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Research Article

Different protein localizations on the inner and outer leaflet of cell-sized liposomes using cell-free protein synthesis

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Abstract

Membranes of living cells possess asymmetry. The inner and outer leaflets of the membrane consist of different phospholipid compositions, which are known to affect the function of membrane proteins, and the loss of the asymmetry has been reported to lead to cell apoptosis. In addition, different proteins are found on the inner and outer leaflets of the membrane, and they are essential for various biochemical reactions, including those related to signal transduction and cell morphology. While *in vitro* lipid bilayer reconstitution with asymmetric phospholipid compositions has been reported, the reconstitution of lipid bilayer where different proteins are localized in the inner and outer leaflet, thereby enables asymmetric protein localizations, has remained difficult. Herein, we developed a simple method to achieve this asymmetry using an *in vitro* transcription–translation system (IVTT). The method used a benzylguanine (BG) derivative-modified phospholipid, which forms a covalent bond with a snap-tag sequence. We show that purified snap-tagged protein can be localized to the cell-sized liposome surface via an interaction between BG and the snap-tag. We then show that IVTT-synthesized proteins can be located at the lipid membrane and that different proteins can be asymmetrically localized on the outer and inner leaflets of liposomes.

Key words: liposome; flow cytometer; asymmetry; *in vitro* transcription and translation.

Introduction

Living cells possess a number of different molecules in their membranes. One of the major components of the cell membrane is the phospholipid bilayer, which maintains biomolecular content inside the cell. This phospholipid composition is known to be asymmetric; the inner and outer leaflets of the membrane consist of different phospholipid compositions with important biological roles. For example, for the mammalian plasma membrane, phosphatidylserine (PS) is enriched in the inner leaflet, and exposure of PS to the outer leaflet leads to apoptosis. Several reports have also shown that asymmetric phospholipid composition is important for membrane protein topology and function (1–3). Asymmetry also exists for peripheral membrane proteins. Different proteins exist on the inner and outer leaflets of the membrane, which is essential for various biochemical reactions, including those related to signal transduction (4) and cell shape determination (5).

To study the importance of these asymmetries, researchers have been working on mimicking asymmetric localization *in vitro*. For example, methods for generating lipid vesicles

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(liposomes) with an asymmetric phospholipid composition have been reported (6,7). Membrane protein insertion efficiency was reported to be affected by the asymmetry of phospholipid composition (7). Another report shows that asymmetry affects the functioning of ion channels (8).

Mimicking the asymmetric localization of proteins on the lipid membrane has also been tested. For example, actin filament formation has been studied at the inner or outer leaflet of the liposome to understand membrane–cytoskeleton interactions *in vitro* (9). It has been shown that localizing a large amount of protein to the outer leaflet of the liposome membrane resulted in membrane tabulation and deformation (10,11). Methods to localize proteins on a single side (leaflet) of the liposome have been reported. However, thus far, it remains difficult to localize different proteins on the inner and outer leaflet of the liposome *in vitro*. Once this method has been established, the effect of asymmetric protein localization on membrane morphology can be studied more in detail. In addition, reconstituting signal transduction pathways that require various peripheral and integral membrane proteins in their correct topology may become possible.

In this study, we construct a system to place different proteins on the inner and outer leaflet of a cell-sized liposome using cell-free protein synthesis. This placement was achieved by using a benzylguanine derivative (BG)-modified phospholipid, which forms a covalent bond with a snap-tag sequence (12,13), a cell-sized liposome (14) and an *in vitro* transcription–translation system (IVTT) (15,16). The snap-tag sequence has a size of approximately 20 kDa and is a mutant sequence of human DNA repair protein O^6-alkylguanine DNA alkyltransferase, and BG acts as a suicide inhibitor of this enzyme (12). A previous report (10) showed the localization of snap-tagged protein on a BG-modified phospholipid containing liposome. However, the results were not described quantitatively, and more importantly, asymmetric localization of different proteins in the outer and inner leaflet was not achieved. Herein, we first show green fluorescent protein (GFP) localization to the cell-sized liposome surface via the BG and snap-tag interaction and quantify the density and amount of localized protein. Second, we show that IVTT can be used to provide the protein to be localized on the lipid membrane, and finally, we show that different proteins, GFP and mCherry, can be localized specifically on the inner and outer leaflets of liposomes, respectively.

Materials and methods

BG-modified lipid synthesis

To a mixture of distilled water (10 μl) and DMSO (70 μl), a 100 mg/ml chloroform solution of DSPE-(PEG2000)-amine (20 μl, 720 nmol) was added. The mixture was sonicated for 10 min, and the chloroform was removed using a centrifugal evaporator. Then, a 50 mM DMSO solution of BG-GLA-NHS (20 μl, 1 μmol) and 100 mM aqueous $NaHCO_3$ (20 μl, 2 μmol) were added. After incubation with shaking at room temperature for 1 h, 0.38% formic acid and acetonitrile were added to quench the reaction. The desired product was purified with a reverse-phase HPLC (XBridge C8, 2.5 μm, 4.6 × 20 mm, flow rate 1.5 ml min^{-1} with a linear gradient of 0–100% methanol in 0.38% formic acid over 15 min) to afford BG-DSPE (460 nmol; 64% yield). The product was identified by MALDI-TOF-MS (calculated *m*/z value = 3138.91 for $[M+H]^+$ in which the number of ethylene glycol units is 45, observed *m*/z value = 3138.82).

Reagents and materials

The IVTT system used in this study is a reconstituted IVTT (the PURE system (17)), which was modified to synthesize more proteins than the original one (15,16). The DNA encoding the snap-tag fused to the N-terminus of GFP (pET-snap-GFP) and mCherry (pET-snap-mCherry) was constructed using a GeneArt Seamless Cloning and Assembly Enzyme Mix (Thermo Fisher Scientific, Waltham, MA, USA) using pSNAP-tag (New England Biolabs, Ipswich, MA, USA), pET-GFPuv5 (18) and mCherry sequences (Takara Bio USA Inc., Mountain View, CA, USA) as templates. Both snap-GFP and snap-mCherry are encoded under the control of the T7 promoter and Shine-Dalgarno sequence. Templates for IVTT were prepared by polymerase chain reaction (PCR) amplification of pET-GFPuv5, pET-snap-GFP and pET-snap-mCherry plasmids, respectively, using T7F (5′-TAATACGACTCACTATAGGG) and T7R (5′-GCTAGTTATTGCTCAGCGG) primers. PCR was performed using Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions unless otherwise noted. The PCR product was purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. SNAP-Cell 430 used for the competition experiment was obtained from New England Biolabs. Egg phosphatidylcholine (PC) (Coatsome NC50) was obtained from the NOF Corporation (Tokyo, JAPAN). 1, 2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-(PEG2000)-amine) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl-(polyethylene glycol)-2000] (biotin-DSPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The biotin-DSPE solution was prepared as previously described (19). BG-GLA-NHS was obtained from New England Biolabs. Streptavidin-R-phycoerythrin was from Sigma Aldrich (St. Louis, MO, USA). Snap-GFP and snap-mCherry were over expressed in *Escherichia coli* and purified using Ni-NTA superflow (QIAGEN) according to the manufacturer's instructions.

Protein synthesis inside liposomes

Liposomes with egg PC containing reconstituted IVTT were prepared using the water-in-oil (W/O) emulsion/transfer method (14) as previously described (20,21). Briefly, 20 μl of reconstituted IVTT was supplemented with template DNA, 200 mM sucrose, 0.8 U/μl RNase inhibitor and 1.5 μM ovalbumin Alexa Fluor 647 conjugate (OA647, Thermo Fisher Scientific, Waltham, MA, USA); 200 μl of liquid paraffin (Wako, Osaka, Japan) containing 1.7 mg of egg PC in the absence or presence of 5 μg of BG-DSPE was then added. The fluorescence signal of OA647 was used to calculate the aqueous volume of each vesicle (see below). The mixtures were vortexed for 30 s to form W/O emulsions that were then equilibrated on ice for 10 min. The solution was gently placed on top of 200 μl of the outer solution (see below for the composition) and centrifuged at 18 000 × *g* for 30 min at 4°C. The pelleted liposomes were collected through an opening at the bottom of the tube. Proteins were synthesized by incubating the liposomes at 37°C. The outer solution contained the low-molecular weight components of reconstituted IVTT [0.3 mM concentrations of 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 10-formyl-5,6,7,8-tetrahydrofolic acid, 280 mM potassium glutamate, 18 mM $Mg(OAc)_2$ and 100 mM HEPES (pH 7.6)].

Binding of purified snap-GFP to BG-modified liposomes

Preparation of the liposomes without IVTT was performed essentially the same as described above except that 100 mM

HEPES (pH 7.6), 300 mM sucrose, 700 mM glucose and 1.5 μM OA647 was used as the inner solution, whereas 100 mM HEPES (pH 7.6) and 1 M glucose was used as the outer solution. When necessary, snap-mCherry was added to the inner solution (see Supplementary Figure S2). The liposome solution was diluted 5-fold with the outer solution (100 mM HEPES-KOH (pH 7.6), 1 M glucose), and the snap-GFP solution was added at each final concentration, followed by incubation for 0.5 h at 37°C. Following these preparations, the liposomes were centrifuged once at 8000 ×*g* for 5 min to replace the supernatant with fresh outer solution and remove unbound snap-GFP.

Protein synthesis both inside and outside liposomes

The liposome encapsulating the IVTT solution and snap-mCherry-encoding DNA were prepared as described above, and this liposome solution was centrifuged at 8000 ×*g* for 5 min at 4°C. The supernatant was then replaced with fresh reconstituted IVTT supplemented with snap-GFP encoding DNA and 0.8 U/μl of RNase inhibitor. Proteins inside and outside the liposomes were synthesized simultaneously by incubation at 37°C. For Proteinase K (Nacalai Tesque, Kyoto, Japan), the treatment liposome was incubated with 7.1 μg/ml protease for 0.5 h at 37°C.

Fluorescence cytometer analysis and confocal microscopy

The fluorescent signals from the liposomes were measured by FACS (FACSAriaIII or FACSVerse; BD Biosciences, Franklin Lakes, NJ, USA). Before fluorescence cytometer (FCM) analysis, the liposome suspension was diluted 10- or 40-fold in dilution buffer (liposomes with IVTT were diluted with 100 mM HEPES-KOH (pH 7.6), 280 mM potassium glutamate, 19 mM $Mg(OAc)_2$ and 200 mM glucose, whereas liposomes without IVTT were diluted with 100 mM HEPES-KOH (pH 7.6) and 1 M glucose).

The vesicle volumes were calculated from the OA647 fluorescence intensity obtained with FCM using the equation $\log(N_{A647}) = 0.8431\ \log(FI_{647}) + 1.7696$, where N_{A647} (molecule) is the number of Alexa Fluor 647 molecules in the liposomes and FI_{647} is the OA647 fluorescence intensity. The equation was obtained as follows. First, a correlation between the amount of Alexa Fluor 647, and its fluorescence intensity was obtained by measurements of the calibration beads (Quantum Alexa Fluor 647 MESF Beads, Bang Laboratories, Technology Drive Fishers, IN, USA) by FCM. Second, by knowing the number of Alexa Fluor 647 conjugated to each ovalbumin molecule (2 Alexa Fluor 647 molecules/ovalbumin) and the concentration of OA647 inside the liposome (1.5 μM = 903 molecules/fL), we obtained the aqueous volume of the liposome (V (fL) = N_{A647}/2/903). The number of snap-GFP molecules on the liposome surface was obtained using the equation $\log(N_{GFP}) = 0.83\ \log(FI_{GFP}) - 2.5802$, where N_{GFP} (molecules) is the number of snap-GFP molecules, and FI_{GFP} is the snap-GFP fluorescence obtained with FCM. The equation was obtained by encapsulating known concentrations (0.8, 1.6 and 5.4 μM) of snap-GFP in the liposome together with 1.5 μM OA647 and measuring the resulting liposome by FCM.

Assuming a sphere with a radius of r, the volume V and surface area A of the sphere can be written as $4/3\pi r^3$ and $4\pi r^2$, respectively. Using these two relationships, we obtain the following equation:

$$\frac{V}{\frac{4}{3}\pi} = \left(\frac{A}{4\pi}\right)^{3/2}$$

When plotting the relationship between V and A in a log–log plot, a slope of 3/2 = 1.5 is predicted to appear (Figure 1C).

Fluorescence images of the liposome membrane were obtained using a confocal laser scanning microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany) and a 63× oil immersion objective.

Results and discussion

Cell-sized liposome with BG-modified phospholipids

BG-modified 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(poly ethylene glycol)-2000] (BG-DSPE) was synthesized as described in the Materials and methods section (see Supplementary Figure S1). BG-DSPE was added to a final concentration of 0.3% (w/w) to egg-derived phosphatidylcholine (egg PC), and the cell-sized liposome was prepared using a water-in-oil emulsion transfer method as described previously (20,21). As a control, the liposome was prepared with commercially available modified phospholipid, biotinylated DSPE (biotin-DSPE) (Figure 1A). Inside the liposome, ovalbumin conjugated with Alexa Fluor 647 (OA647) was incorporated, which was used as an indicator of the aqueous volume of the liposome. First, cell-sized liposomes were prepared, and then, purified proteins were added such that only the outer leaflet of the liposome was decorated with the proteins. We used a FCM to analyze the fluorescence of cell-sized liposomes (Figure 1).

When a biotin-modified liposome (egg PC + 0.3% (w/w) biotin-DSPE) was mixed with GFP fused to a snap-tag (snap-GFP), the liposome showed no detectable fluorescence, whereas a strong increase in the fluorescence signal was observed when the liposome was mixed with R-phycoerythrin conjugated with streptavidin (PE-SA) (Figure 1B (vii and viii)). On the other hand, when a BG-modified liposome (egg PC + 0.3% (w/w) BG-DSPE) was mixed with snap-GFP, the liposome showed strong fluorescence, whereas no detectable increase in the fluorescence signal was observed when the liposome was mixed PE-SA (Figure 1B (ix and x)). No detectable fluorescence was observed when modified lipids were absent or when no fluorescent proteins were added ((i)–(vi)). These results suggest that BG-modified liposomes were prepared successfully, and the snap-tagged protein was localized on BG-modified liposomes.

It should be noted that in the log–log plot, the liposome population was distributed along the line with a slope of 1.5 (dashed line), indicating that GFP or PE fluorescence is proportional to the surface area of the vesicle (21,22). A detailed mathematical description is provided in the Materials and methods section.

Quantitative analysis of snap-tagged GFP binding to BG-modified liposomes

To investigate the amount of BG on the surface of the liposome, the BG-modified liposome was mixed with different concentrations of purified snap-GFP (Figure 2A). First, cell-sized liposomes were prepared, and then, purified snap-GFP was added such that only the outer leaflet of the liposome was decorated with snap-GFP. An increase in GFP fluorescence was observed with increasing concentrations of snap-GFP (Figure 2A). The fluorescence signal was saturated above 3 μM snap-GFP (Figure 2B). From the GFP fluorescence intensity obtained by FCM at this saturation condition, the density of the BG-DSPE was calculated. First, the aqueous volume of the liposome was calculated from the OA647 fluorescence intensity. Second, assuming that the

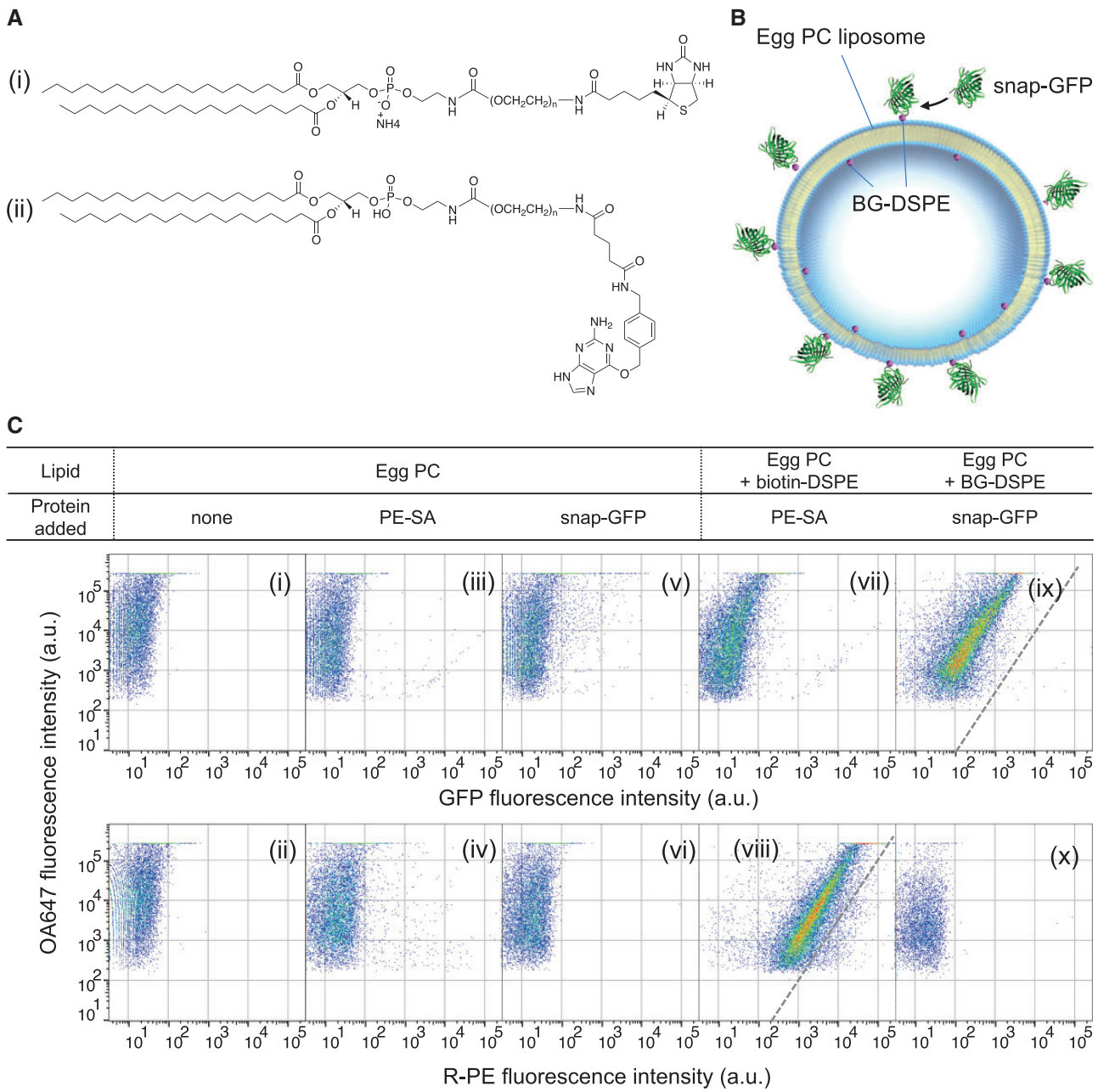

Figure 1. Protein localization on the surface of cell-sized liposomes. (**A**) Chemical structures of (i) biotin-DSPE and (ii) BG-DSPE. (**B**) Schematic of protein localization on the surface of cell-sized liposomes via the BG and snap-tag interaction. (**C**) FCM analysis of egg PC liposomes and egg PC with biotin or BG-DSPE added to cell-sized liposomes. The 2D plot shows the relationship between OA647 fluorescence intensity, representing the aqueous volume of the liposome and GFP or PE fluorescence intensity. Dashed lines show a line with a slope of 1.5 in the log–log plots.

liposome is a sphere, the surface area was calculated from the liposome volume. Third, the number of snap-GFP molecules on each liposome was calculated from the GFP fluorescence obtained by FCM. Finally, we found the density of snap-GFP to be 7700 molecules/μm^2 on average at the outer leaflet. The number of membrane proteins of the *E. coli* cell is approximately 1.7×10^5 molecules/μm^2 (23), and thus, the amount of snap-GFP is 20-fold lower than that of *E. coli* cells. Nevertheless, large amount of proteins can be localized on the liposome surface.

The number of phospholipid molecules in the lipid bilayer is approximately 8.3×10^6 molecules/μm^2 (per layer) (23). We added 0.3% (w/w) BG-DSPE, and assuming that the weight of the BG-DSPE is 4-fold greater than that of the egg PC constituting lipids, 6200 BG molecules/μm^2 are present. As we found 7700 molecules of snap-GFP/μm^2, nearly 100% of BG molecules were occupied with snap-GFP molecules.

In addition to quantitative analysis, we investigated the specificity of snap-GFP binding to BG (Figure 2C). We performed a competition experiment, where 1 μM snap-GFP was mixed with a 10 μM concentration of one of the substrates of snap-tag, SNAP-Cell 430 (diethylaminocoumarin conjugated with BG), and then added it to the BG-modified liposome. We see a fluorescence signal similar to the background when SNAP-Cell 430 was mixed with snap-GFP prior to addition to the liposome (Figure 2C). This result indicates the specific binding of snap-tag GFP to BG on the liposome surface.

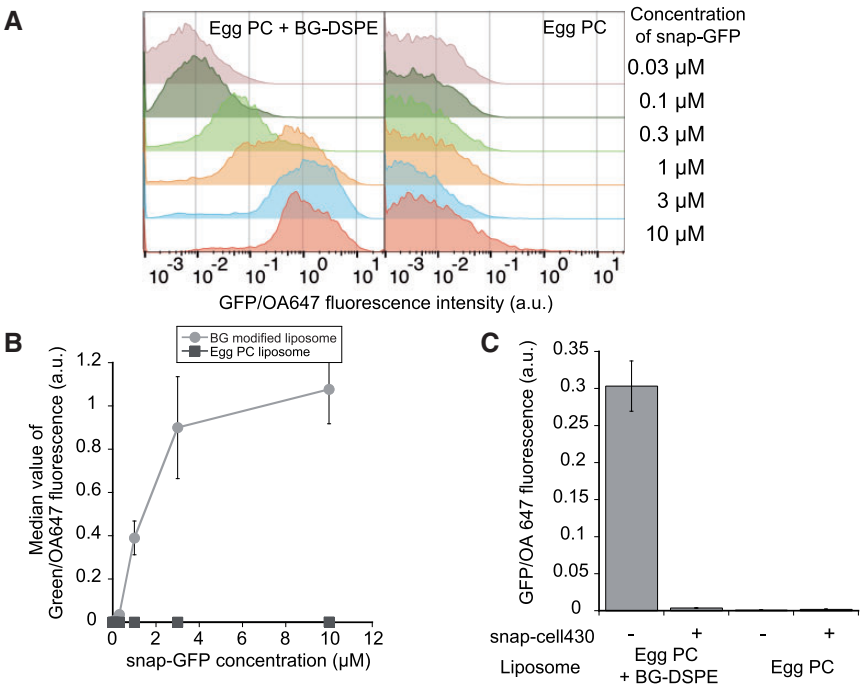

Figure 2. Snap-GFP binding to BG-modified liposomes. (**A**) Histogram of the GFP/OA647 fluorescence intensity obtained by FCM. (**B**) Relationship between the median value of (A) and the snap-GFP concentration. (**C**) Competition experiment: 1 μM snap-GFP was mixed with (+) or without (−) 10 μM SNAP-Cell 430 and added to egg PC or BG-modified liposomes. The resulting liposomes were analyzed by FCM, and the median value was measured. The average and standard error of the triplicates are shown.

Cell-free synthesis of GFP using BG-modified liposomes

Thus far, purified snap-GFP, which was over-expressed and purified from *E. coli* cells, was used. Protein preparation in this way is time-consuming and laborious, and moreover, it is difficult to locate different proteins inside and outside of the liposome. The water-in-oil (W/O) emulsion/transfer method (14), which we used in the current study, is among the most frequently used methods to encapsulate a protein of interest inside cell-sized liposomes. With this method, an aqueous solution consisting of proteins is first mixed with phospholipid dissolved oil to form W/O emulsions. This emulsion containing oil phase is then placed on top of a water phase and centrifuged. When the W/O emulsions transfer the oil–water interface, W/O emulsions are transformed into unilamellar vesicles. During this process, not all W/O emulsions are transformed into vesicles. Many of them collapse at the oil–water interface.

When BG-DSPE is present in the oil phase and a snap-tagged protein is present in the water phase, most of the snap-tagged proteins are expected to bind BG-DSPE inside the W/O emulsion, however, snap-tagged protein bound BG-DSPE molecule can also be present at the oil–water interface before centrifugation. In addition, because of the collapse of the emulsions during centrifugation, snap-tagged protein bound BG-DSPE molecule will also be present in the outer solution of the liposome, which can get inserted into the outer leaflet of the liposome. For this reason, it is difficult to localize the purified protein to only the inner leaflet of liposomes. Indeed, while we aimed to localize a snap-tagged protein only at the inner leaflet, we found the presence of snap-tagged protein at the outer leaflet of the liposome (see Supplementary Figure S2). We thus used cell-free protein synthesis to supply the proteins.

In this study, we used an *E. coli*-based reconstituted IVTT (15), where the minimal components required for protein synthesis are highly purified individually and reconstituted to perform protein synthesis *in vitro*. Reconstituted IVTT was encapsulated into cell-sized liposomes, and protein synthesis was performed. Two different proteins, GFP and snap-GFP, were synthesized inside the liposome. The FCM data show that GFP fluorescence was detected for both GFP and snap-GFP, indicating that they were synthesized inside the liposome (Figure 3A).

Different from Figure 1C, in the log–log plot of Figure 3A, the liposome population was distributed along the line with a slope of 1 (dashed line), indicating that GFP fluorescence is proportional to liposome size. This result is because the amount of DNA, which determines the amount of synthesized GFP and snap-GFP, incorporated in the liposome is proportional to liposome size.

The liposomes were further analyzed by confocal fluorescence microscopy. We clearly detected the localization of GFP fluorescence with snap-GFP, whereas no clear localization was observed with GFP (Figure 3B). OA647 was encapsulated together with the IVTT as a marker of the aqueous volume of the liposome, showing a homogeneous distribution inside the liposome regardless of the synthesized protein. These results indicated that the snap-GFP synthesized within the liposomes was properly folded and localized on the lipid membrane.

The second order rate constant of BG and snap-tag interaction has been reported as 2.8×10^4 ($M^{-1}s^{-1}$) (13). As 6200 BG molecules/μm^2 are present, this value corresponds to 16 μM BG in a liposome with 30 fL, which is an average size of liposomes. When the BG concentration >> synthesized protein concentration, we obtain a pseudo first order rate constant of $0.45\,s^{-1} = (2.8 \times 10^4\,M^{-1}s^{-1} \times 16\,\mu M)$, and thus, the half-life of the snap-tagged protein is $1.5\,s = (\ln(2)/0.45)$. This calculation indicates that snap-tagged proteins are localized within a few second after their synthesis.

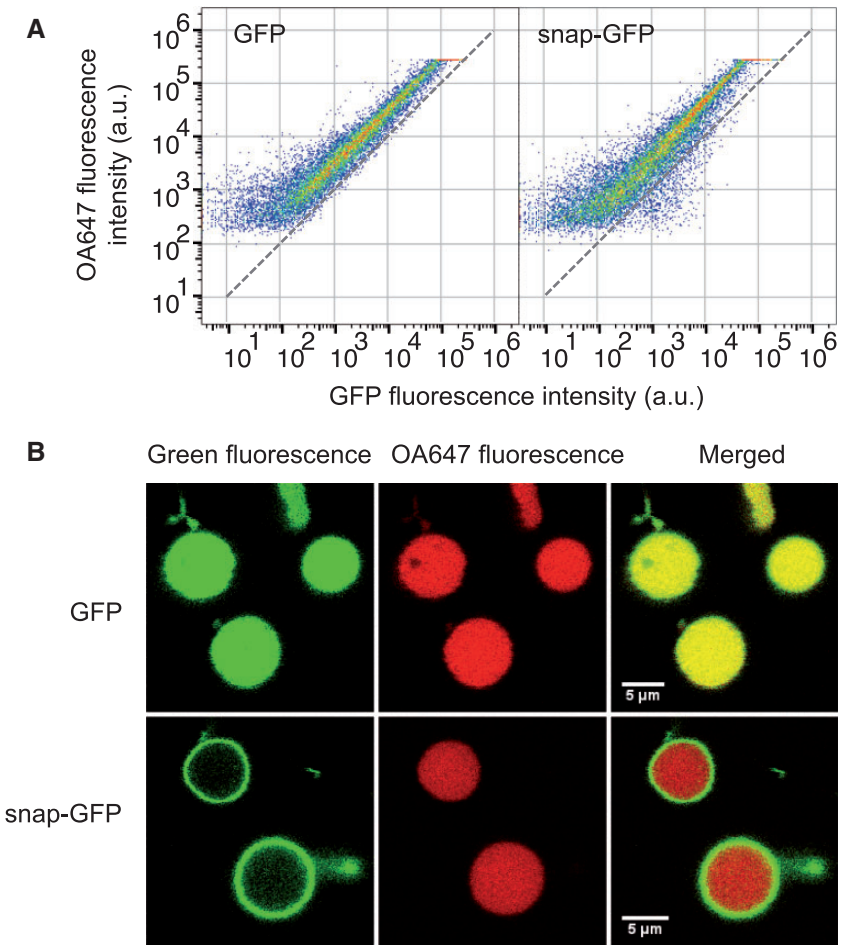

Figure 3. GFP and snap-GFP synthesis using reconstituted IVTT inside cell-sized liposomes. Protein synthesis was performed using 5 nM DNA and incubation at 37°C for 2 h. (**A**) The 2D plot shows the relationship between OA647 fluorescence intensity, representing the aqueous volume of the liposome and GFP fluorescence intensity. Dashed lines show a line with a slope of 1 in the log–log plots. (**B**) Confocal images of liposomes with GFP-encoding DNA and snap-GFP-encoding DNA.

Asymmetric localization of different proteins on cell-sized liposomes using cell-free synthesis

Reconstituted IVTT was used to prepare cell-sized liposomes with asymmetric protein membrane localization. mCherry fused to snap-tag (snap-mCherry) and snap-GFP were synthesized on the inside and outside of the liposome, respectively (Figure 4A). The resulting liposomes were analyzed by FCM and confocal microscopy (Figure 4B and C).

FCM data show that both mCherry and GFP fluorescence are present on the cell-sized liposome, indicating the presence of two fluorescent proteins (Figure 4B). Furthermore, the confocal image shows that both fluorescence signals are localized on the lipid membrane (Figure 4C). To investigate the membrane localization of snap-GFP and snap-mCherry, protease treatment was used. Proteinase K was added to the liposome solution, which is expected to degrade proteins localized at the outer leaflet of the liposome membrane but not those at the inner leaflet. Figure 4B shows that only the GFP fluorescence decreased upon the addition of Proteinase K, indicating the asymmetric localization of proteins on the lipid bilayer.

Conclusion

Cell-free protein synthesis is a powerful tool not only for protein production but also for protein characterization and engineering (24–27). Herein, we show that a cell-free system can be adopted to create cell-sized vesicles with asymmetric protein localization, partially mimicking living cells. Nickel (Ni) ion- and biotin-modified phospholipids are commercially available (both from Avanti Polar lipids). These lipids, in principle, can be used to prepare liposomes with different proteins on the inner and outer leaflets of the liposome. However, there have been no reports on generating liposomes with asymmetric protein localization, even with these lipids. Herein, we used BG and snap-tag, which form a covalent bond; this interaction is different from the Ni ion and polyhistidine tag interaction or biotin–streptavidin interaction. Snap-tag is a monomeric protein, whereas streptavidin is a homotetramer, and thus, protein localized on the lipid via streptavidin will be in very close proximity, which might result in unwanted interactions. The Ni ion and polyhistidine tag interaction is affected by reagents in the IVTT, including reducing reagents, and amino acids, whereas the BG and snap-tag interaction is not. Nevertheless, under an appropriate condition, Ni ion- and biotin-modified phospholipids can be used together with BG to localize a variety of proteins to the lipid surface.

It has been shown that localizing a large amount of protein to the membrane resulted in membrane tabulation and deformation (10,11). While previous studies have shown the crowding environment only on the outer leaflet of the membrane, our

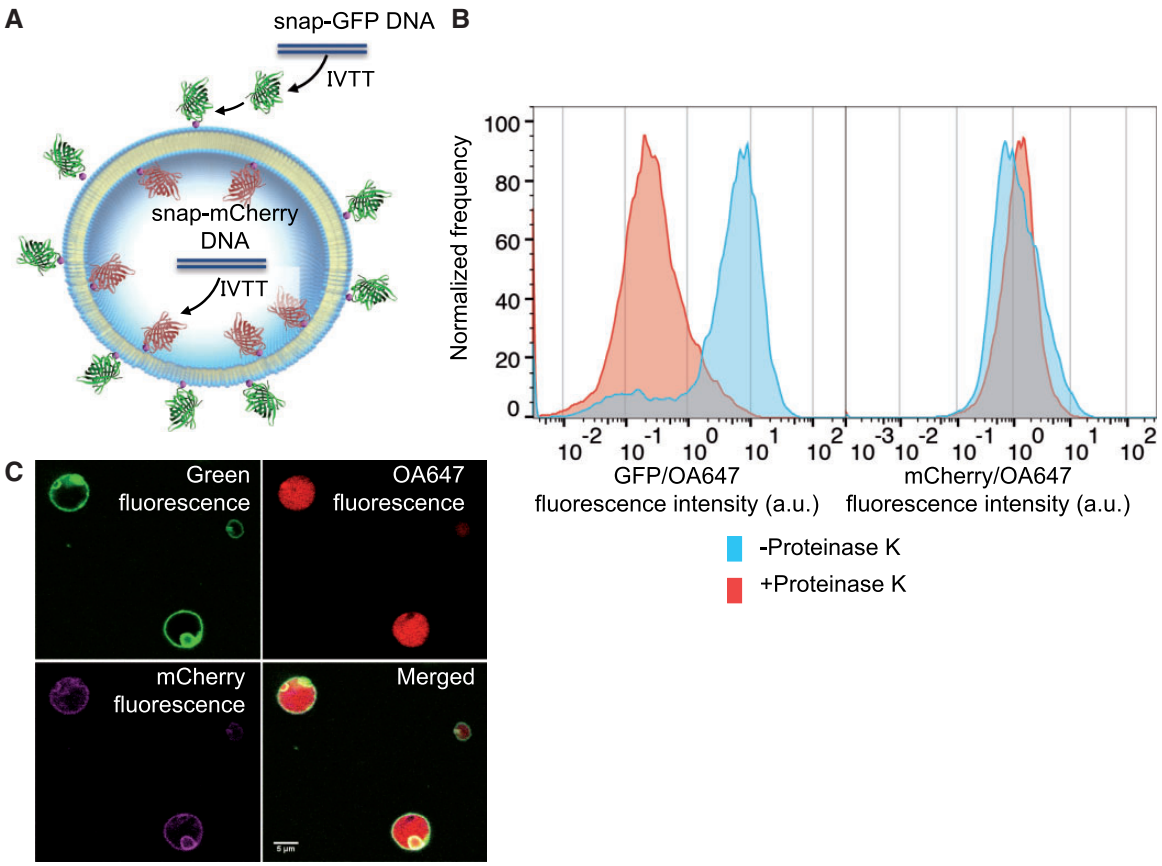

Figure 4. Asymmetric localization of proteins on the inner and outer leaflets of cell-sized liposomes. (**A**) Schematic of the asymmetric localization. (**B**) A 2D plot shows the relationship between OA647 fluorescence intensity, representing the aqueous volume of the liposome and GFP or mCherry fluorescence intensity. (**C**) Confocal images of liposomes. Protein synthesis was performed using 5 nM and 0.15 nM DNA and incubation at 37°C for 2 h. Proteinase K treatment was performed with 7.1 μg/ml protease for 0.5 h at 37°C.

method enabled the investigation of crowding on both the inner and outer leaflets. In addition, BG molecules at the outer and inner leaflet might be used to locate peripheral and integral membrane proteins in the lipid bilayer more effectively with a defined topology. While there are a few reports on incorporating the translocation machinery that brings the membrane proteins to the bilayer into IVTT (28–30), IVTT lacks this machinery in most cases. Although it does not directly promote the insertion of the proteins into membranes, the BG and snap-tag interaction might be useful for localizing membrane proteins in their functional form by rapidly bringing the synthesized protein into close proximity to the lipid bilayer. In addition, proteins localized at the outer leaflet can be molecules such as antibodies that enable the immobilization of liposomes at the desired surface. Lastly, together with membrane protein synthesis technology inside cell-sized liposomes (31–33), this artificial cellular system is another step forward toward synthetic cell construction.

SUPPLEMENTARY DATA

Supplementary Data are available at SYNBIO Online.

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Conflict of interest statement. None declared.

References

1. Henderson,J.C., Zimmerman,S.M., Crofts,A.A., Boll,J.M., Kuhns,L.G., Herrera,C.M. and Trent,M.S. (2016) The power of asymmetry: architecture and assembly of the gram-negative outer membrane lipid bilayer. *Annu. Rev. Microbiol.*, 70, 255–278.
2. Marquardt,D., Geier,B. and Pabst,G. (2015) Asymmetric lipid membranes: towards more realistic model systems. *Membranes (Basel)*, 5, 180–196.
3. Murate,M., Abe,M., Kasahara,K., Iwabuchi,K., Umeda,M. and Kobayashi,T. (2015) Transbilayer distribution of lipids at nano scale. *J. Cell Sci.*, 128, 1627–1638.
4. Hubbard,S.R. and Till,J.H. (2000) Protein tyrosine kinase structure and function. *Annu. Rev. Biochem.*, 69, 373–398.
5. Salbreux,G., Charras,G. and Paluch,E. (2012) Actin cortex mechanics and cellular morphogenesis. *Trends Cell Biol.*, 22, 536–545.
6. Pautot,S., Frisken,B.J. and Weitz,D.A. (2003) Engineering asymmetric vesicles. *Proc. Natl. Acad. Sci. USA*, 100, 10718–10721.

7. Kamiya,K., Kawano,R., Osaki,T., Akiyoshi,K. and Takeuchi,S. (2016) Cell-sized asymmetric lipid vesicles facilitate the investigation of asymmetric membranes. *Nat. Chem.*, 8, 881–889.
8. Iwamoto,M. and Oiki,S. (2015) Contact bubble bilayers with flush drainage. *Sci. Rep.*, 5, 9110.
9. Vogel,S.K. and Schwille,P. (2012) Minimal systems to study membrane-cytoskeleton interactions. *Curr. Opin. Biotechnol.*, 23, 758–765.
10. Rudd,A.K., Valls Cuevas,J.M. and Devaraj,N.K. (2015) SNAP-tag-reactive lipid anchors enable targeted and spatiotemporally controlled localization of proteins to phospholipid membranes. *J. Am. Chem. Soc.*, 137, 4884–4887.
11. Stachowiak,J.C., Schmid,E.M., Ryan,C.J., Ann,H.S., Sasaki,D.Y., Sherman,M.B., Geissler,P.L., Fletcher,D.A. and Hayden,C.C. (2012) Membrane bending by protein-protein crowding. *Nat. Cell Biol.*, 14, 944–949.
12. Keppler,A., Gendreizig,S., Gronemeyer,T., Pick,H., Vogel,H. and Johnsson,K. (2003) A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. *Nat. Biotechnol.*, 21, 86–89.
13. Gautier,A., Juillerat,A., Heinis,C., Correa,I.R. Jr, Kindermann,M., Beaufils,F. and Johnsson,K. (2008) An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol.*, 15, 128–136.
14. Pautot,S., Frisken,B.J. and Weitz,D.A. (2003) Production of unilamellar vesicles using an inverted emulsion. *Langmuir*, 19, 2870–2879.
15. Shimizu,Y., Inoue,A., Tomari,Y., Suzuki,T., Yokogawa,T., Nishikawa,K. and Ueda,T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.*, 19, 751–755.
16. ——, Kanamori,T. and Ueda,T. (2005) Protein synthesis by pure translation systems. *Methods*, 36, 299–304.
17. Kazuta,Y., Matsuura,T., Ichihashi,N. and Yomo,T. (2014) Synthesis of milligram quantities of proteins using a reconstituted *in vitro* protein synthesis system. *J. Biosci. Bioeng.*, 118, 554–557.
18. Ito,Y., Suzuki,M. and Husimi,Y. (1999) A novel mutant of green fluorescent protein with enhanced sensitivity for microanalysis at 488 nm excitation. *Biochem. Biophys. Res. Commun.*, 264, 556–560.
19. Nishimura,K., Matsuura,T., Sunami,T., Fujii,S., Nishimura,K., Suzuki,H. and Yomo,T. (2014) Identification of giant unilamellar vesicles with permeability to small charged molecules. *RSC Adv.*, 4, 35224–35232.
20. ——, ——, Nishimura,K., Sunami,T., Suzuki,H. and Yomo,T. (2012) Cell-free protein synthesis inside giant unilamellar vesicles analyzed by flow cytometry. *Langmuir*, 28, 8426–8432.
21. ——, Hosoi,T., Sunami,T., Toyota,T., Fujinami,M., Oguma,K., Matsuura,T., Suzuki,H. and Yomo,T. (2009) Population analysis of structural properties of giant liposomes by flow cytometry. *Langmuir*, 25, 10439–10443.
22. Soga,H., Fujii,S., Yomo,T., Kato,Y., Watanabe,H. and Matsuura,T. (2014) *In vitro* membrane protein synthesis inside cell-sized vesicles reveals the dependence of membrane protein integration on vesicle volume. *ACS Synth. Biol.*, 3, 372–379.
23. Phillips,R., Kondev,J. and Theriot,J. (2009) *Physical Biology of the Cell*. Garland Science, New York.
24. Endo,Y. and Sawasaki,T. (2006) Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.*, 17, 373–380.
25. Yokoyama,S. (2003) Protein expression systems for structural genomics and proteomics. *Curr. Opin. Chem. Biol.*, 7, 39–43.
26. Swartz,J.R. (2001) Advances in *Escherichia coli* production of therapeutic proteins. *Curr. Opin. Biotechnol.*, 12, 195–201.
27. Carlson,E.D., Gan,R., Hodgman,C.E. and Jewett,M.C. (2012) Cell-free protein synthesis: applications come of age. *Biotechnol. Adv.*, 30, 1185–1194.
28. Matsubayashi,H., Kuruma,Y. and Ueda,T. (2014) In *vitro* synthesis of the E. coli sec translocon from DNA. *Angew. Chem. Int. Ed. Engl.*, 53, 7535–7538.
29. Ohta,N., Kato,Y., Watanabe,H., Mori,H. and Matsuura,T. (2016) *In vitro* membrane protein synthesis inside Sec translocon-reconstituted cell-sized liposomes. *Sci. Rep.*, 6, 36466.
30. Wuu,J.J. and Swartz,J.R. (2008) High yield cell-free production of integral membrane proteins without refolding or detergents. *Biochim. Biophys. Acta*, 1778, 1237–1250.
31. Uyeda,A., Nakayama,S., Kato,Y., Watanabe,H. and Matsuura,T. (2016) Construction of an *in vitro* gene screening system of the *E. coli* EmrE transporter using liposome display. *Anal. Chem.*, 88, 12028–12035.
32. Fujii,S., Matsuura,T., Sunami,T., Nishikawa,T., Kazuta,Y. and Yomo,T. (2014) Liposome display for *in vitro* selection and evolution of membrane proteins. *Nat. Protoc.*, 9, 1578–1591.
33. ——, ——, ——, Kazuta,Y. and Yomo,T. (2013) *In vitro* evolution of alpha-hemolysin using a liposome display. *Proc. Natl. Acad. Sci. USA*, 110, 16796–16801.