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In vitro directed evolution of alpha-hemolysin by liposome display

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We have developed a method to enable *in vitro* directed evolution that can be applied to membrane proteins. This method, termed liposome display, uses liposomes as compartments in which membrane proteins are synthesized and as scaffolds for membrane protein integration. Thus, the synthesized membrane proteins are displayed on the surface of the liposome and exhibit their functions. A randomly mutated DNA library of the membrane protein was generated, encapsulated in the liposomes at the single-molecule level, and used to generate a liposome library. Liposomes displaying the desired membrane protein function were selected, thus accumulating the DNA molecule encoding the desired membrane protein. We have applied this method to alpha-hemolysin, a membrane protein derived from *Staphylococcus aureus*. Alpha-hemolysin forms a nanopore in the membrane, which allows the penetration of small molecules. We aimed to improve this nanopore activity by using the liposome display method. Consequently, alpha-hemolysin evolved and attained a higher specific affinity for the liposome membrane. In this review, we describe the essential characteristics of liposome display and the properties of the evolved alpha-hemolysin obtained by this method.

Key words: fluorescence-activated cell sorter, membrane protein, PURE system

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In vitro directed protein evolution

Directed protein evolution is an engineering method used to obtain a protein with desired properties by generating a mutant library and selecting the fittest among them. Many proteins have been engineered by this method, and useful mutants with higher thermostability, structural stability, or ligand specificities have been generated [1,2]. Moreover, the analysis of the obtained mutant can reveal the relationship between sequence and function to further understand the target protein. Directed protein evolution can be classified in to an *in vivo* method that uses living cells or an *in vitro* method that uses cell-free translation systems. The *in vitro* method is useful for handling proteins that affect the growth of cells, such as toxic proteins. Moreover, the *in vitro* method enables us to manage larger DNA libraries and achieve more rapid generation of the desired proteins than the *in vivo* method.

Although many successful methods for *in vitro* directed protein evolution have been reported, existing methods are limited to soluble globular proteins such as antibodies and enzymes. However, no methods for the directed evolution of membrane proteins *in vitro* have been developed. Membrane proteins constitute 20–25% of all genes and more than 50% of current pharmaceutical targets [3,4]. Therefore, the directed evolution method, which enables the production of a useful membrane protein and the further understanding of molecular mechanisms, is becoming an important technique.

When establishing the directed protein evolution method *in vitro*, linking the genotype and phenotype is an important requirement. For example, ribosomes are used as linkage

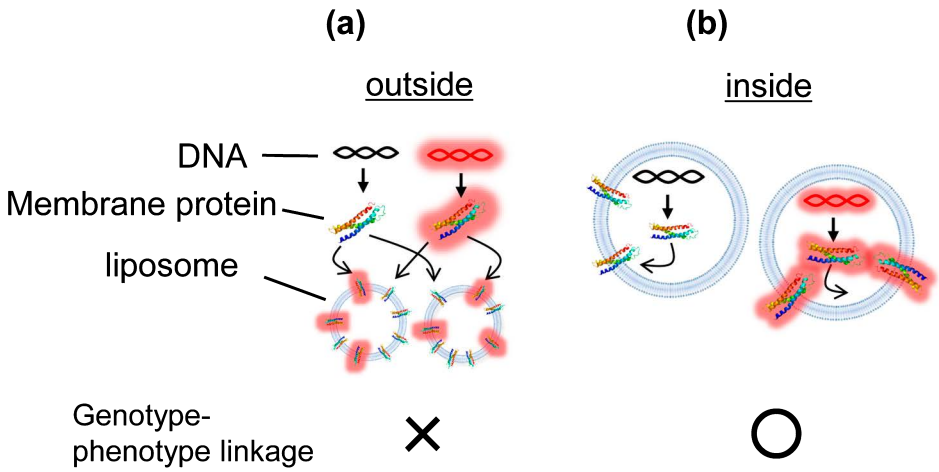

Figure 1 Two strategies for constructing the proteo-liposomes by using a cell-free translation system and liposomes. (a) Proteins are synthesized outside of the liposome, which does not allow the linkage of the genotype and phenotype. (b) When proteins are synthesized inside the liposome, the genotype and phenotype are linked by the liposome compartment.

molecules in the ribosome display method to connect the mRNA with the translated protein of each mRNA [5]. Puromycin and RepA are also used as linkage molecules in mRNA display and CIS display, respectively [6,7]. Water in oil (w/o) emulsions and liposomes are used for linking the genotype and phenotype [8,9]. Within these compartments, proteins are synthesized from DNA or RNA, enabling the co-localization of DNA (RNA) and the proteins and linking the genotype and phenotype.

In vitro membrane protein synthesis

We attempted to prepare a functional membrane protein by using a cell-free translation system while linking the genotype and phenotype. It is well known that membrane proteins tend to form aggregates while being synthesized in cell-free translation systems. Thus, detergents or lipids are usually mixed into the reaction mixture, enabling membrane proteins to fold in their native states in micelles or liposomes and exhibit their functions [10,11]. However, in these systems, the DNA (RNA) molecules encoding the membrane protein are synthesized outside of liposomes (micelles), thus the generated liposomes (micelles) will hold the mixture of various membrane proteins. Therefore, this system does not satisfy the genotype-phenotype linkage requirement (Fig. 1a). To develop a system applicable to directed membrane protein evolution, we conducted the translation of membrane proteins inside the liposome, using the liposome as a compartment, and linking the genotype and phenotype (Fig. 1b).

Recently, giant lipid vesicles encapsulating a cell-free translation system and DNA and/or RNA have been constructed, and this approach is becoming a growing field in synthetic biology and bioengineering [12,13]. As this vesicle is reconstituted by defined components and exhibits protein translation activity inside the cell-sized compartment, this vesicle is called an ‘artificial cell’. By designing the components, gene-duplication systems, two-stage genetic networks, and controllable gene expression systems have been constructed [14–16]. In our previous study, we applied these techniques to a membrane protein, alpha-hemolysin, derived from *Staphylococcus aureus* [17,18]. Alpha-hemolysin is synthesized as a soluble monomer, binds to the lipid membrane, and assembles into a heptameric nanopore, which allows the penetration of molecules smaller than 3 kDa [12]. We aimed to improve the nanopore formation ability of the alpha-hemolysin.

Liposome display

We have developed a method to construct a library of liposomes that exhibits the function of the membrane proteins encoded by the DNA encapsulated inside and a screening system to isolate the liposomes that possess DNA with the desired properties. Because the membrane protein is synthesized inside the liposome and displayed on the surface of the liposome membrane, we called this method ‘liposome display’. In this review, we will describe this method, using alpha-hemolysin as the target membrane protein (Fig. 2a).

We constructed a randomly mutagenized DNA library of alpha-hemolysin and encapsulated it in liposomes with a cell-free translation system, the PURE system [19]. The DNA concentration was set to 5 pM, which is low enough to let zero or one DNA molecules be encapsulated in most of the liposomes (Fig. 2b). If more than two DNA molecules were encapsulated, the phenotype of that liposome would represent the sum of those DNA properties. Therefore, encapsulating a single DNA molecule achieves the proper linkage of the genotype with the phenotype, enabling directed evolution with high selection efficiency.

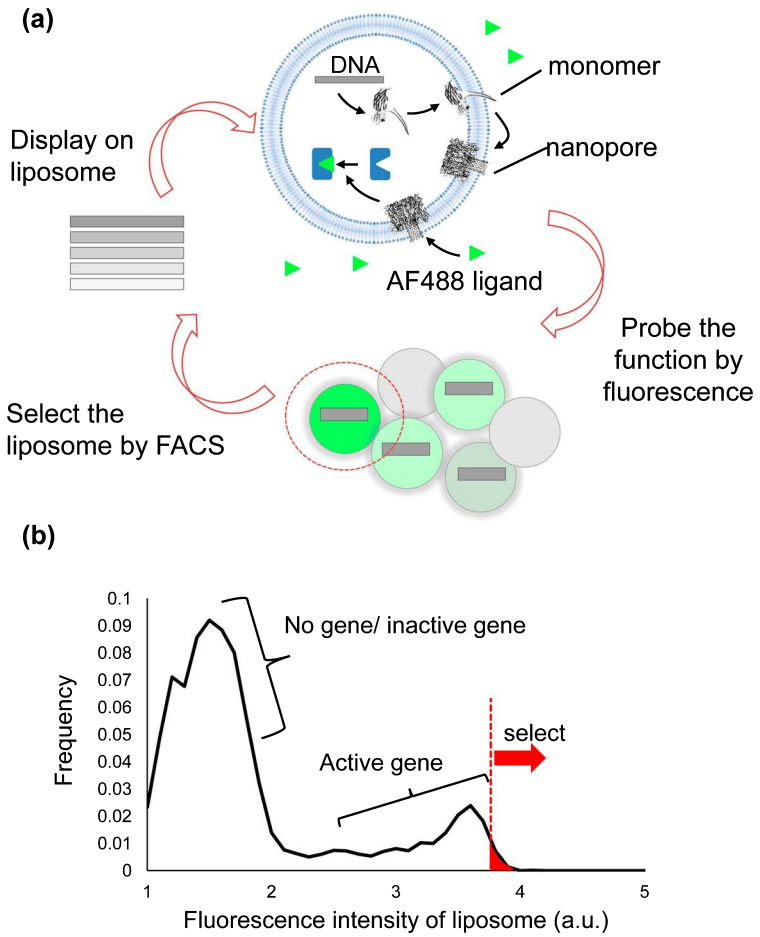

Figure 2 Schematic of the liposome display experiment of alpha-hemolysin. (a) Randomly mutagenized DNA libraries are encapsulated inside the liposomes with the cell-free translation system. Within the liposome, alpha-hemolysin is synthesized, binds to the membrane, and forms nanopores. The AF488 ligand penetrates through the nanopore and is captured by the HaloTag protein. Then, the liposome with the highest AF488 fluorescence intensity is selected using FACS. The DNA inside the selected liposome is purified, amplified, and encapsulated inside the liposome again for the next liposome display experiment. (b) Histogram of the fluorescence intensity of AF488 in the liposome. Most liposomes did not encapsulate DNA or encapsulated inactive DNA and thus did not exhibit the enrichment of AF488. Another liposome population, which shows an enrichment of AF488, represents the liposome encapsulating active DNA.

We also encapsulated a fluorescent probe in the liposomes so that we could estimate the aqueous volume of each liposome. The size distribution of the liposomes ranges from 0.1–500 fL when constructed by a water-in-oil emulsion transfer method [20–22]. As more DNA molecules will be encapsulated in large liposomes than in small liposomes, we need to determine the liposome volume to verify that the liposome encapsulates the DNA at the single-molecule level. Moreover, as the membrane curvature is known to affect the binding of membrane proteins [23–25], identifying the liposome size will enable us to use a constant membrane curvature in each experiment. Therefore, we encapsulated transferrin conjugated with Alexa Fluor 647 in liposomes, measured the fluorescence intensity using a fluorescence-activated cell sorter (FACS), and converted the intensity into the aqueous volume of the liposome.

Next, we developed a method to probe the number of nanopores formed in the liposome membrane. The HaloTag ligand Alexa Fluor 488 (AF488) was externally applied to the liposome, penetrated through the nanopore in the liposome membrane, and was captured by the HaloTag protein that was encapsulated in the liposome. The complex of the HaloTag protein and AF488 cannot pass through the nanopore due to its size, enabling the enrichment of AF488 in the liposome. Using this system, we succeeded in detecting and quantitatively probing the number of nanopores of alpha-hemolysin by measuring the fluorescence of AF488.

We encapsulated the DNA library in liposomes, probed the nanopore activity using the AF488 ligand, and selected the liposome possessing the highest AF488 fluorescence intensity, which was expected to possess the highest nanopore activity. We purified the DNA from the selected liposome, amplified it by PCR, and encapsulated it in liposomes again for the next round of selection.

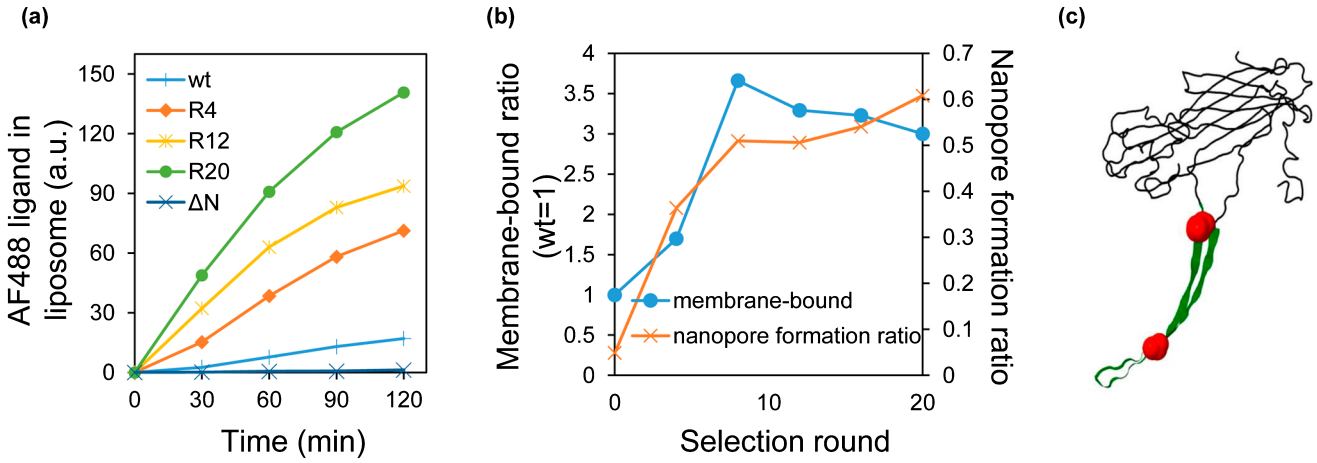

Figure 3 Evaluation of the obtained alpha-hemolysin mutant. (a) Time course of AF488 ligand accumulation using the DNA pools obtained after 4 (R4), 12 (R12), or 20 (R20) rounds of selection. The wild-type (wt) and a functionally deficient alpha-hemolysin mutant (ΔN) were used as the controls [32]. (b) The relative amount of membrane-bound alpha-hemolysin and the nanopore formation ratio were plotted against the number of selection experiments. Zero on the horizontal axis represents the activity of the wild-type. (c) The fixed mutations in the mutant obtained after 20 cycles of liposome display are marked on the structure of the alpha-hemolysin monomer. The red balls represent the mutated amino acids, and the green ribbon structure represents the stem domain of alpha-hemolysin.

Evaluation of the mutant obtained by liposome display

We repeated the liposome display experiments 20 times and succeeded in obtaining a mutant with an increased nanopore activity compared with the wild-type. When we measured the influx of AF488 of the alpha-hemolysin gene pool obtained after 4, 12, and 20 liposome display rounds, the velocity of the AF488 accumulation increased with the number of rounds, and the activity was 12 times higher than the wild type after 20 experiments (Fig. 3a).

To verify and evaluate the increased nanopore activity of the mutants, we measured the membrane binding efficiency (membrane bound/total synthesized) and the nanopore formation ratio (nanopore formed/total membrane bound monomer) by using the DNA obtained after 4, 8, 12, 16, and 20 rounds of liposome display experiments (Fig. 3b). The membrane binding efficiency increased to 3 times higher than that of the wild type, and the nanopore formation ratio increased from 5% (wild-type) to 60%. We also measured the amount of protein synthesized by each DNA and determined that the amount did not increase or decrease during the liposome display experiments [18].

Then, we identified the sequence of the alpha-hemolysin mutant and mapped the most frequently observed mutations, the so-called fixed mutations, in the structure of alpha-hemolysin (Fig. 3c). There were two fixed mutations both of which were located in the stem domain, which covers only 13% of the alpha-hemolysin sequence, suggesting that stem domain is strongly involved in the evolved properties of the mutant alpha-hemolysin. Although the stem domain has been thought of as an important domain for the nanopore formation of alpha-hemolysin [26], our results showed a concrete amino acid substitution that improves nanopore formation.

When we evaluated the lipid specificity of the evolved alpha-hemolysin mutant, we found that it adapted specifically to the conditions where the liposome display experiment was performed. Alpha-hemolysin has been known to exhibit nanopore activity in a lipid composition-specific manner [27]. In our experiment, we used 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol. In addition to this composition, we used liposomes composed of only the POPC, 1-2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) to analyze the nanopore activity of the obtained alpha-hemolysin mutant. As a result, the evolved alpha-hemolysin showed increased nanopore activity relative to wild-type with liposomes that contained phosphocholine and did not show such an increase with POPG or POPE (Fig. 4a, b). We also evaluated the hemolytic activity, which is the original function of alpha-hemolysin [28]. There was no difference in the hemolytic activity between the wild-type and the obtained mutant (Fig. 4c). This result indicated that even the lipid specificity of membrane proteins can be modified by the liposome display method.

Perspectives

We have developed a liposome display method and enabled the directed evolution of membrane proteins entirely *in vitro*. In our previous study, we showed that five out of seven of the membrane proteins derived from *Escherichia coli* were integrated in the liposome membrane [18]. These

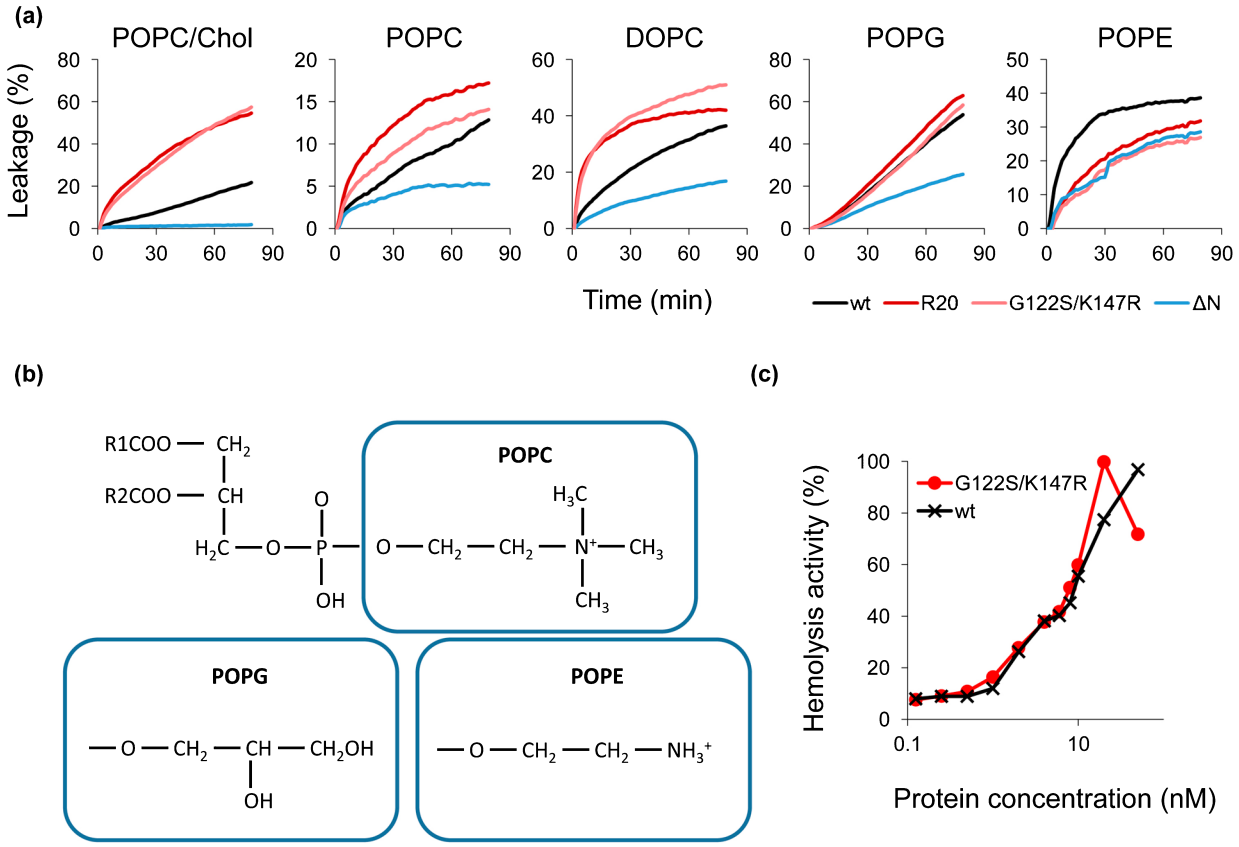

Figure 4 The lipid-specific evolution of alpha-hemolysin. (a) Time course of the leakage assay using the liposome encapsulating a self-quenching carboxyfluorescein (100 mM). As the leakage of carboxyfluorescein causes the dequenching of the fluorescence, the pore-forming activity of alpha-hemolysin can be measured by spectrophotometer. Liposomes with the lipid composition of POPC:cholesterol = 1 : 1 (wt/wt), POPC, DOPC, POPG, and POPE were constructed. The DNA of the wild-type (wt), the pool obtained after 20 cycles of liposome display (R20), the mutant with two amino acid substitutions shown in Figure 3c (G122S/K147R), and the ΔN mutant were used to prepare the alpha-hemolysin protein. Alpha-hemolysin protein was added to the liposome, and the leakage of carboxyfluorescein was measured. (b) The structural formulas of POPC, POPG, and POPE are shown. R1 and R2 represent palmitoyl and oleoyl fatty acid hydrocarbon chains, respectively. The common structure is omitted in POPG and POPE, and the features of each lipid are framed in a blue box. (c) The hemolytic activity was measured for several concentrations of alpha-hemolysin protein. The wild type and the G122S/K147R mutant were applied to rabbit erythrocytes, and the degree of hemolysis was measured.

five proteins included both alpha-helices and beta-sheets, which are the two major membrane integration domains. As the components inside and outside the liposome can be modified based on the measurement system of the target membrane protein, this method can be applied to other membrane proteins. For example, we have succeeded in detecting the transporter activity of the membrane protein by using the same liposome display scheme [29]. Given its high degree of controllability, the creation of useful bio-sensors or nanopores for next-generation DNA sequencing techniques will be made possible by liposome display [30,31]. In addition, as the entirely *in vitro* liposome display method enables to generate mutants adapted to the selection conditions, such to a specific lipid composition, this method will be useful in the further understanding of the sequence-function relationship of membrane proteins.

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