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Cell-Free Protein Synthesis inside Giant Unilamellar Vesicles Analyzed by Flow Cytometry

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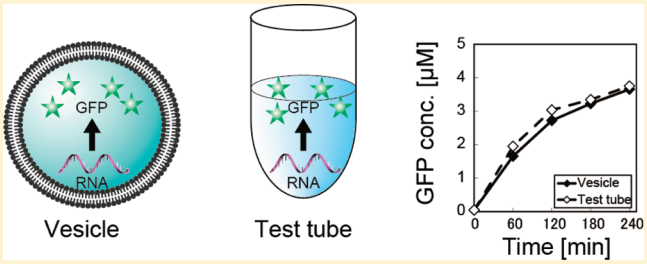
Supporting Information

ABSTRACT: Lipid vesicles have been used as model cell systems, in which an in-vitro transcription−translation system (IVTT) is encapsulated to carry out intravesicular protein synthesis. Despite a large number of previous studies, a quantitative understanding of how protein synthesis inside the vesicles is affected by the lipid membrane remains elusive. This is mainly because of the heterogeneity in structural properties of the lipid vesicles used in the experiments. We investigated the effects of the phospholipid membrane on green fluorescent protein (GFP) synthesis occurring inside cell-sized giant unilamellar vesicles (GUV), which have a defined quantity of lipids relative to the reaction volume. We first developed a method to distinguish GUV from multilamellar vesicles using flow cytometry (FCM). Using this method, we investigated the time course of GFP synthesis using one of the IVTT, the PURE system, and found that phospholipid in the form of GUV has little effect on GFP synthesis based on three lines of investigation. (1) GFP synthesis inside the GUV was not dependent on the size of GUV (2) or on the fraction of cholesterol or anionic phospholipid constituting the GUV, and (3) GFP synthesis proceeded similarly in GUV and in the test tube. The present results suggest that GUV provides an ideal reaction environment that does not affect the internal biochemical reaction. On the other hand, we also found that internal GFP synthesis is strongly dependent on the chemical composition of the outer solution.



1. INTRODUCTION

It is becoming increasingly evident that the intracellular environment is very different from the conditions under which biochemical reactions are measured and analyzed in vitro. For example, the intracellular space of the cell is filled with macromolecules at high volume occupancy,[1] and these crowding conditions are known to affect biochemical reactions.[2] All present cells have a cell boundary, the presence of which may be one of the parameters that affect the intracellular reactions, because the reactant molecules should frequently collide with the boundary consisting of lipid molecules.[3] One strategy to investigate how lipid molecules in the form of a cell-sized boundary affect intracellular reactions is to carry out biochemical reactions inside artificial lipid vesicles (liposomes) and compare the results with those obtained in the test tube.[4]

A number of researchers, including our group,[5] have attempted to construct model cell systems, with an in-vitro transcription−translation system (IVTT) encapsulated in lipid vesicles.[6] In these studies, interesting characteristic features were observed. For example, Nomura et al.[6a] showed that green fluorescent protein (GFP) synthesis proceeded faster in lipid vesicles than in the test tube, while the final yield was nearly identical. Other reports also suggested that protein synthesis in lipid vesicles proceeds differently from that in the test tube,[5a,d,6c,e] although the mechanisms underlying the alterations in the reaction remains unclear in most of these studies.

Despite a large number of previous studies, we still do not have a quantitative understanding of how protein synthesis inside the vesicles is affected by the lipid membrane. This is mainly due to the heterogeneity in structural properties of the lipid vesicle used in the experiments. It is well-known that structural properties, such as size, lamellarity, and internal substructures of the lipid vesicles, vary significantly depending on the preparation methods used.[4a] Previous studies have used various lipid vesicles, including giant multilamellar vesicles

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(MLV), which are known to have a complex internal substructure,[5b] making it difficult to assess the effects of lipids quantitatively. Large unilamellar vesicles (LUV) a few 100 nm in diameter have also been used;[6c] however, this is different from the size of a living cell, and encapsulation of IVTT at this scale is still a matter of debate.[7]

Giant unilamellar vesicles (GUV) measuring greater than 1 μm are ideal for studying how the lipid membrane affects intracellular reactions. This is because GUV have a size that is more typical of living cells and because of the lack of an internal substructure. Therefore, we can study the extent of the effect by defining the quantity of lipids relative to the reaction volume in each vesicle. There are several strategies to prepare GUV,[4a,8] which have been used to investigate protein synthesis in lipid vesicles,[6a,b,d,f,9] but the vesicles generated still include a fraction of multilamellar vesicles in the population and they show a large degree of heterogeneity in size.[8] Therefore, it is first necessary to develop a method to distinguish unilamellar from multilamellar vesicles and to selectively analyze the reaction inside the GUV. The size heterogeneity can be utilized to study the effects of relative lipid quantity. Nevertheless, to explore this aspect, it is necessary to analyze a large number of vesicles to obtain statistical data.

In this study, we encapsulated the PURE system, a reconstituted in-vitro transcription−translation system, together with the RNA encoding GFP in GUV prepared by the water-in-oil (w/o) emulsion transfer method,[6f,10] and the internal reaction was analyzed by flow cytometry (FCM). FCM allows the measurement of multiple properties of a large number of vesicles (>50 000 vesicles/s) including the internal aqueous volume, lamellarity, and the time course of GFP synthesis inside,[4b,11] thus allowing us to study the correlations of these parameters. To investigate whether lipid molecules affect the internal GFP synthesis, we analyzed the reaction in GUV of different size and phospholipid compositions. GUV with different sizes have different surface-to-volume ratios, and carrying out protein synthesis in such GUV enabled us to investigate the effects of lipid concentration on internal protein synthesis. We found that the time course as well as the final yield of GFP did not differ among the GUV with inner aqueous volumes of 1−100 fL (1.24−5.76 μm in diameter). The reaction was nearly identical in GUV and in the test tube. Furthermore, increasing the cholesterol or charged lipid composition did not affect GFP synthesis. While GFP synthesis was unaffected by the lipid molecules in the form of GUV, we found that the internal GFP synthesis is strongly dependent on the chemical composition of the outer solution.

2. EXPERIMENTAL SECTION

In-Vitro Transcription and Translation System. GFP synthesis was carried out using a reconstituted in-vitro transcription−translation system (PURE system[12]). The composition of components was as described previously.[5b] For GFP synthesis, aliquots of 20 μL of IVTT containing 4 units of RNasin (Promega), 1 μM transferrin Alexa Fluor 647 conjugate (TA647; Invitrogen), 200 mM sucrose, and RNA encoding GFP were prepared and incubated at 37 °C. The GFP DNA fragment was amplified by PCR with PYRObest DNA polymerase (Takara) according to the manufacturer's instructions using pETG5tag[5a] as a template with the primers sense 02 (5′-CTCCTTTCAGCAAAAAACCCCTCAAGACCC-3′) and antisense 02 (5′-CCCGCGAAATTAATACGACTCACTATAGGG-3′). The resulting PCR products were used directly for in-vitro transcription by adding 150 μg of PCR fragments to 800 μL mixtures consisting of 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 0.4 mM NTPs, and 20 μg of T7 RNA polymerase and incubated at 37 °C for 5 h. RNA was purified using an RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The GFP used was GFPuv5, which was constructed previously.[13]

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG), and 18:1−12:0 (Square-685) PC were purchased from Avanti Polar Lipids, and cholesterol was from Nacalai Tesque. R-Phycoerythrin and transferrin Alexa Fluor 647 conjugate (TA647) were from Invitrogen.

Preparation of Giant Unilamellar Vesicles. We used a modified version of the protocol presented previously.[6f,8] POPC, POPG, and cholesterol were dissolved individually in chloroform at a concentration of 100 mg/mL. Liquid paraffin (product number 128-04375; Wako Pure Chemical Industries) was then added to bring the lipid concentration to 5 mg/mL and heated at 80 °C for 20 min to completely dissolve the lipids and evaporate the chloroform. The liquid paraffin solutions were then mixed to prepare mixtures of phospholipids and cholesterol. This was designated as the oil phase.

Aliquots of 30 μL of the inner solution (see below for the composition) were added to 300 μL of the oil phase. These mixtures were vortexed for 30 s to form w/o emulsions that were then equilibrated on ice for 10 min. Then, 250 μL of the oil phase was placed gently on top of 225 μL of the outer solution, and centrifuged at 18000*g*, 4 °C, for 30 min. The pelleted vesicles were collected through a hole opened at the bottom of the tube. When the composition of the outer solution was changed, the vesicles were pelleted by centrifugation at 18000*g* for 10 min at 4 °C, and the supernatant was replaced with the desired solution. This procedure was repeated twice to replace the outer solution.

The inner solution was the PURE system supplemented with 200 mM sucrose, 1 μM TA647, template RNA or DNA, and 0.2 U/μL RNasin. The outer solution consisted of the small molecular weight components of the PURE system (0.3 mM each amino acid, 3.75 mM ATP, 2.5 mM GTP, 1.25 mM CTP and UTP, 1.5 mM spermidine, 25 mM creatine phosphate, 1.5 mM dithiothreitol (DTT), 0.01 μg/μL N5-formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid (FD), 280 mM potassium glutamate, 18.9 mM $Mg(OAc)_2$, 100 mM HEPES) supplemented with 200 mM glucose. Glucose was added at the same molarity as the sucrose in the inner solution to adjust the osmolarity. When investigating the relationship between lipid quantity and aqueous volume of each vesicle (Figure 2), 0.2% 18:1−12:0 (Square-685) PC (molar ratio) was included in the lipid mixture, and the IVTT was supplemented with 400 nM R-phycoerythrin instead of TA647.

Flow Cytometric Analysis. Four fluorescent signals from GFP, R-phycoerythrin, TA647, and Square-685 were measured by FCM (BD FACSAria Cell Sorter). We obtained 100 000 data samples for each measurement. Briefly, GFP was excited with a 488 nm semiconductor laser, and the emission was detected through a 530 ± 15 nm bandpass filter. R-phycoerythrin was excited with a 488 nm semiconductor laser, and the emission was detected through a 585 ± 21 nm bandpass filter. TA647 and Square-685 were excited with a HeNe laser (633 nm), and the emission was detected through a 660 ± 10 nm bandpass filter. Prior to the measurement, vesicles were diluted to an appropriate concentration with the dilution buffer (50 mM HEPES-KOH, pH 7.6, 13 mM $Mg(OAc)_2$, 100 mM potassium glutamate, and 200 mM glucose). Unilamellar vesicles were defined as those in the region that satisfied $\log(FS) > 1.5 \times \log(SS) - 1$, where FS and SS are forward scattering and side scattering intensities, respectively. Fluorescence intensities of GFP and TA647 were converted to the number of GFP and TA647 molecules using the strategies described previously.[5] All calculations were performed with programs developed in our laboratory.

Microscopic Observation. Micrographs were obtained using an inverted light microscope (IX70; Olympus) with a ×100 oil-immersion objective lens and a digital color CCD camera (VB-7000; Keyence). Bright-field images were obtained by differential interference contrast observation. Fluorescence images of the orange and green marker molecules (R-phycoerythrin and GFP, respectively) were

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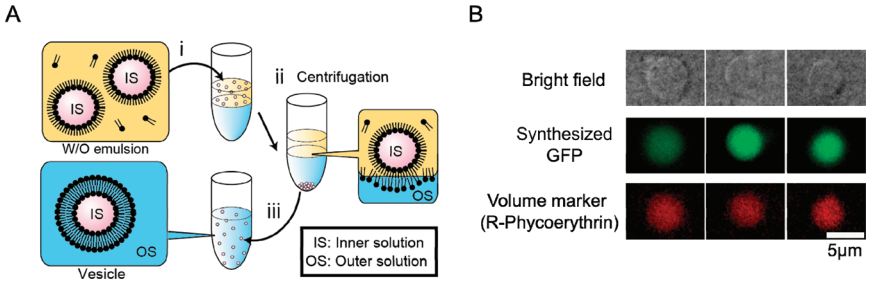

Figure 1. Preparation of GUV and micrographs. (A) Schematic of GUV preparation method. (i) Inner solution (IS) was dispersed in the oil phase consisting of phospholipids to prepare the w/o emulsion, and overlaid on the outer solution (OS). (ii) The w/o emulsion droplets were transformed into vesicles by centrifugation. (iii) Vesicles were collected from the bottom of the tube. (B) Micrographs of the vesicles after GFP synthesis. R-Phycoerythrin was used to visualize the internal aqueous solution of the vesicles.

obtained through corresponding filters and dichroic mirror units (Cy3-4040C; Semrock and NIBA; Olympus).

3. RESULTS

GFP Synthesis inside Vesicles. Vesicles carrying the IVTT (PURE system[12]) were prepared as shown schematically in Figure 1A. The method was developed based on the strategies described previously.[6f,8] Briefly, the IVTT (inner solution) was dispersed in the oil phase containing phospholipids to prepare w/o emulsion droplets. This emulsion was placed on top of the water phase (outer solution) and centrifuged to obtain vesicles. The components constituting the IVTT used can be classified into two groups: macromolecules (e.g., ribosomes, tRNAs, and protein components) and small molecular weight compounds (e.g., NTPs and amino acids) (see Experimental Section for details). In the outer solution, all small molecular weight compounds were included at the same concentrations as with the IVTT, whereas none of the macromolecules were included. Note that the IVTT (inner solution) contained 200 mM sucrose and the outer solution contained 200 mM glucose. The difference in specific gravity facilitated vesicle formation at the water/oil interface by centrifugal force (Figure 1A). The vesicles were prepared at 4 °C to prevent GFP synthesis from occurring during the preparation steps and then placed at 37 °C to initiate the reaction. To investigate the effects of lipid on the translation process and to exclude the possibility of detecting the effect on transcription, GFP synthesis was performed with RNA. Protein synthesis occurs only inside the vesicles, as there were no macromolecules in the outer solution. Note that including the macromolecules in the outer solution did not affect the reaction inside the vesicles (Figure S1). Therefore, they were omitted from the outer solution for simplicity. Figure 1B shows a micrograph of the vesicles after 3 h of incubation at 37 °C. The green fluorescence indicated synthesis of GFP.

Identification of GUV by FCM. The major portion of vesicles prepared by the w/o emulsion transfer method was determined previously to be unilamellar by FCM.[8] To examine whether the vesicles encapsulating IVTT prepared in this study are also unilamellar, vesicles were prepared using lipids supplemented with 18:1−12:0 (Square-685) PC and the IVTT supplemented with R-phycoerythrin. 18:1−12:0 (Square-685) PC and R-phycoerythrin were used to determine the relative membrane quantity and aqueous volume of each vesicle, respectively. The measure of vesicle volume is based on the observation that the encapsulation efficiency is nearly 100%[10,14] (i.e., the concentration of volume marker before and after encapsulation is nearly identical). Vesicles were then analyzed by FCM. A characteristic pattern was seen in the 2D density plot of the intensities from the relative membrane quantity and volume (Figure 2A). The ridge line of the most

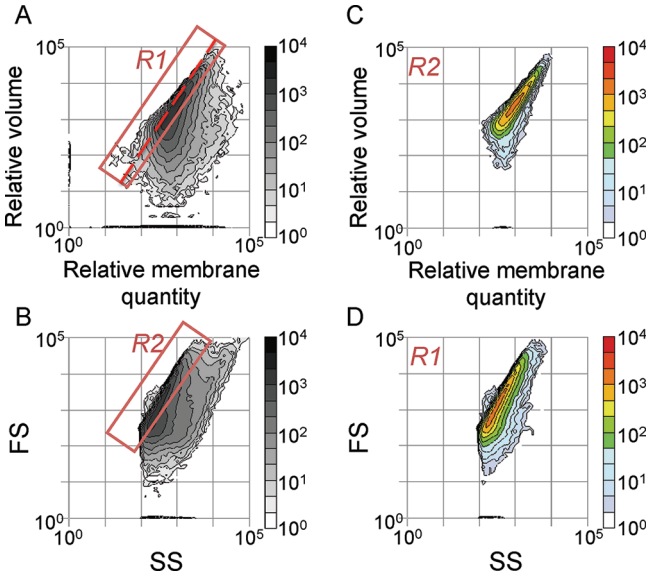

Figure 2. Identification of GUV from the FCM data. (A) 2D density plots of the relative aqueous volume and lipid membrane quantity of each vesicle, which are determined as the fluorescence intensities of R-phycoerythrin and 18:1−12:0 (Square-685) PC, respectively. Vesicles in region R1 are denoted as giant unilamellar vesicles (GUV) in accordance with our previous study.[8] The dashed line is the ridge line of the most frequent vesicle population, which has a slope of 1.5. (B) 2D plots of forward scattering (FS) and side (SS) scattering light intensities of the vesicles obtained simultaneously with (A). R2 is the region where vesicles in R1 appear predominantly. (C) 2D density plots of the relative aqueous volume and lipid membrane quantity of the vesicles in R2. (D) 2D density plots of FS and SS of the vesicles in R1.

frequent vesicle population (dashed line) had a slope of 1.5 on a logarithmic scale. As a slope of 1.5 represents the scaling exponent of the volume-to-surface ratio of a sphere,[8] vesicles scattered along this line are nearly spherical in shape. According to our previous study,[8] we refer to the vesicles in region R1 of Figure 2A as giant unilamellar vesicles (GUV). We then investigated where the GUV appeared in the two-dimensional (2D) plot of forward scattering (FS) and side scattering (SS) light intensities (Figure 2B). Comparison of the data shown in parts B and D of Figure 2 indicates that GUV appear in a specific region of the 2D plot. We then investigated the

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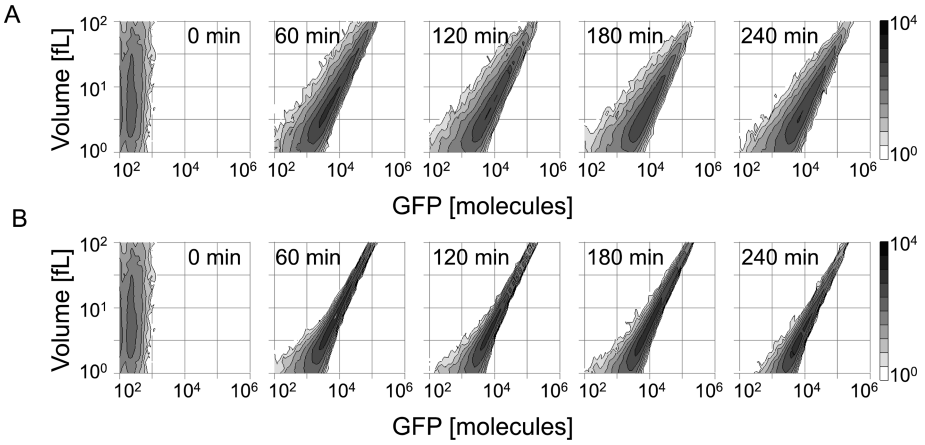

Figure 3. 2D density plots of vesicles obtained by FCM. (A) Results of in-vesicle GFP synthesis are shown. IVTT with 500 nM RNA encoding GFP was encapsulated into vesicles, incubated at 37 °C, and subjected to FCM analysis at the times indicated in the figure. (B) Results of GFP synthesis in bulk are shown. IVTT with 500 nM GFP encoding RNA was incubated at 37 °C, encapsulated into vesicles at the times indicated in the figure, and subjected to FCM analysis. The lipid composition used to prepare the GUV was POPC:cholesterol = 9:1 (weight ratio).

correspondence of these populations in reverse: where the vesicles present in the region R2 (Figure 2B) of the 2D plot of FS and SS light intensities appear in the 2D plot of surface area and the volume of the vesicles. The results indicated that 90% of the vesicles in R2 were in R1 (Figure 2A,C), and therefore vesicles that appear in R2 are considered to be GUV. In this way, we identified the GUV without using fluorescence signals. Note that the fraction of vesicles in R2 was typically between 20% and 40%, a substantial fraction of the total population. We analyzed GFP synthesis within the GUV, i.e., vesicles appearing only in region R2 in the FS and SS plot.

Quantitative Analysis of GFP Synthesis in GUV Using FCM. We analyzed the time course of GFP synthesis using 500 nM RNA in GUV. Here, transferrin conjugated with alexa647 (TA647), which exhibits red fluorescence, was included in the IVTT as a volume marker. By measuring the red and the green fluorescence intensities of each vesicle, we obtained the internal aqueous volume (vertical axis, Figure 3) and the number of GFP molecules (horizontal axis, Figure 3), respectively. Figure 3A shows the time evolution of the 2D density plot, indicating the relationship between synthesized GFP and vesicle volume. These vesicles showed increased green fluorescence signal over time, indicating the occurrence of GFP synthesis. To compare the GFP synthesis in GUV with that in the test tube, GFP synthesis was performed in test tubes at identical RNA concentrations. Aliquots of the reaction mix sampled at various incubation time points were then encapsulated into vesicles using the method shown in Figure 1A, and the resulting GUV was analyzed by FCM (Figure 3B).

As shown in Figure 3A,B, the number of GFP molecules and the size of each GUV were known. Thus, we estimated the GFP concentration in each GUV and calculated the mean GFP concentration at each time point. Figure 4A shows the time course data. GFP synthesis in GUV was almost identical to that in the test tube. Identical experiments were performed at different RNA concentrations (56 and 167 nM), and similar results were obtained (Figure 4A, 2D density plots are shown in Figure S2). These results indicate that the presence of lipid molecules has little effect on GFP synthesis. The results obtained in GUV and in the test tube were also similar when GFP synthesis was performed with DNA (Figure S3),

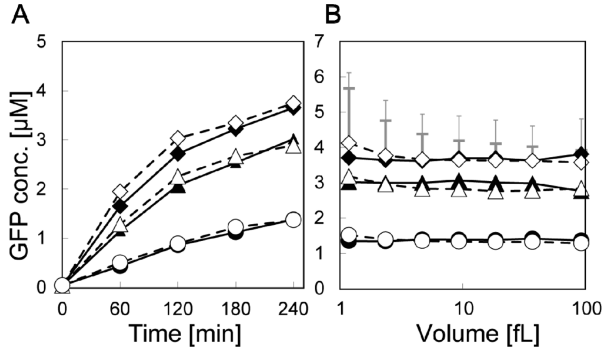

Figure 4. Comparison between GFP synthesis in the vesicles and in bulk. (A) Time course of GFP synthesis in vesicles (●, ▲, ♦) and in bulk (○, △, ◇), at 56 (○, ●), 167 (△, ▲) and 500 nM (◇, ♦) RNA. The mean GFP concentrations are plotted. (B) Yield of GFP after 4 h of incubation at 37 °C and its dependency on vesicle size. The symbols are the same as in (A). The mean GFP concentrations at each vesicle size are plotted. Error bars with thin and thick lines are the standard deviation of the GFP concentrations in vesicles and in bulk, respectively. For clarity, we show the bars of only 500 nM RNA, but similar results were obtained with other RNA concentrations. Reactions in vesicles show large variability than that in the test tube. 2D density plots used to obtain the data presented here are shown in Figure S2. The lipid composition used to prepare the GUV is POPC:cholesterol = 9:1 (weight ratio).

indicating that the presence of lipid molecules also has little effect on transcription.

GFP Synthesis in GUV of Different Sizes. The surface-to-volume ratio increases as the vesicle size decreases; i.e., the smaller the vesicle, the larger the nominal lipid concentration for the molecules inside the vesicle. If lipid molecules have an effect on GFP synthesis, we would expect a pronounced effect in smaller GUV. Therefore, the vesicle size dependencies of the yield of GFP synthesis were investigated (Figure 4B, time course data are shown in Figure S4). Figure 4B shows the relationship between the yield of GFP after 4 h of reaction and GUV size. The yield after 4 h of incubation was dependent on the RNA concentration used; however, at identical RNA concentration, the yield of GFP did not differ among vesicles of different size, regardless of whether synthesis was done in the vesicles or in the test tube. These results further indicate that

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the presence of lipid molecules in the form of GUV has little effect on GFP synthesis.

Effects of Lipid Composition on Internal GFP Synthesis. The results shown in Figures 3 and 4 were obtained with GUV composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC):cholesterol = 9:1 (weight ratio). Phospholipid was reported previously to affect biochemical reactions.[5d,15] For example, Bui et al.[15c] reported that adding POPC-based LUV to GFP synthesis using IVTT increased the yield of fluorescence of GFP by 1.5-fold relative to that in the test tube. Addition of cholesterol further increased the yield. Another study indicated that adding charged lipids inhibited GFP synthesis.[15b,d] These observations prompted us to explore the effects of GUV lipid composition on internal GFP synthesis.

First, when the fraction of cholesterol was altered from POPC:cholesterol = 9:1 to 10:0 or 8:2, we did not observe any significant effect on GFP synthesis (Figure 5A, 2D density plots

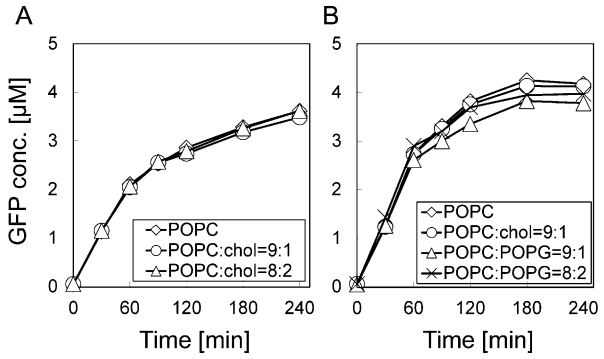

Figure 5. Time courses of GFP synthesis in vesicles of different lipid compositions. (A) Time courses of GFP synthesis with different cholesterol concentrations and (B) POPG concentrations. 2D density plots used to obtain the data presented here are shown in Figures S5 and S6. The RNA concentration used is 500 nM.

are shown in Figure S5). Second, when POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]) was added at different concentrations, we also did not see any effect on GFP synthesis (Figure 5B and Figure S6). Thus, GFP synthesis proceeded similarly irrespective of alterations in the lipid composition, indicating that the lipid composition has little effect on internal GFP synthesis, at least over the ranges of cholesterol and POPG concentrations examined here.

Effects of Membrane Permeability on Internal GFP Synthesis. We showed that GFP synthesis proceeds inside the GUV as in the test tube (Figure 4A) and that the reaction inside the GUV is unaffected by the compartment size (Figure 4B) or the lipid composition (Figure 5). Next, we investigated how the intravesicular reaction is affected by the permeability properties of the lipid bilayer. As the lipid bilayer is known to allow the selective passage of small and/or hydrophobic molecules,[16] we investigated the possibility that the permeability of the GUV may affect the internal reaction.

We altered the concentrations of small molecular weight compounds between the inner and outer solutions of the GUV, thereby creating osmotic stress, and investigated its effect on internal GFP synthesis. Note that macromolecules such as proteins were unlikely to be permeable for several reasons. First, the size distribution of the vesicles in GUV did not change during 4 h of incubation (Figure S7A), indicating that TA647 (molecular weight 80 kDa) used as a volume marker present in the IVTT was not permeable. Second, GFP synthesis proceeded in GUV as in the test tube (Figure 4), irrespective of the presence or absence of macromolecules in the outer solution (Figure S1). If macromolecules including proteins were permeable, then GFP synthesis would not have occurred as in the test tube when macromolecules were absent in the outer solution as the activity of IVTT is sensitive to the macromolecule concentration.[17]

We then investigated the permeability of small molecular weight components (Figure 6). For this purpose, GUV were

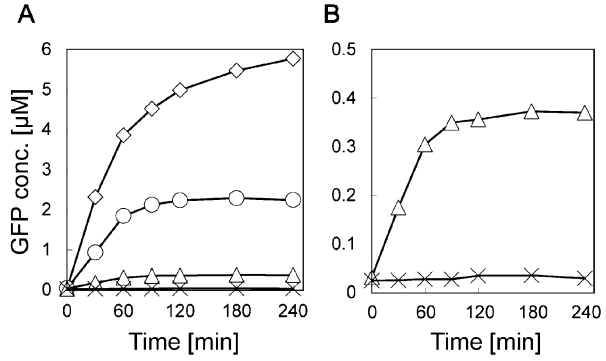

Figure 6. Effects of outer solution on GFP synthesis inside the GUV. The lipid composition used to prepare the GUV is POPC:cholesterol = 9:1 (weight ratio). The concentrations of small molecular weight components (e.g., amino acids and NTPs) in the inner and outer solutions were altered. (A) Time course of the reaction with balanced inner and outer solutions with the concentration of the original solution (◇), that when only the concentration of outer solution was reduced to 1/4 of the original (○), that when the concentrations of outer and inner solution were both reduced to 1/4 (×), and that when only the concentration of inner solution was reduced to 1/4 of the original (△) are shown. (B) Magnification of the data when the concentrations of outer and inner solutions were both reduced to 1/4 (×) and that when only the concentration of inner solution was reduced to 1/4 of the original (△) are shown. The RNA concentration used is 500 nM.

first prepared by the w/o emulsion transfer method as shown in Figure 1A. At this stage, the concentrations of small molecular weight components in the inner and outer solutions of the GUV were balanced. The GUV were then transferred into an outer solution in which the concentrations of small molecular weight components (e.g., 20 amino acids and 4 nucleotides), except sucrose were reduced to 1/4 of those in the original outer solution described in the Experimental Section. When GFP synthesis was carried out under these conditions, the yield decreased to 1/3 relative to that in the original outer solution (Figure 6A). This result suggested that internal small molecular weight components leaked from inside to outside of the GUV. When we reduced the concentration of small components to 1/4 of the original both inside and outside the GUV, GFP synthesis did not occur. GFP synthesis proceeded when these GUV were transferred into the original outer solution (Figure 6B). This result suggested the influx of small molecular weight components from outside to inside of the GUV resumed GFP synthesis. Exchanging the outer solution for fresh original outer solution prior to initiating incubation at 37 °C had little effect on the internal GFP synthesis (Figure S8), indicating that exchanging the outer solution itself neither destroys nor affects the internal reaction.

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4. DISCUSSION

We investigated the effects of the phospholipid membrane on GFP synthesis occurring inside cell-sized GUV using one of the IVTT, the PURE system. We first developed a method using FCM to distinguish GUV from other vesicles and analyzed the intravesicular GFP synthesis. The results obtained by three lines of investigation, which were consistent with each other, indicated that lipids in the form of GUV had little effect on GFP synthesis. (1) We found that the GFP synthesis inside the GUV was not dependent on either the size (Figure 4) (2) or on the fraction of cholesterol or anionic phospholipid constituting the GUV (Figure 5) and (3) GFP synthesis in GUV proceeded similarly to that in the test tube (Figure 4).

Previously, we studied protein translation by IVTT encapsulated in MLV,[5a,d] which had an internal substructure that made it difficult to estimate the effective lipid concentration inside the vesicle. We found in our previous study that GFP synthesis was unaffected by the volume of the MLV, and the effect of increasing the fraction of POPG to 20% was negligible, while increasing up to 39% or 58% inhibited the synthesis.[5d] Therefore, the previous results were consistent with those of the present study despite the difference in the vesicles used and the presence of the internal substructure.[5b]

Next, we discuss the extent of interaction between the lipid membrane and the intravesicular reactions in the form of GUV containing the IVTT. The effective lipid concentrations inside the GUV, defined by the number of moles of lipid in the inner membrane leaflet over the vesicle volume, are 5.2 and 2.4 mM for 10 and 100 fL, respectively. Bui et al.[15c] investigated the effects of lipids on the *Escherichia coli* extract-based cell-free translation system by adding LUV to the solution at a lipid concentration between 3 and 6 mM. The results indicated that POPC-based LUV increase the yield of GFP fluorescence by 1.5-fold relative to that in the test tube, and the addition of cholesterol further increases the yield. These results are different from those of the present study, in which GFP synthesis proceeded similarly in both GUV and in the test tube (Figure 4). There are several possible explanations for the difference in results between the two studies, including the difference in IVTT (we used IVTT consisting of only defined components, i.e., the PURE system,[12] whereas Bui et al. used *E. coli* cell extract) and vesicle preparation methods used. While further comprehensive studies are required using fully defined IVTT and lipid compartments, our results suggest that phospholipid in the form of GUV may have less effect on protein synthesis than in the other form.

Although the average dynamics of GFP synthesis inside GUV were nearly identical to those in the test tube (Figure 4), the number of GFP molecules in the vesicles (Figure 3, upper) showed greater variability than in the test tube (Figure 3, lower). Saito et al.[6d] recently studied GFP synthesis in phospholipid vesicles prepared by the w/o emulsion transfer method and reported large variability among vesicles, while the average profile was similar to that in bulk. Our results are consistent with this observation. While further investigations are required, the variability may be due to the heterogeneity in the permeability properties of each vesicle or phospholipids modulating the activity of the intravesicular molecules. The variability in number of synthesized GFP is unlikely to be caused by the stochastic nature of the encapsulation process.[5c,6c] The component with the lowest concentration in the PURE system used here is nucleoside diphosphate kinase (NDK), present at 16 nM,[5b] at which concentration a vesicle with a volume of 1 or 100 fL would hold on average 9.6 or 960 NDK molecules, respectively. Therefore, all components including NDK should have an identical concentration in all GUVs.

Our results also suggested that protein synthesis inside GUV is very sensitive to the concentrations of small molecular weight components in the outer solution. Leakage of small molecular weight components from inside to outside the GUV reduced, while permeation into GUV increased the yield of internal GFP synthesis (Figure 6). On the other hand, GFP synthesis proceeded inside the GUV as in the test tube when the concentrations of small molecular weight components in inner and outer solutions were balanced (Figure 4A). In general, smaller and more hydrophobic molecules tend to have higher permeability.[16] It is not clear exactly which molecule(s) among those included in the IVTT permeated through the membrane, but the discrepancies among previous studies may be partially due to the permeability of the vesicles. For example, Yamaji et al.[6b] reported that the yield of GFP synthesis in the vesicles was lower than that in bulk. Based on our results, this observation can be explained by the permeability of the vesicle membrane. Small chemical components, including amino acids and NTPs, were added only to the inside of the vesicles, and thus leakage of these compounds must have occurred during synthesis, which resulted in a lower yield of GFP inside the vesicles. While the possibility of permeability affecting the GFP synthesis inside the vesicles has been suggested previously,[6f] we directly demonstrated here that the chemical composition of the external solution strongly affects GFP synthesis in GUV. Furthermore, while the permeation of nutrients across the bilayer into the interior space has been shown to induce intravesicular polymerization of nucleic acids,[18] we have shown that the permeability of the membrane can allow protein synthesis—a much more complex reaction—to occur.

In summary, we investigated the time course of GFP synthesis with IVTT inside GUV prepared by the w/o emulsion transfer method. Our results suggest that GUV provide an ideal reaction environment that does not affect the internal biochemical reaction. Our strategy of synthesizing protein in GUV together with analysis using FCM may be a useful platform to study various properties of the reactions in cell-size microcompartments.

■ ASSOCIATED CONTENT

Supporting Information

Effects of the presence of macromolecules in the outer solution during intravesicular GFP synthesis; 2D density plots of vesicle volume and number of GFP molecules at different RNA concentration and lipid composition; comparison between GFP synthesis in the vesicles and in bulk using 10 nM DNA encoding the GFP as a template; time courses of GFP synthesis in GUV of different sizes; size distribution of GUV during GFP synthesis; time courses of GFP synthesis when the outer solution was exchanged. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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