, (b) multilayer stack of membranes in the presence of curvature fluctuations to an increase in the mean bilayer spacing, (b') curvature fluctuation in a free-standing bilayer segment, (c) multilayer stack of membranes in the absence of curvature fluctuations, (d) *Expansion model*: SUVs (as nuclei) produced on the electrode by hydration of lipid film grow up to GVs. (e) *Swelling model*: the lipid thin film is swelled to increase the curvature of lipid film. Model (e) refers to the previous reports by Angelova and her co-workers. The argument on curvature fluctuations refers to the work of Heimburg.[22]

shown). Moreover, an addition of 50 mol % cholesterol drastically inhibited the formation behavior and sometimes resulted in no formation of GVs. Monotonous decreases in both k_{Gr} and $(1/P)_{TMA\text{-}DPH}$ for GVs were observed with the incremental change in cholesterol concentration (Figure 3b). Therefore, it was concluded that the addition of cholesterol likely inhibits the formation behavior by the decrease in membrane permeability to solvent, although the effect of the phase separation of POPC/Chol should be investigated further.

Possible Mechanism of GV Formation with Electroformation Method. Previously, the mechanism of the GV electroformation has been investigated, based on "*the swelling model*" in which the swelling of the membrane visible under the photomicroscope is dominant in GV formation (Figure 4b−e).[7,8] In the case of the swelling model, the growth rate constant of GV formation can be described as

$$\tau^{-1} = L(3R_g T c_{i0}/R_0 + 4K_a/R_0^2) \quad (2)$$

where L is the hydraulic permeability, R_g is the gas constant, and R_0 is the radius of a free-tension GV for which GV gets its spherical shape.[7,8] According to Angelova et al., eq 2 is reasonable to describe the GV growth *at the latter stage* ($\Delta R = R - R_0 \ll R_0$). In the latter stage, R_0 is nearly constant, and the k_{Gr} value can be independent of the radius of GVs. Zeidel and his co-workers have shown that the permeability to water was proportional to the reciprocal of anisotropy from the permeation experiment using small unilamellar vesicles (SUVs) of POPC, DLPC, POPC/Chol, and Sphygomyeline/Chol binary system,[20] suggesting that the L value is proportional to the membrane fluidity. In addition, K_a is roughly proportional to the reciprocal of membrane fluidity (Table 1). Therefore, τ^{-1} ($= k_{Gr}$) *at the latter stage* is supposed to be proportional to the membrane fluidity of GVs. The k_{Gr} value was, therefore, plotted against the membrane fluidity for GVs (Figure 5). The k_{Gr} values were well correlated with the membrane fluidity of GVs regardless to the type of phospholipids (r = 0.938). It is thus considered that the growth process of GVs visible under the photomicroscope depended on their membrane property.

On the other hand, the mechanism of the GV electroformation *at the initial stage* has not been discussed on the basis of

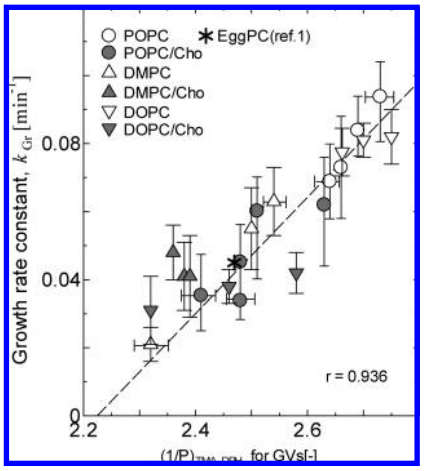

Figure 5. Correlation of the growth rate of GVs with their membrane fluidity. (asterisk) Data from previous literature.[7]

the membrane property because the large variation of the diameter of the GV at the initial stage breaks the condition ($\Delta R = R - R_0 \ll R_0$) to analyze the kinetics of GV formation with eq 1. We considered *the expansion model* in which SUVs, as nuclei, produced on the electrode, grow up to GVs (Figure 4b−d). Since the diameter change from SUVs as nuclei to GVs results in a decrease in membrane fluidity,[16] a decrease in the k_{Gr} value should occur during the expansion of SUVs to GVs according to Figure 5. This implies that the GV formation process involves some kinetic process, not consistent with the analysis as a single kinetic process like in Figure 2. As another possibility, an AC field-induced fusion event of SUVs is considered to mediate the GV formation at the initial stage. Montes et al. reported that the AC electric field (below 1 V, 500 Hz) induced the fusion of the erythrocyte ghost to form GVs.[10] In our experiments, the fusion events between GVs during its formation were avoided. However, if the fusion-mediated growth of GV has occurred, the interaction between SUVs produced by the hydration of lipid film should be dominant in the GV formation process. We observed no fusion of SUVs near the electrode under the AC electric field of our condition (data not shown). Therefore, it is considered that the fusion event of SUVs did not contribute to the initial stage of

the GV electroformation. Furthermore, it is considered that the expansion model (Figure 4b− d) is an unreasonable model for the mechanism of GV electroformation.

The lipid swelling is prerequisite for the GV formation, as shown in Figure 4a to b or c. The activation energy obtained from Figure 3a is considered to be an index for the energy necessary for the formation of GVs, including the lipid swelling by the curvature fluctuation of the lipid membrane (Figure 4b'). The undulation energy density of the curvature fluctuation of the lipid membrane in a bilayer stack, ΔG_u, was described by the equation[29]

$$\Delta G_u = (3\pi/128)(k_B T)^2/(hd)^2 K_a \quad (3)$$

where d is the spacing between membranes, and h is the width of membrane. The K_a value is the area elastic modulus. At $T < T_m$, the stiff membrane with low membrane fluidity showed a large K_a value, as shown in Table 1. A stiff lipid membrane such as DMPC has a small ΔG_u, which is disadvantageous to the compensation of the activation energy ΔE for the bending of lipid membranes during the GV formation process. In the case of DPPC, it is considered that the extremely small ΔG_u cannot compensate for ΔE for the DPPC membrane (see Table 1 and Figure 4c).

In conclusion, we presented an experimental procedure to analyze the growth behavior of GVs. The membrane fluidity of GVs proportional to the permeability to solvent was well correlated with the growth rate constant of GVs obtained from first-order kinetics. Although further investigation is needed, we quantitatively showed that the swelling model was the reasonable mechanism of GV electroformation.

Materials and Methods

Chemicals. Phospholipids used are POPC, DOPC, DPPC, DMPC, and DSPC, which were purchased from Avanti Polar Lipids (Alabaster, AL, USA). A fluorescent probe, TMA-DPH, was purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents of analytical grade were purchased from Wako Pure Chemicals.

Preparation of GVs by Electric Field. The GVs were prepared according to the previous literature.[1−3] The phospholipids were dissolved in diethylether-chloroform (9:1 v/v) solvent. The phospholipid concentration was 0.3 mg-POPC/mL of solvent. Two drops of this solution (2 μL each, 789 pmol) were deposited on two parallel platinum electrodes (diameter 0.5 mm, separation distance 3 mm) to spread them over along the entire length of each wire. The solvent was then evaporated under nitrogen. One milliliter of distilled water or protein solution was added, and then the electric field of AC 1.5 V (10 Hz) were applied at a temperature of 25−45 °C, using a function generator (SG-4101, Iwatsu Corp., Japan). The temperature of the solution in the cell was controlled by a rubber heater and monitored with a thermo recorder (RT-30S, ESPEC MIC. Corp., Japan). The general observations were performed under a phase contrast inverted microscope from Nikon (Japan). The images were acquired by a charge-coupled device (CCD) video camera (module C2400, Hamamatsu Photonics (Japan)). Images were taken by using an image processor ARGUS 20 and software HPD-CP extended from Hamamatsu Photonics K.K. (Japan). In order to avoid artifacts in the observations, GVs were observed at a constant focus depth. The diameters of the vesicles were adopted as the values parallel to the electrodes.

Preparation of SUVs and GVs with the Hydration Method. SUVs were prepared as described before.[4] In brief, lipids were solved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under vacuum overnight, was hydrated with the appropriate buffer solution (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). The suspension was subjected to five cycles of freezing and thawing and then extruded through a polycarbonate filter (100-nm pore size filter, 11 times) using a Liposofast extruder (Avestin, Ottawa, Canda). The lipid concentration was determined in triplicate by phosphorus analysis.

GVs were prepared according to a previous procedure.[6,11,16] The dried lipid was hydrated by the appropriate solution at 60 °C for 3 h. The floating lipid aggregates were sucked with a glass pipet several times. The lipid aggregates were dispersed, and we could observe GVs more than 1 μm in diameter by using photomicroscopy. The diameter of more than 100 GVs was measured to evaluate the average diameter.

Membrane Fluidity. Membrane fluidity of liposome was determined as fluorescence polarization (P) by measuring the fluorescent intensity of TMA-DPH, according to the conventional method.[30−32] The fluorescence probes were oriented into a lipid bilayer by the following method. A solution of TMA-DPH (in ethanol) was added to a liposome suspension to maintain a lipid/probe molar ratio of 250 ([TMA-DPH]$_{final}$ = 2 μM). The mixture was then incubated at least for 1 h at room temperature with gentle stirring. The probe remaining in the outer aqueous phase of the liposomes was negligible since they have little fluorescence in water. The fluorescence intensity of the samples was measured with a spectrofluorimeter to which excitation and analyzing polarizers (FP-777 JASCO Co. Ltd., Japan) were attached. The sample was excited with vertically polarized light (360 nm) and then the emission intensity at 430 nm of both parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the excited light was recorded. The P value of TMA-DPH was calculated from next equation.

$$P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp) \quad (4)$$

The reciprocal value of polarization ($1/P$) was defined as membrane fluidity.

Area Elastic Modulus of GV Membrane. The measurement of the are elastic modulus of GVs was performed according to a previous report.[13,24,33] In brief, the micropipet suction technique was used to measure the area elastic modulus K_a. The change in the surface area of GV at its suction process into the micropipet (ΔA) was estimated with the image processor. The membrane tension τ through the suction of GV was estimated from the pipet caliber (diameter), the diameter for the spherical segment of the GV sucked into the pipet, and the suction pressure. The area elastic modulus K_a can be given by the relationship: $\tau = K_a\alpha$, where α equals to the value $\Delta A/A_0$ (A_0 is the initial surface area of GV).

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