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Construction of an in vitro gene screening system of the E. coli EmrE transporter using liposome display

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ABSTRACT: Liposome display is a method that enables the directed evolution of membrane proteins in vitro. The method is based on the syntheses of membrane proteins using an in vitro transcription–translation system (IVTT) inside cell-sized phospholipid vesicles from a single copy of template DNA. So far, a large number of membrane proteins have been synthesized by IVTT; however, none of these proteins, except for α-hemolysin, has been tested for use in gene screening with liposome display. Here, using EmrE, a multidrug transporter from Escherichia coli, as a model protein, we developed an in vitro screening system of the transporter gene based on its function, which was made possible by using liposome display. The screening was performed based on two functions of EmrE: substrate transport activity and membrane integration activity. Starting from a mock gene library prepared by mixing an active and an inactive gene, 10- to 35-fold enrichment of the active genes was obtained, which was in the same range as theoretically calculated values. In addition, starting from a random mutagenized gene library of wild-type EmrE, a gene pool exhibiting ethidium bromide (EtBr) transport activity higher than that of the wild-type was obtained indicating the validity of the established screening system.

Directed evolution has succeeded in generating novel and highly functional proteins1,2. The resulting proteins have been used in various applications (e.g., drug synthesis and live imaging) and have also contributed to the elucidation of sequence-function relationships of proteins3,4. However, the available methods deal mostly with globular proteins (e.g., antibodies and enzymes) and not membrane proteins, despite their biological and pharmaceutical importance5,6. Technical difficulties such as the expression of membrane proteins in heterologous host cells and the establishment of high throughput functional screening methods have limited the use of directed evolution to engineer membrane proteins7-10.

Liposome display is a method that enables the directed evolution of membrane proteins entirely in vitro11,12. The method is based on the protein syntheses of membrane proteins from a single copy of template DNA using an in vitro transcription-translation system (IVTT) inside a cell-sized phospholipid vesicle (Fig. 1). By assessing the functions of membrane proteins with a fluorescence activated cell sorter (FACS), liposomes with high fluorescence can be sorted, which contains gene encoding membrane proteins with desired functions. Recently, this method was applied to evolve the pore-forming activity of α-hemolysin, a membrane protein derived from Staphylococcus aureus13. α-Hemolysin is a membrane protein whose properties differ from those of typical membrane proteins14. It is soluble, exists in monomeric form in solution, and forms heptamers only on the surface of lipid bilayers; typical membrane proteins form aggregates in solution and are difficult to solubilize in the absence of detergents or lipids. So far, a large number of membrane proteins have been synthesized with the cell-free protein synthesis system13-15; however, none of these proteins, with the exception of α-hemolysin, have been tested for their use in gene screening with liposome display.

EmrE is a multidrug transporter of Escherichia coli, which transports a variety of toxic cationic compounds (e.g., tetraphenylphosphonium (TPP), ethidium bromide (EtBr) and acriflavine toward the outside of the cell by coupling the compound transport with the reverse transport of protons16,17. This protein has been reported to appear in the insoluble fraction when synthesized using IVTT18. It consists of 110 amino acids, which constitutes its four transmembrane domains, and forms an anti-parallel dimer in the membrane. Due to its small size, dual topology and the ability to recognize variety of toxic compounds, we believe that EmrE can be used as an element in a biosensor to detect various toxic chemicals, i.e., using surface plasmon resonance (SPR) or contact droplet method19,20. However, while the binding of EmrE to TPP exhibits a Kd of 10 nM21, its affinity to EtBr and other compounds was found to be in the submicromolar range22. In addition, membrane integration of EmrE into a synthetic membrane was found to be low23. Directed evolution can contribute to the optimization and evolution of these properties, affinities to ligands and/or membrane integration efficiencies into synthetic membranes, which, for example, would result in the generation of mutants suitable for biosensing applications.

In this study, using EmrE as a model protein, we show that screening of the transporter gene based on its function is made possible through the use of liposome display. The screening was performed based on two functions of EmrE: substrate transport activity and membrane integration activity, both of which were converted to fluorescence signals. Starting from a mock gene library prepared by mixing an active and an inactive gene, 10- to 35-fold enrichment of the active gene was obtained indicating the validity of the established screening system.
obtained, which was in the same range as the theoretically calculated value. In addition, starting from a random mutagenized gene library, three rounds of screening was performed to obtain a gene pool exhibiting EtBr transport activity higher than that of the wild-type, indicating the validity of the screening system.

**EXPERIMENTAL SECTION**

Protein synthesis in liposomes. The IVTT system used in this study was a reconstituted IVTT (the PURE system), which was modified to synthesize more proteins, and its composition is shown in Supplementary Table 1. The DNA encoding EmrE-myc, E14C-myc or EmrE-FLAG were prepared by PCR amplification of the pET-EmrE-myc, pET-E14C-myc or pET-EmrE-FLAG plasmids, respectively, using T7F (5'-TAATACGACCATCTATTAGG) and T7R (5'-GCTAGTTATGTTGCAAGG) primers. The map and the sequences of the plasmids are given in Fig. S1. PCR was performed using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer’s instructions unless otherwise noted. EmrE-myc or EmrE-FLAG has a myc or FLAG-tag at the C-terminus of EmrE. EmrE-FLAG was used as a negative control for the gene screening based on membrane interaction activity. E14C-myc encoding E14C mutant (Glu at position 14 was replaced with Cys) that lacked transport activity was used as a negative control for the gene screening based on the substrate transport activity.

The PCR product was purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Transcription reactions were carried out using a MEGAscript T7 Transcription Kit (Ambion/Thermo Fisher Scientific, Waltham, MA, USA) and the PCR product encoding EmrE-myc. The synthesized RNA was purified using an RNeasy Mini kit (QIAGEN). Concentrations of the RNAs were determined by measuring their absorption at 260 nm.

Liposomes containing the reconstituted IVTT were prepared using the water-in-oil (W/O) emulsion transfer method, as previously described. We used liposomes with a lipid composition of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Avanti Polar lipids, Alabaster, AL, USA); cholesterol (Nacalai Tesque, Kyoto, Japan) = 10:1. Briefly, 20 µL of the reconstituted IVTT was supplemented with template DNA or RNA, 200 mM sucrose, 0.8 µL of the RNase inhibitor, and 1.5 µM transferrin Alexa Fluor 647 conjugate (TA647); 200 µL of liquid paraffin (Wako, Osaka, Japan) containing 1.4 mg of POPC and 0.14 mg of cholesterol was then added. The mixture was vortexed for 30 s to form W/O emulsions that were then equilibrated on ice for 10 min. The solution (see below for the composition) and centrifuged at 18,000 × g for 30 min at 4°C. The pelletified 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 the reconstituted IVTT: 0.3 mM of each amino acid, 3.75 mM ATP, 2.5 mM GTP, 1.25 mM CTP and 280 mM potassium glutamate, 18 mM Mg(OAc)2, and 50 mM HEPES (pH 7.6), supplemented with 200 mM glucose.

**FACS analysis and sorting.** To probe the myc-tagged EmrE displayed on the membrane surface, AlexaFlour488 (AF488) labeled anti-myc-tag antibody (MBL, Nagoya, Japan) was added (final concentration of 5 µg/mL) to the vesicle suspension together with 0.1% BSA; this mixture was incubated at 25°C for 30 min. The unbound antibody was removed by pelleting the vesicles via centrifugation at 8,000 × g for 5 min and by resuspending the pellet in dilution buffer (50 mM HEPES-KOH (pH 7.6), 280 mM potassium glutamate, 18 mM Mg(OAc)2, and 200 mM glucose). The resulting vesicle suspension was analyzed using FACS. To detect the influx of EtBr, the external solution of the vesicle was replaced with a buffer that was identical to the dilution buffer except that pH was adjusted to 8.1 and contained 0.2 µg/mL of EtBr.

The fluorescent signals from the AF488, EtBr and TA647 were measured by FACS (FACSAriaII or FACSVersa; BD Biosciences, Franklin Lakes, NJ, USA). Before the FACS analysis, the liposome suspension was diluted five- or ten-fold in dilution buffer.

We calculated the vesicle volumes from the TA647 fluorescence intensities obtained with FACSArm using the equation \( V = 0.0038 \times F_{\text{TLat}} \), where \( V \) (IL) is the volume of the liposomes and \( F_{\text{TLat}} \) is the TA647 fluorescence intensity. The equation was obtained as follows. Firstly, correlation between the number of AlexaFlour 647 and its fluorescence intensity was obtained by measurements of the calibration beads (Quantum AlexaFlour 647 MESP Beads, Bang laboratories, Technology Drive Fishers, IN, USA) by FACS. Secondly, by knowing the number of AlexaFlour 647 conjugated to each transferrin molecule (2 AlexaFlour647/transferrin), and the concentration of TA647 inside the liposome (1.5 µM), we obtained the above equation.

**Quantitative PCR.** DNA or RNA recovered from each sorting region was purified and subjected to quantitative PCR (qPCR) to estimate their enrichment factors (\( En \)). For EtBr transport activity-based screening, \( En \) was defined as

\[
En = \frac{N_{\text{act}}}{N_{\text{inact}}} = \frac{N_{\text{act}}(\text{active(before sort)})}{N_{\text{act}}(\text{inactive(before sort)})}
\]

where \( N_{\text{act}} \) and \( N_{\text{inact}} \) are the numbers of EmrE-myc and E14C DNA estimated by qPCR, respectively, included in the gene pool obtained after sorting. For membrane integration activity-based screening, \( En \) was defined as

\[
En = \frac{N_{\text{act}}}{N_{\text{inact}}} = \frac{N_{\text{act}}(\text{active(after sort)})}{N_{\text{act}}(\text{inactive(after sort)})}
\]

where \( N_{\text{act}} \) and \( N_{\text{inact}} \) are the numbers of EmrE-myc and EmrE-FLAG DNA estimated by qPCR, respectively, included in the gene pool obtained after sorting. 1/9 is the proportion of an active and an inactive gene present in a mock gene library (i.e., before sorting) and \( N_{\text{act}}(\text{after sort})/N_{\text{inact}}(\text{after sort}) \) is the proportion of an active gene in the gene pool after sorting.

From the sorted samples stained with EtBr, DNA was purified using a MiniElute PCR purification kit (Qiagen), and the resultant product was subjected to qPCR using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TAKARA Bio, Kusatsu, Japan) on a real-time PCR system (MX3000P; Agilent Technologies, Santa Clara, CA, USA). The primer sets used to quantify EmrE-myc and E14C were wF (5'-TTGCTGAATACTGTGGCAGAG) with wR (5'-GTAGGAATATAAGGCCAGGTC) and mF (5'-GGTGCATCTTGTAGCATGC) with wR, respectively. From the sorted samples stained with anti-myc tag antibody, RNA was purified using an RNeasy Mini kit (Qiagen), and the resultant product was subjected to reverse transcription using a PrimeScript™ RT reagent Kit (Perfect Real Time) (TAKARA Bio) with mRT (5'-ATGGTCCTACAGCGCTATTC) primer followed by qPCR. The primer sets used to quantify EmrE-myc and EmrE-FLAG were mF (5'-GATTAGCTTACTGTCTGG) and mR (5'-TCTTATATATTTTGTTCCTCA) with wR (5'-TTTATACGTGCATCCCTTATGTC), respectively. RNA was quantified because the amount of recovered DNA tends to be low in mRNA-stained samples, presumably due to the collapse of liposomes (Fig. S5, Fig. 4) caused by the increase in EmrE synthesis inside the liposome, which is necessary for detection.

**Calculating the theoretical enrichment factor.** Two different DNAs encoding active or inactive proteins termed active and inactive DNA, respectively, are present at concentrations of \( c_{\text{act}} \) and \( c_{\text{inact}} \) (molecule/IL), respectively. As we aim to enrich the active gene present as a minor fraction from a mixture of the two, the experiment is carried out under a condition where an inactive gene is present in excess, i.e., \( c_{\text{act}} < c_{\text{inact}} \). Here, we assume that liposomes that contain more than a single copy of active DNA are sorted regardless of the presence of inactive DNA.

Inside the liposomes with a volume of \( V \) (IL), average number of an active (\( N_{\text{act}}(\text{before sort}) \)) and an inactive DNA (\( N_{\text{inact}}(\text{before sort}) \)) are \( c_{\text{act}}V \) and \( c_{\text{inact}}V \) (copies), respectively. By knowing the average number, the fraction of liposomes carrying \( k \) copies of active DNA (P(\( k \))) can be calculated using the Poisson distribution:

\[
\phi(k) = \frac{e^{-c_{\text{act}}V} (c_{\text{act}}V)^k}{k!}
\]
Inside the sorted liposome, more than a single copy of active DNA is present. The fraction of such liposome can be calculated as
\[
f_k(k > 0) = 1 - P_k(0) = 1 - e^{-c_v \cdot k}
\]
From these equations, the fraction of liposomes carrying \( k \) copies of active DNA \( f_k(k) \) can be calculated as
\[
f_k(k) = \frac{e^{c_v \cdot k \cdot N_{active(before sort)}}}{1 - e^{-c_v \cdot k}} \quad \text{Eq. 3}
\]
Therefore, average number of active DNA \( N_{active(after sort)} \) inside a sorted liposome can be calculated as
\[
N_{active(after sort)} = \sum_{k=1}^{\infty} k f_k(k) = \frac{c_v \cdot N_{active(before sort)}}{1 - e^{-c_v}} \quad \text{Eq. 4}
\]
As there is no bias toward the number of inactive DNA, the average number of inactive DNA after sorting \( N_{inact(after sort)} \) is \( c_v \cdot N_{inact(before sort)} \).

From the definition of enrichment factor (Eqs. 1 and 2), we obtain
\[
E_n(V) = \frac{N_{active(after sort)}}{N_{inact(after sort)}} / \frac{N_{active(before sort)}}{N_{inact(before sort)}}
\]
\[
= \left( \frac{c_v \cdot N_{active(before sort)}}{c_v \cdot N_{inact(before sort)}} \right) / \left( \frac{c_v \cdot N_{active(before sort)}}{c_v \cdot N_{inact(before sort)}} \right) = e^{c_v \cdot V} \quad \text{Eq. 5}
\]

Eq. 5 is shown in Fig. 5B dashed line.

**RESULTS and DISCUSSION**

**Schematic of liposome display with an EmrE transporter.**

Methods used for directed evolution require a link between the genotype and the phenotype. The liposome display is achieved by encapsulating a single copy of DNA encoding the target membrane protein together with the IVTT reconstituted only from defined and purified elements, known as the PURE system \(^{20,27}\) inside the cell-sized liposome, whose size is typically between 1 to 100 fL in volume and 1 to 6 μm in diameter (Fig. 1). Such liposomes were prepared with the water-in-oil emulsion transfer method \(^{20,31}\) and incubated at 37°C to synthesize EmrE and to integrate the synthesized protein into the membrane. The method produces liposomes with volumes of 1 to 100 fL \(^{30,31}\). Encapsulation of a single copy of DNA was achieved by adding a low concentration of DNA (50 pM = 0.03 molecule/μL) to the IVTT; a fraction of the liposome would have no DNA and those with DNA would have nearly a single copy of DNA.

For gene screening, the fraction of the displayed membrane protein must be converted into a fluorescence signal. We took into account the two functions of EmrE, one of which is transport activity (Fig. 1A) and the other is membrane integration activity (Fig. 1B). When screening for transport activity, we used EtBr as a substrate (Fig. 1A). EtBr is a well-known substrate of EmrE \(^{16}\), and once it is incorporated inside the liposome it binds to the ribosome and the tRNAs and exhibits fluorescence \(^{32}\). When screening for membrane integration activity, we used AlexaFluor488 (AF488)-labeled anti-myc tag antibody that binds to the myc-tag fused to the C-terminus of EmrE (Fig. 1B). When EmrE is inserted into the membrane and its C terminus is positioned outside the liposome, it should be recognized by the antibody. As EmrE forms an anti-parallel dimer, one of the subunits should be detectable as long as EmrE is present in its active form.

**Evaluating the activity of EmrE synthesized in cell-sized liposomes.** We first investigated if the EtBr transport activity could be measured quantitatively using our cell-sized liposomes. Fig. 2A shows the EtBr transport at three different time points. EmrE encoded with 1 to 30 nM RNA was used for protein synthesis. After 2 h of protein synthesis inside the liposomes, the liposomes were subjected to an EtBr transport assay. When RNA was present, the liposome population was found to move toward higher EtBr fluorescence signals over time (Fig. 2A). From the FACS data, we calculated the rate of fluorescence increase and plotted its correlation against RNA concentrations. We found that the rate increased linearly up to a concentration of 30 nM RNA (Fig. 2B). The amount of synthesized protein is known to be correlated with the amount of template used for the IVTT (Fig. S2) \(^{33}\). Therefore, the results suggest that the EtBr transport activity is limited by the amount of synthesized EmrE and not by other components such as EtBr. This is an essential property of the assay system that enables gene screening based on transport activity. Note that the observed EtBr transport was confirmed to be coupled with proton transfer, as the transport was inhibited by the addition of nigericin, an ionophore that cancels the proton gradient across the membrane (Fig. 3, Fig. S4).

Linearity was also observed with membrane integration activity (Fig. 2C, Fig. S3). After EmrE synthesis inside the liposomes with 1 to 30 nM RNA for 2, 4 or 20 h, the resulting liposomes were stained with AF488-labeled anti-myc tag antibody. We found that the rate of increase in the AF488 signal had a linear correlation with the RNA concentrations used for the synthesis (Fig. 2C), suggesting the validity of the assay system for use in gene screening based on membrane integration activity.
Figure 1. Schematic of the in vitro gene screening of EmrE using liposome display. This method uses cell-sized liposomes for in vitro membrane protein synthesis, membrane protein integration, and protein function detection for screening. An IVTT and approximately a single molecule of DNA, derived from the DNA library, are encapsulated in liposomes, where a membrane protein is then synthesized. The expressed membrane protein is subsequently integrated into the lipid membrane and displayed on the surface of the liposome. To establish a gene screening system based on the displayed membrane proteins using a fluorescence-activated cell sorter (FACS), the function(s) of these membrane proteins must be converted into fluorescence signal. In the case of EmrE, two different screening schemes can be considered. A) Screening based on EtBr transport activity, and B) screening based on EmrE membrane integration activity. The system is designed such that the liposomes displaying proteins with higher activity exhibit higher fluorescence and can be selectively sorted using FACS. The DNA segment encoding the functional membrane protein can then be isolated from the selected liposomes.

Figure 2. Functional analysis of EmrE synthesized inside cell-sized liposomes. A) EtBr transport by EmrE analyzed by FACS. After 2 h of protein synthesis inside the liposomes at 37°C using RNA encoding EmrE-myc, EtBr was added to the outer solution and the liposomes were analyzed after indicated incubation times. The vertical axis shows the TA647 fluorescence indicative of the relative volume of liposomes, and the horizontal axis shows EtBr fluorescence of the liposomes. B) Correlation between RNA concentrations (horizontal axis) used for EmrE synthesis and the relative transport rate of EtBr (vertical axis). The line shows the linear fit of the plotted data. For liposomes with a given RNA concentration, parts of which are shown in A), the median of the histogram of EtBr fluorescence/TA647 fluorescence was calculated for each time point. The slope calculated from the relationship between time and the median values was defined as the relative EtBr transport rate for liposomes at a given RNA concentration. Error bars indicate the standard error of 3 or 4 samples. C) Correlation between RNA concentrations (horizontal axis) used for EmrE synthesis and relative membrane integration rates (vertical axis). The line shows the linear fit of the plotted data. EmrE was synthesized with different concentrations of RNA inside liposomes and stained with AF488-labeled anti-myc antibody after, 2, 4, 20 h incubation at 37°C. For liposomes with given RNA concentrations, part of which are shown in Fig. S3, the median of the histogram of AF488 fluorescence/TA647 fluorescence was calculated for each time point. The slope calculated from the relationship between time and the median values was defined as the relative membrane integration rate of liposomes with given RNA concentrations. Error bars indicate the standard error of 3 samples. All measurements shown were obtained using FACSVerse.
brane has been quantified to be at most about 300 copies/µm². With samples with nigericin, 15 µM of nigericin was added after 10 min. All measurements shown were obtained using FACSVerse. The median of the histogram of EtBr fluorescence/TAg647 fluorescence was calculated for each time point, which was defined as the relative EtBr transport concentration. Results in which the nigericin was added at time 0 are shown in Fig. S4.

During the experiments described above, we found that more EmrE membrane integration led to the rupture of the liposomes. Fig. 3 shows the histogram of forward scattering intensities of the liposomes after EmrE synthesis with different concentration of RNA, which correlates with the size of the liposomes. With increases in RNA concentrations, we found that the fractions of liposomes with forward scattering intensities larger than 1x10⁴ (a.u.) decreased. Note that the peak values hardly change across the histograms. These results indicate that larger liposomes have the tendency to rupture when more EmrE is expressed inside. We see similar tendency when working with other membrane proteins. While we do not have direct evidence and requires additional experiments, the rupture is presumably due to the deformation of membrane structures by membrane insertion of proteins. This insertion may leads to the destruction of the genotype-phenotype linkages required for directed evolution. Thus, the expression level of membrane proteins inside liposomes should be high enough to enable the detection of their function but low enough to avoid destruction of liposomes.

It is interesting to note that in our system, the number of EmrE inserted in the liposome membrane has been quantified to be at most about 300 copies/µm² at the highest gene concentration used [23], whereas E. coli has 10⁸ copies of membrane proteins/µm² [34]. The rupture of liposome with significantly less number of membrane proteins than that of E. coli is indicating the lack of robustness of the liposome system, which may be overcome by incorporation of membrane protein translocation machineries and/or by the modification of the phospholipid composition.

Figure 3. Inhibition of EmrE activity using nigericin. After 2 h of protein synthesis inside the liposomes at 37°C using 3.2 nM RNA encoding EmrE, the liposome suspension was diluted 10-fold with dilution buffer containing 0.2 µg/mL EtBr. The liposomes were analyzed by FACS after indicated incubation times. For the samples with nigericin, 15 µM of nigericin was added after 10 min. All measurements shown were obtained using FACSVerse. The median of the histogram of EtBr fluorescence/TAg647 fluorescence was calculated for each time point, which was defined as the relative EtBr transport concentration. Results in which the nigericin was added at time 0 are shown in Fig. S4.

Gene screening based on the EtBr transport activity of EmrE. We investigated if gene screening based on EtBr transport was possible using liposome display. For this purpose, we used two DNA sequences: one encoded with wild type EmrE and the other encoded with an E14C mutant (Glu at position 14 was replaced with Cys) that lacked transport activity [21]. A myc-tag was attached to the C-terminus of both sequences, which were denoted as EmrE-myc and E14C-myc DNA, respectively. We used 0.2 µM T7 RNA polymerase, which was 1/10 of the standard condition [35], to reduce the amount of expressed proteins and thus reduce the rupture of large liposomes (Fig. S5).

When EtBr transport activity was measured using a mock gene library prepared by mixing the DNA encoding EmrE-myc and E14C-myc DNA in a 1:9 ratio, 1.53% of the liposomes were found in the P1 gate (Fig. 5A). Here, the fraction of liposomes in the P1 gate was used as an indicator for EtBr transport activity. This value was slightly higher than that observed with E14C-myc alone (0.19%) but much lower than that observed with EmrE-myc (13.1%).

From liposomes prepared using the mock gene library, approximately 10,000 liposomes were sorted, representing the upper 0.8 to 1% of liposomes with the highest fluorescence signals. The liposomes were sorted into three different size groups (P2, P3 and P4). The average volumes of liposomes in these gates were 98.9, 28.7 and 8.2 fl, respectively. As the efficiency of gene screening is expected to depend on the size of the liposomes used (see EXPERIMENTAL SECTION), size dependent sorting was conducted to quantitatively evaluate the validity of the sorting experiments.

DNA was extracted from the sorted liposomes and the amounts of EmrE-myc and E14C-myc DNA were quantified using qPCR. Then, the enrichment factors (En) representing how many folds the proportion of active DNA has increase after sorting from the initial proportion (1/9) was calculated using the following equation. $En = \left[ \frac{N_{EmrE(after\ sort)} / N_{EmrE(before\ sort)}} {N_{E14C(after\ sort)} / N_{E14C(before\ sort)}} \right]$, where $N_{EmrE(after\ sort)}$ and $N_{E14C(after\ sort)}$ are the numbers of active EmrE-myc and E14C-myc DNA, respectively, after sorting; $N_{EmrE(before\ sort)}$ and $N_{E14C(before\ sort)}$ are the numbers of active EmrE-myc and E14C-myc DNA, respectively, before sorting.

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where $N_{\text{after\ sort}}$ and $N_{\text{before\ sort}}$ were the number of x (x = EmrE or E14C) gene after and before sorting, respectively. We found that smaller liposomes showed higher enrichment factors, which was reasonable for this experimental set up (Fig. 5B, black squares). This was confirmed by comparing the experimental and theoretical data (dashed line).

**Gene screening based on the membrane integration activity of EmrE.** Next, we investigated if the gene screening based on membrane integration was possible using liposome display. To this end, we used two types of DNA, which both encoded with wild type EmrE but with a myc-tag or FLAG-tag at its C-terminus, termed EmrE-myc and EmrE-FLAG, respectively. Here, we used 2 µM T7 RNA polymerase, as the concentration used for EtBr transport activity-based sorting was not sufficient for myc-tag detection. However, as shown in Fig. 4, the increase in the expression of EmrE led to the reduction of large liposomes (Fig. S5).

When the membrane integration activity was measured using a mock gene library prepared by mixing the DNA encoding the EmrE-myc and EmrE-FLAG DNA in a 1:9 ratio, 1.22% of the liposomes were found in the P5 gate (Fig. 5C). Here, the fraction of liposomes in the P5 gate was used as an indicator for membrane integration activity. This value was slightly higher than that obtained with EmrE-FLAG alone (0.19%) but much smaller than the value obtained with EmrE-myc (10.3%).

Approximately 10,000 liposomes were sorted from the liposomes prepared using the mock gene library, representing the upper 0.8 to 1% of liposomes with the highest fluorescence signals. The sorting was performed for liposomes of three different sizes (P6, P7 and P8). The average volume of liposomes in each gate was 166, 43.2 and 13.5 fL, respectively.

DNA was extracted from the sorted liposomes and the amounts of EmrE-myc and EmrE-FLAG DNA were quantified using qPCR. Then, the enrichment factors ($En = \left[ \frac{N_{\text{myc(after\ sort)}}}{N_{\text{myc(before\ sort)}}} \right] / \left[ \frac{N_{\text{FLAG(after\ sort)}}}{N_{\text{FLAG(before\ sort)}}} \right]$) were calculated where $N_{\text{x(after\ sort)}}$ and $N_{\text{x(before\ sort)}}$ are the number of x (x = myc or FLAG) gene after and before sorting, respectively. Similar to the results obtained from the screening based on EtBr transport, we found that smaller liposomes showed higher enrichment factors (Fig. 5B, gray squares), consistent with theoretical estimations, indicating that gene screening based on membrane integration was possible using liposome display. The 13.5 fL liposomes (those from P8) should have shown slightly higher enrichment factors compared to the 43.2 fL liposomes. While we do not know the exact reason for this observation, it may have been caused by the rupture of large liposomes (Fig. S5, Fig. 4).

**Gene screening from a random mutagenized gene library.** Finally, we investigated if gene screening based on EtBr transport was possible from a random mutagenized gene library. Random mutagenized DNA library with on average 4.2 amino acid substitutions/clone was prepared using error prone PCR and wild-type EmrE gene as a template. Then liposome display rounds based on the EtBr transport was preformed (Fig. 1A).

From liposomes prepared using the random mutagenized DNA library, approximately 10,000 liposomes were sorted, representing the upper 0.4 to 1% of liposomes with the highest fluorescence signals. The liposomes population with a volume of around 20 fL (median value) was sorted. DNA was extracted from the sorted liposomes, amplified and subjected to the next round of screening. The screening was repeated three times.
Figure 5. Gene enrichment experiments based on the EtBr transport and membrane integration activities of EmrE. A) The 2D FACS data of liposomes treated with EtBr, prepared using the DNA depicted on the top of the plots. The vertical axis shows the TA647 fluorescence indicative of the vesicle volumes, and the horizontal axis shows the fluorescence intensity of EtBr. The P1 gate was used to assess and quantify EtBr transport activity (see text), and the numbers indicate the fraction of liposomes in the P1 gate. The P2, P3 and P4 gates, aiming to sort liposomes with different size, were used. Protein synthesis was carried out as described in the Methods section except for the use of 0.2 μM T7 RNA polymerase and 50 pm DNA, and by incubating the samples for 2 h at 37°C. B) Relationship between enrichment factors and the sorted liposome volumes. The black line shows the data from the EtBr transport activity screening experiment and the gray line shows the data from the membrane integration activity screening experiment. The dashed line shows the theoretical values obtained through Eq. 5. C) The 2D FACS data of liposomes stained with AF488-labeled anti-myc tag antibody, prepared using the DNA depicted on the top of the plots. The vertical axis shows the TA647 fluorescence indicative of the vesicle volume, and the horizontal axis shows the fluorescence intensity of the AF488. The P5 gate was used to quantify membrane integration activity (see text), and the numbers indicate the fraction of liposomes in the P5 gate. The P6, P7 and P8 gates, aiming to sort liposomes with different size, were used for sorting. Protein synthesis was carried out as described in the Methods section except for the use of 2 μM T7 RNA polymerase and 50 pm DNA, and by incubating the samples for 4 h at 37°C. All measurements and sorting were obtained using FACS AriaII.

Then the collected gene pools were subjected to EtBr transport activity assay (Fig. 6). We found a clear increase in the EtBr transport activity with the gene pools after two and three rounds of screening (depicted as R2 and R3, respectively), compared to that of the initial random mutagenized library. These results suggest that in vitro evolution of EmrE is possible using liposome display. By analyzing the sequence of the clones in the R2 and R3 gene pools, we identified various mutations. Among them some mutations (L7H, G17D, V69A, P86S and M91L) were found in two clones (Fig. S6). Further rounds of screening are expected to enrich and clarify mutations exhibiting beneficial effects on the EmrE activity.

CONCLUSION

Liposome display enables the directed evolution of membrane proteins entirely in vitro. Here, we have shown that the gene screening of the multidrug transporter EmrE is possible based on two properties of the molecule: substrate transport activity and membrane integration activity.

Limitations of liposome display include (i) the conversion of membrane protein function to fluorescence signal and (ii)
membrane rupture caused by membrane protein integration. More and more fluorescence substrates for functional assays of transporters are becoming available. In addition, as the liposome display technology consists only of defined components, we may convert the non-fluorescent substrate transport to the expression of a reporter gene (e.g., GFP, β-galactosidase) inside liposomes, thereby generating a fluorescence signal. Membrane rupture may be eliminated by incorporating the Sec translocon [35,36], the membrane protein transport machinery inside the liposome, as it assists in the proper integration of membrane proteins. Because liposome display has such a high degree of controllability, i.e., the ability to modify functional screening and/or protein synthesis conditions, the technology should be applicable for the screening and evolution of various transporters and receptors.

There is great interest in engineering membrane proteins for improved expression and stability [38], as it is thought to contribute in characterization of the membrane proteins including three-dimensional structure analysis via crystallization. While there are reports of in vivo selection systems [38,39], there are no reports of an in vitro selection system that enable the evolution of an expression level and/or stability of membrane protein of interest. As we can readily display and detect the membrane proteins on the lipid membrane in a high-throughput format, we believe that liposome display can contribute in the rapid evolution of membrane protein for structural studies.

ASSOCIATED CONTENT

Supporting Information
Supplementary Table and Supplementary Figures. These materials are available free of charge via the Internet at http://pubs.acs.org.

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