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Femtoliter compartment in liposomes for in vitro selection of proteins

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Abstract

The aqueous compartment in liposomes provides a reaction resembling the cell and therefore is used as a microcompartment in which to study enzymatic reactions. However, regardless of their method of preparation, the heterogeneity in size of cell-size liposomes limits their potential uses. We established a strategy to estimate the internal aqueous volume of cell-size liposomes using a fluorescence-activated cell sorter (FACS). Reactions inside individual liposomes can be measured in a high-throughput format provided that the encapsulated proteins give rise to a fluorescent signal such as by exhibiting fluorescence themselves or by catalyzing production of a fluorescent compound. The strategy of volume estimation was applied to in vitro selection experiments. The green fluorescent protein (GFP) gene was encapsulated into liposomes together with an in vitro translation system. Here liposomes carrying a single copy of the gene were identified using the internal aqueous volume information of individual liposomes, and those exhibiting higher green fluorescence intensity were sorted by the FACS machine. This system was able to enrich those encoding GFP with higher fluorescence intensity over those with lower intensity. These results suggest the possibility of performing evolutionary experiments in an environment that mimics the cell.

Keywords: Liposomes; Fluorescence-activated cell sorter; In vitro translation; Directed evolution

Liposomes are a powerful tool for various applications, such as drug and gene delivery [1], and also are a well-defined model in which to examine the structure and function of biological membranes [2]. In addition, liposomes are of particular interest as microreactors in which to study enzymatic reactions inside vesicles [3,4]. As a reaction environment, liposomes differ from test tubes in their size (typically on the zeptoliter to femtoliter scale). Moreover, liposomes are also different from microchambers [5] or emulsions [6], which can create an aqueous volume of a size similar to liposomes, because the reaction takes place in an environment surrounded by lipid molecules. Hence, liposomes provide a biologically relevant reaction environment.

The size of liposomes has a significant effect on the complex biochemical reactions inside the vesicles [7]. Protein synthesis involves various biochemical reactions involving a large number of components (e.g., chemicals, proteins, RNA), and all of the components are present in different concentrations. For such reactions to occur in liposomes, all components must be present. However, when liposomes become smaller, the fraction of liposomes carrying all of the required components decreases. For example, aminoacyl–tRNA synthetases are present at approximately 10 nM [8], corresponding to only three molecules on average per liposome 1 μm in diameter, and the average number of molecules per liposome becomes less than one for smaller liposomes. Therefore, cell-size liposomes are crucial to carry out reactions such as protein synthesis.

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On the other hand, regardless of their method of preparation, cell-size liposomes are heterogeneous in size, limiting their potential uses. Liposomes are known to be a kinetically, rather than a thermodynamically, stable state of lipids [9]. Consequently, preparation of liposomes requires input of external energy, and the mean size is dependent not only on the chemical structure of the lipid but also on the strategy used for liposome preparation [4]. This is more the case for cell-size liposomes, whereas with those smaller than 100 nm the extrusion method can be used to prepare liposomes of homogeneous size [10]. Therefore, to analyze enzymatic reactions in each individual liposome, it is necessary to measure not only the yield of the product but also the internal aqueous volume, which then gives the concentration of the product in each liposome.

Previously, we established a strategy to synthesize protein in its functional form in cell-size liposomes by encapsulating an in vitro translation system together with the DNA encoding the protein of interest [11,12]. In these studies, a fluorescence-activated cell sorter (FACS),[1] which can analyze more than 50,000 events per second depending on the performance of the FACS machine used, was used to detect the synthesized product by synthesizing green fluorescent protein (GFP). The FACS allows analysis of individual liposomes with regard to various properties in a high-throughput format. For example, the FACS can yield multidimensional information such as size and shape represented by forward-scattering (FS) or side-scattering (SS) intensities [13]. Further possibilities include measurement of the production of certain compounds inside the vesicles, internal aqueous volume, and lipid contents of each liposome. These can be achieved so long as each property is linked to fluorescence intensity. For example, use of fluorescently labeled lipid will enable measurement of the lipid content of each individual liposome [14].

In the current study, we first established a strategy to evaluate the efficiency of protein synthesis reaction in each individual liposome using an internal aqueous fluorescence marker, allowing estimation of the internal aqueous volume of each liposome. That is, we determined the concentration of synthesized GFP in individual liposomes by FACS analysis. Furthermore, we also showed that the strategy for measuring the internal aqueous volume of each liposome can be further adapted to carry out in vitro selection experiments. Although the chemical nature of liposomes is of great interest, we report here that liposomes can be used for in vitro evolution of proteins, which can be achieved only by knowing the internal aqueous volume of each liposome. This system allows linkage between the genotype and the phenotype by using liposomes. Consequently, evolution of proteins is possible in an environment where the protein is expressed and its function is evaluated inside the lipid vesicles.

[1] *Abbreviations used:* FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; FS, forward-scattering; SS, side-scattering; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-phosphatidylcholine; SLPC, 1-stearoyl-2-linoleoyl-*sn*-phosphatidylcholine; DSPE–PEG5000, distearoyl phosphatidyl ethanolamine–polyethyleneglycol 5000; PE, phycoerythrin; EGFP, enhanced green fluorescent protein; w/o, water-in-oil; w/o/w, water-in-oil-in-water.

Materials and methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC), 1-palmitoyl-2-linoleoyl-*sn*-phosphatidylcholine (PLPC), 1-stearoyl-2-oleoyl-*sn*-phosphatidylcholine (SOPC), and 1-stearoyl-2-linoleoyl-*sn*-phosphatidylcholine (SLPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan), and distearoyl phosphatidyl ethanolamine–polyethyleneglycol 5000 (DSPE–PEG5000) was kindly supplied by NOF (Tokyo, Japan). Phycoerythrin (PE) was purchased from Molecular Probes (Eugene, OR, USA). In vitro translation was carried out using PURE system Classic II (Post Genome Institute, Tokyo, Japan). The plasmids pETG5tag and pETG2tag encode T7 promoter, Shine-Dalgarno sequence, and T7-tag sequence (MTGGQQMGR) followed by GFPuv5 and GFPuv2, respectively. GFPuv5 [15] and GFPuv2 [16] are improved variants of GFPuv (Clontech, Palo Alto, CA, USA), carrying S208L mutation and F64L/S65T/S208L/I167T mutations, respectively (numbering based on wild-type GFP). Plasmids were prepared using Qiagen MidiPrep Kits (Hilden, Germany) in accordance with the manufacturer's recommendations.

Preparation of liposomes

Liposomes were prepared based on the freeze-dried empty liposomes method [17], and the procedures were the same as those reported previously [12] except that the experiments were carried out at room temperature unless stated otherwise. Briefly, the lipid mixture (1.2 mmol, molar ratio of POPC/PLPC/SOPC/SLPC/cholesterol/DSPE–PEG5000 = 129:67:48:24:180:14) dissolved in dichloromethane/diethyl ether (1:1, v/v) was subjected to rotary evaporation in a pear-shaped flask under vacuum to yield a thin lipid film. Then 100 ml of deionized water was added to the film under argon gas. After 15 min, the lipid film was vortexed to disperse the liposomes. The liposome dispersion was homogenized on ice by sonication with an ultrasonic disrupter (Tomy Seiko, Tokyo, Japan) and was extruded through a polycarbonate filter with a pore size of 0.4 μm (Whatman, Brentford, UK). The solution was then transferred to an Eppendorf tube and lyophilized in a freeze dryer (Labconco, Kansas City, MO, USA). The freeze-dried empty liposomes were stored at −20 °C under argon gas.

In vitro translation

In vitro translation was carried out using PURE system Classic II in accordance with the manufacturer's recommendations. The most outstanding property of this system is the good yield of protein when using low concentrations of DNA. We tested various in vitro translation systems, including those prepared in our laboratory [18,19], and these usually required a DNA concentration of 500 ng in a 50-μl reaction mixture for sufficient protein production. However, with PURE system, the DNA concentration could be reduced to 1 ng/μl without a decrease in the yield of protein, presumably due to the low RNase activity (data not shown).

Synthesis of GFP using PURE system was detected with an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Reactions in liposomes were carried out essentially as described previously [12]. Briefly, aliquots of 25 μl of the reaction mixture, consisting of PURE system, 75 μg/ml (300 nM) PE, and plasmid DNA, were added to the lyophilized liposomes at 4 °C. Subsequently, 0.75 μl of RNase (0.25 mg/ml) was added to prevent protein synthesis occurring outside the liposomes. To initiate the translation reaction, liposomes were transferred to and incubated at 37 °C for 4 h.

FACS analysis and sorting

The liposomes were analyzed with an EPICS ALTRA HyPerSort FACS (Beckman Coulter, Fullerton, CA, USA) equipped with a 488-nm water-cooled argon ion laser (Coherent Japan, Tokyo, Japan) at 20 mW. Prior to the analysis, the liposome dispersion prepared above was diluted approximately 2000-fold with isotonic buffer (IsoFlow, Beckman Coulter) to allow a rate of analysis of less than 2000 events per second. The analytical conditions were nearly the same as those described previously [12]. GFP fluorescence signals were measured using a 488-nm band-block filter, a 550-nm dichroic lens, and a bandpass filter at 525 ± 20 nm. Red fluorescence signals were measured using a 488-nm band-block filter, a 550-nm dichroic lens, and a bandpass filter at 575 ± 15 nm. FS, SS, and fluorescence data were collected with a logarithmic amplifier.

Estimation of internal aqueous volume and GFP concentration in each liposome from the red and green fluorescence intensities, respectively

The red fluorescence intensities obtained for each liposome were converted to the numbers of PE molecules based on the correlation between the two determined using a QuantiBrite PE Quantitation Kit (BD Biosciences, Franklin Lakes, NJ, USA), giving the equation

$$\log(N_{\mathrm{PE}}) = 0.91\log(\mathrm{FI_R}) + 1.60, \tag{1}$$

where N_{PE} is the number of PE molecules and $\mathrm{FI_R}$ is the red fluorescence intensity obtained by FACS analysis.

The green fluorescence intensities obtained for each liposome were converted to the number of GFP molecules based on the correlation between the two using BD Living Colors Enhanced GFP (EGFP) Calibration Beads (BD Biosciences), giving the equation

$$\log(N_{\mathrm{EGFP}}) = 0.99\log(\mathrm{FI_G}) + 2.16N_{\mathrm{GFPuv5}} = 1.57N_{\mathrm{EGFP}}, \tag{2}$$

where, N_{GFPuv5} and N_{EGFP} are the numbers of GFPuv5 and EGFP molecules, respectively, and $\mathrm{FI_G}$ is the green fluorescence intensity obtained by FACS analysis. It should be noted that there is a difference in fluorescence intensity between EGFP and GFPuv5 and that the values obtained with EGFP calibration beads were calibrated based on this difference.

It should also be noted that calculations were performed only for liposomes exhibiting red or green fluorescence intensity greater than that in preparations encapsulating only the buffer.

Plasmid purification and quantitative PCR

After sorting liposomes by FACS, plasmid DNA was purified using a LaboPass PCR purification kit (Hokkaido System Science, Sapporo, Japan). More than 90% of plasmid DNA could be recovered using this kit; thus, the loss of DNA was negligible during this step. Quantitative PCR was carried out using SYBR premix EXtaq (Takara Shuzo, Tokyo, Japan) on an ABI 7700 sequence detector in accordance with the manufacturer's recommendations. Primers used for specific amplification of pETG5tag were gfp_f (5′-TAGTTCCCGTCATCTTTG) and g5_r (5′-ACTTGTCACTACTCTGAC), and those used for specific amplification of pETG2-tag were gfp_f and g2_r (5′-ACTTGTCACTACTTTCTCT). Primers designed for pETG5tag were confirmed to amplify only the correct cognate and not the others (and vice versa).

Results and discussion

Estimating the internal aqueous volume of each liposome by FACS analysis

Regardless of their method of preparation, cell-size liposomes are heterogeneous in size [4], limiting their potential uses. Therefore, we established a strategy to estimate the internal aqueous volume of cell-size liposomes using a FACS machine. This was achieved by encapsulation of red fluorescent protein (PE) as an internal aqueous marker using the freeze-dried empty liposome method [17]. Briefly, aqueous solutions containing PE were added to dried lipid films (for details, see Materials and methods). Liposomes carrying PE were then analyzed by FACS, showing a large population of those present with increased red fluorescence intensity (Fig. 1B) as compared with those prepared with encapsulation of buffer alone (Fig. 1A).

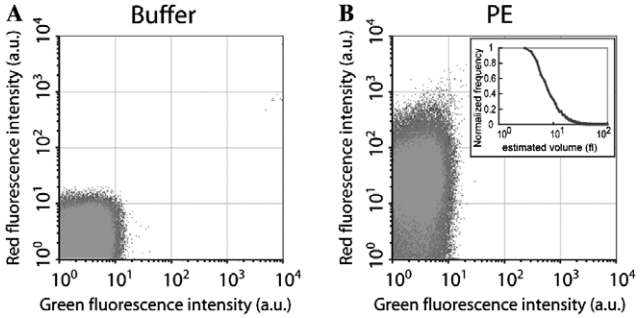

Fig. 1. Internal aqueous volume of individual liposomes measured by FACS. Density plots of green (horizontal) and red (vertical) fluorescence intensity analysis of liposomes prepared by encapsulating buffer IsoFlow used for FACS sheath fluid (no fluorescence) (A) and 0.3 μM PE (B). Inset in panel B shows the distribution of the internal aqueous volume of liposomes estimated from the red fluorescence intensity. Liposomes with internal aqueous volumes less than 2.6 fl could not be detected because their red fluorescence intensity was not greater than that of liposomes encapsulating the buffer alone.

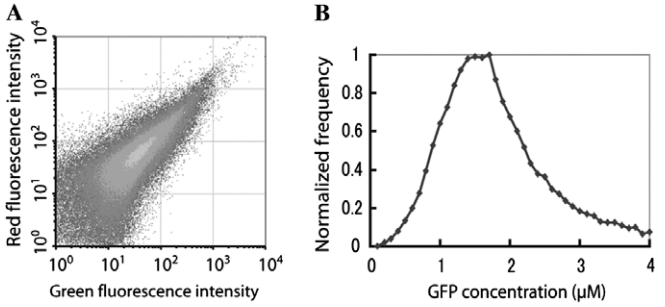

Fig. 2. GFP concentration determination in individual liposomes measured by FACS. (A) Density plots of green (horizontal) and red (vertical) fluorescence intensities of liposomes after encapsulating 1.5 μM GFP and 0.3 μM PE. (B) Distribution of GFP concentration in individual liposomes estimated from panel A using Eqs. (1) and (2) (see Materials and methods).

The correlation between the number of PE molecules and red fluorescence intensity measured by FACS was identified by analyzing PE-coated beads carrying different copy numbers of PE molecules on each bead (Eq. (1)). Knowing this correlation, the fluorescence intensity of each liposome can then be converted to the number of PE molecules inside each liposome. When the concentration of PE is known, the internal aqueous volume of each liposome can be estimated. The inset in Fig. 1B shows the distribution of the calculated internal aqueous volume of liposomes, with an average volume of 9.5 fl. It should be noted that the calculations were done only for liposomes exhibiting red or green fluorescence intensity greater than that in preparations encapsulating the buffer alone. The broad distribution of internal aqueous volume shown in the inset suggests that the volume can differ by more than 10-fold; thus, not only the yield of the product but also the internal aqueous volume must be considered in studies of enzymatic reactions within cell-size liposomes.

To verify the accuracy of the internal aqueous volume estimation, 1.5 μM GFP was encapsulated into liposomes together with the internal marker, namely PE (0.3 μM). If the volume estimation is appropriate, the concentration of GFP in individual liposomes should be 1.5 μM. The results of FACS analysis of these liposomes are shown in Fig. 2A. Based on these results, first, the numbers of GFP molecules in individual liposomes were estimated using the correlation between the number of GFP molecules and green fluorescence intensity determined by FACS (Eq. (2)). Second, the internal aqueous volumes of individual liposomes were estimated from the red fluorescence intensity using Eq. (1). From these two values, the distribution of GFP concentration in individual liposomes was obtained (Fig. 2B), showing a peak at 1.5 μM, consistent with the amount that had been encapsulated. These results suggest that the volume estimation is accurate and that the concentration of fluorescent compound in individual liposomes can be estimated from the fluorescence intensity.

Protein synthesis in vitro and in liposomes

Using the strategies described above, it is possible to detect the enzymatic reaction in individual liposomes in a high-throughput format so long as the encapsulated proteins give rise to a fluorescent signal such as by exhibiting fluorescence themselves or by catalyzing the production of a fluorescent compound. To verify the utility of our system, protein synthesis was carried out using the GFP gene as a template in liposomes. Here two GFP variants, GFPuv2 [16] and GFPuv5 [15] (encoded on plasmids pETG2tag and pETG5tag, respectively), were used. In vitro translation reaction of these two GFP variants in a test tube showed that GFPuv5 exhibits eightfold higher fluorescence intensity than does GFPuv2 when excited at 488 nm (Fig. 3A), consistent with the results obtained from purified proteins (data not shown). Using these two plasmids as templates, GFP synthesis reaction was carried out in liposomes and subsequently analyzed by FACS (Figs. 3B–D), first to investigate whether the synthesized GFP can be detected and second to verify whether the difference in fluorescence intensity between the two GFP variants can be detected. PE was added during the translation reaction to estimate the internal aqueous volume of each liposome as well as the average number of plasmids encapsulated in each liposome (for details, see above). On FACS analysis, liposomes expressing GFPuv5 clearly showed dots with high green fluorescence intensity (Fig. 3D), whereas those with GFPuv2 did not (Fig. 3C). These results indicated that GFP synthesis reaction could be detected in liposomes and that the difference in fluorescence intensity between GFPuv2 and GFPuv5 could be detected.

The synthesized protein concentration in each liposome was estimated from the results shown in the inset in Fig. 3D using the intensities of both green and red fluorescence. This was done using Eqs. (1) and (2), which convert the green and red fluorescence intensities to the numbers of GFP and PE molecules in each liposome, respectively. GFP synthesis in liposomes exhibits a broad distribution with an average concentration of 310 nM, which is approx-

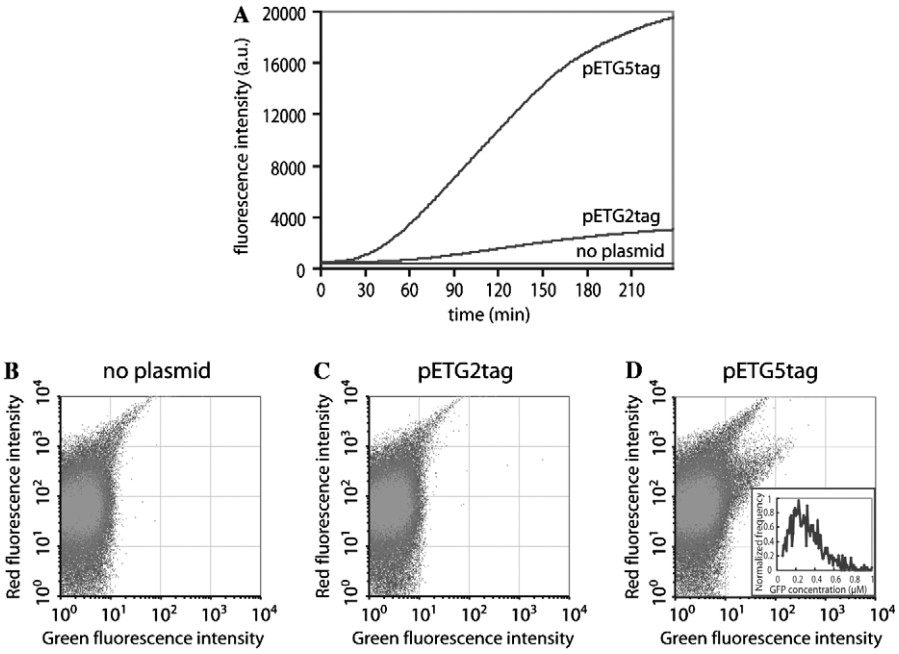

Fig. 3. In vitro translation of GFP in test tubes (A) and in liposomes (B–D). (A) In vitro translation reactions in test tubes were carried out at 37 °C for 4 h with a plasmid concentration of 1 ng/μl. (B–D) Density plots of green (horizontal) and red (vertical) fluorescence intensities of liposomes after in vitro translation at 37 °C for 4 h without plasmid (B), with 1 ng/μl pETG2tag (C), and with 1 ng/μl pETG5tag (D). The inset in panel D shows the distribution of synthesized GFPuv5 concentration in individual liposomes. The distribution was obtained from liposomes exhibiting red or green fluorescence intensities different from those in preparations encapsulating the in vitro translation system in the absence of the plasmids.

imately fivefold lower than that in the test tube under identical conditions. Although there are various possible explanations for the decrease in translation efficiency inside liposomes, such as the inhibitory effect of lipids and the permeability of the liposomes, yields of expressed proteins are sufficient to identify the differences in fluorescence intensity of GFPuv2 and GFPuv5.

Scheme of the in vitro selection of proteins using liposomes as a microcompartment

We have described a strategy to estimate the internal aqueous volume of liposomes by FACS (Fig. 1). Furthermore, synthesis of two GFP-exhibiting differences in fluorescence intensity could be detected, and the concentration of synthesized GFP in each liposome could be estimated (Fig. 3). These strategies were applied to carry out in vitro selection of proteins using liposomes as a microcompartment and the FACS as a detector (Fig. 4).

In vitro selection experiments seek to alter or improve the properties of the target molecule by consecutive rounds of diversification and selection. Typically, a gene encoding the protein of interest is mutated to generate a library of mutant genes and proteins are analyzed based on the properties of interest. Proteins exhibiting the desired properties are selected, and the genes encoding them are amplified and brought to further rounds of mutagenesis, selection, and amplification. The evolution of proteins differs from that of RNA or DNA in that proteins cannot be amplified themselves; thus, selection based on their properties must simultaneously select the genes encoding the proteins. Hence, the genotype and phenotype must be linked physically. By establishing the linkage, the properties of proteins, including binding affinity, catalytic activity, and stability, can be evolved [20].

Here this linkage was achieved by encapsulating a single copy of a gene into each liposome together with the in vitro translation system (Fig. 4). Under these conditions, expressed proteins and the corresponding genes are segregated from the others by being surrounded by liposomes. Moreover, so long as the protein function is correlated with the fluorescence intensity, by exhibiting fluorescence itself or by catalyzing the production of a fluorescent compound(s), sorting those with higher fluorescence intensity by FACS will result in a pool of genes encoding the proteins exhibiting high-fluorescence intensity (Fig. 4). Hence, in this system, the gene of interest (genotype) will be enriched based on the function of the expressed protein of interest (phenotype); thus, the genotype and phenotype are linked by being encapsulated in the aqueous compartments of liposomes.

The selection scheme consists of three steps (Fig. 4). The first step is the encapsulation of plasmid DNA together with the in vitro translation and transcription system into liposomes. Ideally, a single plasmid should be encapsulated in each liposome to establish a one-to-one linkage between the genotype and the phenotype. However, the number of plasmids incorporated into each liposome can vary for two reasons: (i) because of differences in the internal aqueous volume among individual liposomes (liposomes show

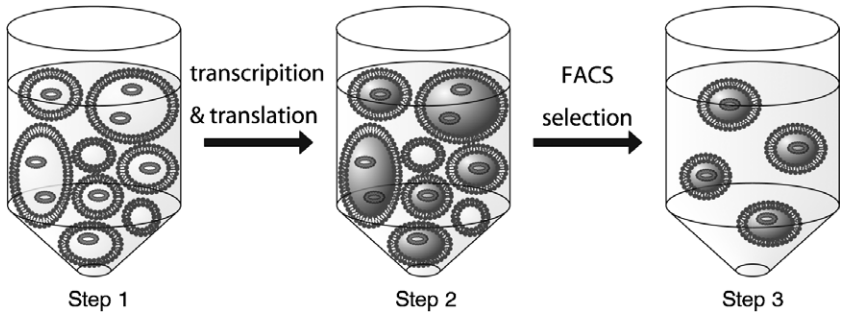

Fig. 4. Scheme of in vitro selection using liposomes and FACS. The scheme consists of three steps. In the first step, a plasmid DNA library encoding the protein of interest is encapsulated into liposomes with the in vitro translation system and PE, which is used to measure the internal aqueous volume of each liposome. This step is carried out at 4 °C. Liposomes are then transferred to 37 °C for transcription and translation reaction. In the second step, liposomes harboring mutants with activities that produce fluorescence (e.g., exhibit fluorescence themselves or catalyze the production of fluorescent compounds) will give rise to liposomes with detectable fluorescence intensity. In the third step, liposomes exhibiting greater fluorescence intensity and appropriate internal aqueous volume are collected by FACS. In this way, genes encoding proteins of interest can be enriched.

large differences in their sizes Fig. 1B) and (ii) because incorporation of the plasmid is an independent and stochastic event and thus follows the Poisson distribution. To overcome these problems, we estimated the internal aqueous volume of liposomes to determine the average number of plasmids in each liposome using PE as an internal aqueous volume marker. After the encapsulation step, the translation reaction is initiated by transferring the reaction mixture from 4 to 37 °C. In the second step (after the incubation step), proteins with activities to produce fluorescence (e.g., those that exhibit fluorescence themselves or catalyze the production of fluorescent compounds) will give rise to liposomes with detectable fluorescence intensity. In the third step, liposomes with an appropriate volume (carrying a single copy of the plasmid on average) and higher fluorescence intensity are collected using FACS. In this way, a pool of DNA can be subjected to the screening step, and the selected pool of DNA can be brought to the next round of selection.

Gene enrichment experiments

Gene enrichment experiments were carried out to evaluate the selection scheme described above. First, the plasmids pETG2tag and pETG5tag were mixed at a ratio of 0.85:0.15 (verified by quantitative PCR) and used as the template for in vitro translation reaction in liposomes. The plasmid concentration was 1 ng/μl, that is, the concentration needed to achieve an average of one copy of plasmid per liposome 2 μm in diameter. Fig. 5A shows the results of FACS analysis of these liposomes. Again, PE was added to the translation mixture to estimate the internal aqueous volume of each liposome, as depicted on the right vertical axis of Fig. 5A. Subsequently, six different regions (R1–R6) were sorted independently two or three times by FACS, and the plasmids contained were recovered for further evaluation.

GFPuv2 and GFPuv5 differ by only three amino acid residues, and these mutations are located very close to each other on the DNA sequence. Therefore, primers that can specifically amplify DNA encoding GFPuv2 or GFPuv5 were designed such that the 5′ end of the primers would anneal near the sequences that differ between the two variants. Using these primers for quantitative PCR, the numbers of copies of pETG2tag and pETG5tag were determined from a mixture of the two (Table 1). Moreover, the number of sorted liposomes from each region was counted during sorting by FACS. Using these numbers, the average number of plasmids in each liposome and the enrichment factor of plasmid pETG5tag over pETG2tag for each region, R1–R6, were estimated. The values obtained for each sorting region are summarized in Table 1. Furthermore, Fig. 5B shows the correlation between the average plasmid copy number in each liposome and the enrichment factor obtained for each sorted region. As expected, much higher enrichment factors were seen in the regions with liposomes possessing lower plasmid copy numbers. These results clearly demonstrated the potential for using liposomes in combination with FACS to evolve proteins so long as the protein function is correlated with the fluorescence intensity.

The fraction of the liposomes sorted in regions R1 and R2, carrying a single copy of the GFP gene, is 0.04% of the total number of events. Therefore, a gene library with a diversity of 10^5 can be screened using our current setup. These numbers can be increased by using more advanced FACS machines, such as BD FACSAria (BD Biosciences), but a more fundamental approach would be to understand the cause of the liposome's heterogeneity in size and establish a strategy to prepare cell-size liposomes with homogeneous size, although this is not yet feasible [4].

The main issue to establish the scheme shown in Fig. 4 is the variety in the size of liposomes that results in variations in the number of plasmids incorporated into each liposome. We have shown, by encapsulating PE as a measure of the internal aqueous volume of liposomes, that those with a single copy of the plasmid can be identified and that indeed a gene encoding GFP with higher fluorescence intensity can be enriched. To obtain further insight into these results, theoretical enrichment factors from each sort-

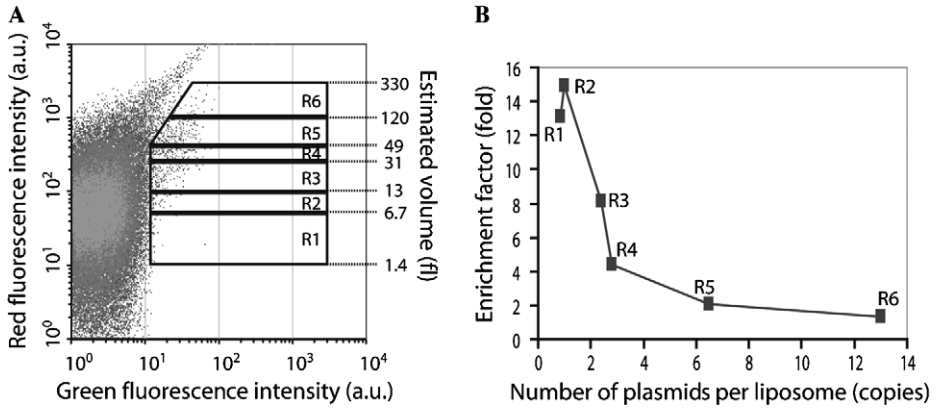

Fig. 5. Gene enrichment experiments using liposomes with FACS. (A) Density plots of green (horizontal) and red (vertical) fluorescence intensities of liposomes after in vitro translation at 37 °C for 4 h using a mixture of plasmids pETG2tag and pETG5tag (pETG2tag/pETG5tag = 0.85:0.15) as the template. Regions R1–R6 were sorted independently two or three times to obtain plasmids for further analysis. The right vertical axis shows the estimated volume of each liposome calculated from the red fluorescence intensity using Eq. (1). (B) Correlation between plasmid copy number per liposome and enrichment factor obtained from regions R1 to R6.

Table 1
Results of the gene enrichment experiments

	R1	R2	R3	R4	R5	R6
Copy number of pETG2tag (copies)[a]	130	760	23,000	31,000	220,000	110,000
Copy number of pETG5tag (copies)[a]	300	2000	33,000	24,000	82,000	26,000
Total number of liposomes[b]	500	2700	24,000	20,000	47,000	10,000
Ratio of the collected liposomes (%)[c]	0.014	0.029	0.074	0.064	0.138	0.029
Enrichment factor (fold)[d]	12.5	14.8	8.1	4.4	2.0	1.4
Plasmid copy number per liposome (copies)	0.9	1.0	2.4	2.8	6.5	13.0

[a] Copy numbers of plasmids pETG2tag and pETG5tag were determined by quantitative PCR.
[b] The number of liposomes sorted from each region is the number of events counted by FACS during the sorting process.
[c] The ratio of the collected liposomes is the ratio between the number of collected liposomes from each region and the number of total events required for the collection. It should be noted that the required numbers of total events were different among the sorted regions.
[d] The enrichment factor is defined as (pETG5tag copy number/pETG2tag copy number)/(0.15/0.85).

ed region were calculated based on the experimental conditions used. To evaluate the experimental results, these values were then compared with those obtained experimentally.

Theoretical enrichment factors were calculated (for details, see Appendix A) based on three assumptions: (i) the ratio of the initial plasmid mixture is pETG5tag/pETG2tag = 0.15:0.85; (ii) 8 molecules of GFPuv2 give a fluorescence intensity equivalent to that of one molecule of GFPuv5; and (iii) liposomes carrying at least a single copy of pETG5tag, or more than eight copies of pETG2tag even in the absence of pETG5tag, can be sorted by FACS. Based on these assumptions, equations were derived and the theoretical enrichment factors were calculated (for details, see Appendix A). As can be seen in Fig. 6, theoretical enrichment factors showed a good correlation with the experimentally determined values. Ideally, theoretical and experimental values should be identical; however, our results showed that the slope was 0.25, indicating that the theoretically determined enrichment factors were underestimated or that the experimentally determined enrichment factors were overestimated. This can be explained by the encapsulation efficiency of the

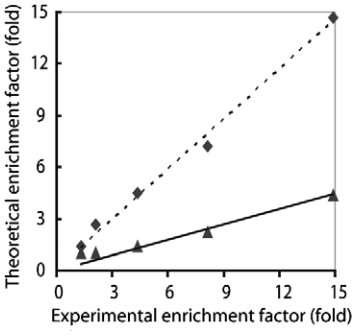

Fig. 6. Correlation between experimentally and theoretically determined enrichment factors obtained from each sorted region. Theoretical values were calculated using the equation given in Appendix A. Calculated values assuming plasmid encapsulation efficiencies of 100% (triangles) and 27% (diamonds) are plotted against values determined experimentally. Data from region R1 were omitted from the plot because this region includes liposomes below the limit of detection.

plasmid DNA that varies significantly between 10 and 80% depending on the lipid composition and the encapsulation method used [21,22]. This is thought to be due to both the size of the plasmids and the negative charge of the nucleic acids. In fact, the values become nearly iden-

tical when assuming an encapsulation efficiency of 27% (Fig. 6). Nevertheless, it is important that a linear correlation appears in Fig. 6 because this provides insight into the results obtained in future selection experiments. For example, when carrying out the same experiments with GFP variants that differ in their fluorescence intensity by only 2- or 3-fold, the enrichment factors are expected to be 2.7- or 8-fold, respectively. It should also be noted that although the maximum enrichment factor we have obtained experimentally was 14.8-fold (Table 1), the linearity suggests that this is what should be expected under our experimental setup. That is, unless the differences in fluorescence intensities of the two GFP variants are greater or the sensitivity of the FACS machine is improved, it is not possible to obtain greater enrichment factors.

Finally, these results indicate that red fluorescence intensity can be used as a measure of the internal aqueous volume of the liposomes and that efficient gene enrichment can be achieved in this way.

Significance of using liposomes as a microcompartment

Liposomes have been used for various applications, such as drug or gene delivery systems and enzyme replacement therapy [1]. Here, we have presented another application using liposomes as a microcompartment for in vitro selection of proteins. This system is significant for a number of reasons. First, use of an in vitro translation system expands the possibility of protein expression beyond using living cells. Second, in contrast to most other selection tools, this system allows the direct screening of enzymatic reactions. Phage display, ribosome display, and mRNA display have been used successfully as tools for in vitro evolution of proteins [20]. However, although there are some exceptions [23], they have been used mostly to improve the binding interactions of the target proteins to a given molecule. This is because the screening is based on binding to an immobilized ligand on a solid surface. On the other hand, liposomes provide a compartmentalized aqueous core, and so long as the protein function is correlated with the fluorescence intensity (as is the case with fluorescent proteins or fluorescent products produced by enzyme catalysis), genes encoding proteins with the desired properties can be enriched by FACS. Third, the use of liposomes as a microcompartment mimics the environment of cells and allows selection to occur in an environment surrounded by lipid.

In vitro compartmentalization [6,24] uses water-in-oil (w/o) emulsions as an aqueous core for in vitro translation reaction; as such, genotype–phenotype linkage occurs by encapsulating both the gene and the encoded proteins. This method allows direct screening for catalysis, and evolution experiments of enzymes have been reported using an elegant strategy by encapsulating microbeads into the w/o emulsions. Furthermore, FACS has been used to sort the emulsions with desired fluorescence intensity using water-in-oil-in-water (w/o/w) emulsions [25–27]. Although the w/o/w emulsions have great potential for high-throughput screening, they do not provide a biologically relevant environment such as that achieved by lipid vesicles. We believe that our system can be applied to the alteration of proteins that interact with lipids such as membrane proteins. Furthermore, the possibility of integrating further functions into the vesicles, such as selective permeability with integration of pore proteins [28] and a change in the lipid composition [29,30], is another interesting feature that is possible only with liposomes. Moreover, using this system, evolution of self-replicating molecules may be possible to experimentally construct artificial cells [31–34].

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Appendix A

Theoretical enrichment factors were calculated as shown below.

The probability of a single liposome before the sorting process possessing n copies of plasmid of which x is pETG5tag ($c(n,x)$) can be expressed as

$$c(n,x) = a(n)\cdot b(x:n,p) = \frac{\mathrm{e}^{-y}y^n}{n!}\cdot\binom{n}{x}p^x(1-p)^{n-x},$$

where $a(n)$ is the probability of a single liposome harboring n copies of the plasmid when the average is y copies and $b(n)$ is the probability of x copies of n being the plasmid pETG5tag when the proportion of that to pETG2tag is p. $a(n)$ and $b(x\colon n, p)$ are assumed to follow the Poisson distribution and binomial distribution, respectively.

Assuming that liposomes carrying at least a single copy of plasmid pETG5tag or more than eight copies of pETG2-tag even in the absence of pETG5tag are sorted according to their green fluorescence intensity by FACS, the fraction of liposomes being sorted (d) can be expressed as

$$d = 1 - \sum_{n=0}^{7} c(n,0).$$

Therefore, the probability of a sorted liposome possessing n copies of plasmids of which x are pETG5tag ($q(n,x)$) can be expressed as

$$q(n,x) = \frac{c(n,x)}{d},$$

and the average number of copies of plasmids pETG5tag (N_{G5}) and pETG2tag (N_{G2}) found in a single sorted liposome can be expressed as

$$N_{G5} = \sum_{n=1}^{\infty} \sum_{x=1}^{n} x \cdot q(n,x)$$

$$N_{G2} = \sum_{n=1}^{\infty} \sum_{x=1}^{n} (n-x) \cdot q(n,x) + \sum_{n=8}^{\infty} n \cdot q(n,0).$$

Finally, the theoretical enrichment factor is defined as

$$\frac{N_{G5}/N_{G2}}{0.15/0.85}.$$

References

[1] D. Felnerova, J.F. Viret, R. Gluck, C. Moser, Liposomes and virosomes as delivery systems for antigens, nucleic acids, and drugs, Curr. Opin. Biotechnol. 15 (2004) 518–529.

[2] P.L. Luisi, P. Walde, Giant Vesicles, John Wiley, Chichester, UK, 2000.

[3] D.T. Chiu, C.F. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B.P. Modi, A. Moscho, R.A. Garza-Lopez, O. Orwar, R.N. Zare, Chemical transformations in individual ultrasmall biomimetic containers, Science 283 (1999) 1892–1895.

[4] P. Walde, S. Ichikawa, Enzymes inside lipid vesicles: preparation, reactivity, and applications, Biomol. Eng. 18 (2001) 143–177.

[5] Y. Rondelez, G. Tresset, K.V. Tabata, H. Arata, H. Fujita, S. Takeuchi, H. Noji, Microfabricated arrays of femtoliter chambers allow single molecule enzymology, Nat. Biotechnol. 23 (2005) 361–365.

[6] A.D. Griffiths, D.S. Tawfik, Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization, EMBO J. 22 (2003) 24–35.

[7] J. Drott, L. Rosengren, K. Lindstrom, T. Laurell, Pore morphology influence on catalytic turn-over for enzyme activated porous silicon matrices, Thin Solid Films 330 (1998) 161–166.

[8] Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, T. Ueda, Cell-free translation reconstituted with purified components, Nat. Biotechnol. 19 (2001) 751–755.

[9] R.G. Laughlin, Equilibrium vesicles: fact or fiction? Colloids Surf. A 128 (1997) 27–38.

[10] B. Mui, L. Chow, M.J. Hope, Extrusion technique to generate liposomes of defined size, Methods Enzymol. 367 (2003) 3–14.

[11] W. Yu, K. Sato, M. Wakabayashi, T. Nakaishi, E.P. Ko-Mitamura, Y. Shima, I. Urabe, T. Yomo, Synthesis of functional protein in liposome, J. Biosci. Bioeng. 92 (2001) 590–593.

[12] K. Ishikawa, K. Sato, Y. Shima, I. Urabe, T. Yomo, Expression of a cascading genetic network within liposomes, FEBS Lett. 576 (2004) 387–390.

[13] K. Vorauer-Uhl, A. Wagner, N. Borth, H. Katinger, Determination of liposome size distribution by flow cytometry, Cytometry 39 (2000) 166–171.

[14] K. Sato, K. Obinata, H. Sugawara, I. Urabe, T. Yomo, in: A.J. Ijspeert, T. Masuzawa, S. Kusumoto (Eds.), Lecture Notes in Computer Science, Springer, Berlin, 2006, pp. 330–337.

[15] M. Suzuki, Y. Ito, H.E. Savage, Y. Husimi, K.T. Douglas, Protease-sensitive signalling by chemically engineered intramolecular fluorescent resonance energy transfer mutants of green fluorescent protein, Biochim. Biophys. Acta 1679 (2004) 222–229.

[16] Y. Ito, M. Suzuki, Y. Husimi, A novel mutant of green fluorescent protein with enhanced sensitivity for microanalysis at 488 nm excitation, Biochem. Biophys. Res. Commun. 264 (1999) 556–560.

[17] H. Kikuchi, N. Suzuki, K. Ebihara, H. Morita, Y. Ishii, A. Kikuchi, S. Sugaya, T. Serikawa, K. Tanaka, Gene delivery using liposome technology, J. Control. Release 62 (1999) 269–277.

[18] T. Matsuura, M. Yamaguchi, E.P. Ko-Mitamura, Y. Shima, I. Urabe, T. Yomo, Importance of compartment formation for a self-encoding system, Proc. Natl. Acad. Sci. USA 99 (2002) 7514–7517.

[19] T. Matsuura, A. Plückthun, Selection based on the folding properties of proteins with ribosome display, FEBS Lett. 539 (2003) 24–28.

[20] H. Leemhuis, V. Stein, A.D. Griffiths, F. Hollfelder, New genotype–phenotype linkages for directed evolution of functional proteins, Curr. Opin. Struct. Biol. 15 (2005) 472–478.

[21] P.A. Monnard, T. Oberholzer, P. Luisi, Entrapment of nucleic acids in liposomes, Biochim. Biophys. Acta 1329 (1997) 39–50.

[22] G. Gregoriadis, B. McCormack, M. Obrenovic, R. Saffie, B. Zadi, Y. Perrie, Vaccine entrapment in liposomes, Methods 19 (1999) 156–162.

[23] A. Fernandez-Gacio, M. Uguen, J. Fastrez, Phage display as a tool for the directed evolution of enzymes, Trends Biotechnol. 21 (2003) 408–414.

[24] D.S. Tawfik, A.D. Griffiths, Man-made cell-like compartments for molecular evolution, Nat. Biotechnol. 16 (1998) 652–656.

[25] K. Bernath, M. Hai, E. Mastrobattista, A.D. Griffiths, S. Magdassi, D.S. Tawfik, In vitro compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting, Anal. Biochem. 325 (2004) 151–157.

[26] A. Aharoni, G. Amitai, K. Bernath, S. Magdassi, D.S. Tawfik, High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments, Chem. Biol. 12 (2005) 1281–1289.

[27] E. Mastrobattista, V. Taly, E. Chanudet, P. Treacy, B.T. Kelly, A.D. Griffiths, High-throughput screening of enzyme libraries: in vitro evolution of a β-galactosidase by fluorescence-activated sorting of double emulsions, Chem. Biol. 12 (2005) 1291–1300.

[28] V. Noireaux, A. Libchaber, A vesicle bioreactor as a step toward an artificial cell assembly, Proc. Natl. Acad. Sci. USA 101 (2004) 17669–17674.

[29] M.E. Weber, P.H. Schlesinger, G.W. Gokel, Dynamic assessment of bilayer thickness by varying phospholipid and hydraphile synthetic channel chain lengths, J. Am. Chem. Soc. 127 (2005) 636–642.

[30] S. Raffy, J. Teissie, Control of lipid membrane stability by cholesterol content, Biophys. J. 76 (1999) 2072–2080.

[31] V. Noireaux, R. Bar-Ziv, J. Godefroy, H. Salman, A. Libchaber, Toward an artificial cell based on gene expression in vesicles, Phys. Biol. 2 (2005) 1–8.

[32] D. Deamer, A giant step towards artificial life? Trends Biotechnol. 23 (2005) 336–368.

[33] J.W. Szostak, D.P. Bartel, P.L. Luisi, Synthesizing life, Nature 409 (2001) 387–390.

[34] P.L. Luisi, F. Ferri, P. Stano, Approaches to semi-synthetic minimal cells: a review, Naturwissenschaften 93 (2006) 1–13.