Gene Expression within Cell-Sized Lipid Vesicles

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Functional protein synthesis was observed in cell-sized lipid vesicles following encapsulation of a gene-expression system. Expression of rsGFP (red-shifted green fluorescent protein) within individual vesicles was observed by fluorescence microscopy. Interestingly, at the early stage of the reaction, the expression efficiency inside the vesicle was remarkably higher than that in the solution outside. The synthesized rsGFP in individual vesicles is safe from attack by proteinase K added to the external aqueous solution. Studies on cell-sized vesicles expressing protein should contribute to a fundamental understanding of certain aspects of living systems and will be useful for practical applications, such as the construction of microreactors.

Introduction

A cell enclosed by a biomembrane is the common unit structure shared by all living organisms. This suggests that compartmentalization with a boundary between the inner space and the outer environment is indispensable for life on Earth. The biochemical and biophysical properties of the microenvironment inside such a compartment are probably also advantageous for life, judging from the fact that most living cells range from 1 – 100 μm in size, despite the great diversity in the size of the whole organism. Thus, it is reasonable to expect that encapsulation of a set of biomolecules reacting reciprocally in the primitive solution on Earth, such as RNAs, proteins, etc., might have been the last important step leading to the advent of a living system capable of reproducing itself.[1] Vesicles (liposomes) formed from phospholipids have frequently been used as models for living cellular structures, since they make an ideal tool for investigating enclosed systems containing ongoing biochemical processes, including the replication of RNAs,[2] the polymerase chain reaction,[3] and polypeptide synthesis.[4] Thus, tests on the encapsulation of gene-expression systems inside cell-sized lipid vesicles would serve as an essential step in understanding the formation of protocells.[5]

Recently, we reported that DNA molecules can be efficiently entrapped within microscopically observable cell-sized vesicles prepared by using a "natural" swelling method.[6] We have also verified that encapsulated T7 DNA molecules are transcribed into RNAs inside such giant vesicles,[7] while another research team succeeded in this using micropipette techniques.[8] As an extension of these studies, we examined the efficient expression of a rsGFP (red-shifted green fluorescent protein) gene in a giant vesicle under the control of a T7 promoter. Compartmentalization had dramatic effects: 1) gene expression takes place more efficiently inside vesicles than outside, and 2) vesicles can protect internal gene products from attack by an external proteinase K.

Results and Discussion

Figure 1 shows the experimental setup. To obtain a giant vesicle of about 5 μm, we used a natural swelling method that results in the formation of cell-sized vesicles of DOPC/DOPG (10:1 molar ratio) without aggregation, even under salty conditions, as previously described.[9] The fluorescence properties of individual cell-sized vesicles containing a cell-free gene expression system were investigated by recording their microscopic fluorescence images. Figure 2 shows microscopic images of an individual giant vesicle with rsGFP gene expression. Specific rsGFP fluorescence was emitted from the inside of a hollow vesicle; this is evidence of the functional folding of rsGFP. Dark-field microscopy gives high-contrast light-scattering images of colloidal particles, such as the membrane shape of the spherical vesicle, in solution in real time. As shown in the dark-field images in this figure, the interior of the vesicle was not crowded with dense colloids, while the external solution contained inter-

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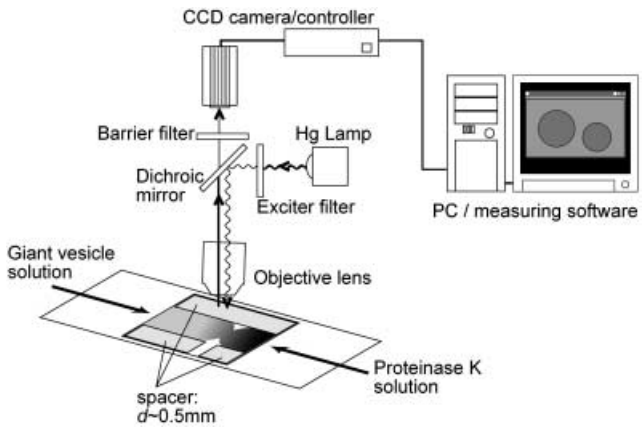

Figure 1. *Schematic illustration of the equipment used to measure the time-course fluorescence of vesicular images. Images of individual vesicles were captured on a personal computer, and the fluorescence intensities were measured.*

spersed colloidal particles. From the integrated intensity of fluorescence for the same area inside and outside the vesicles, the level of expression was evaluated, as shown in Figure 3a. The graph summarizes the degree of expression inside and outside the vesicle under different experimental conditions. In the absence of plasmid DNA as a control, the fluorescence intensity remained low. An amino acid mixture devoid of methionine was used in a second control experiment, since protein synthesis in prokaryotes starts with formylmethionine. Thus, the fluorescence intensity remained at a basal low level, since no rsGFP protein was expressed. The intensity of internal fluorescence was greater than that on the exterior 3 h after the start of hydration; this indicated that the concentration of expressed rsGFP inside the vesicles was higher than that in the external environment (Figure 2a). To observe the internally expressed rsGFP by distinguishing it from that of the external solution, we performed the measurement on a cross-section of the cell-sized vesicle using of laser-scanning microscopy (LSM) (Figure 4). From this measurement, it became clear that the concentration of internal rsGFP was about ten times higher than that in the external solution (see Supporting Information). The enhancement of expression inside the vesicle may be due to the effective accumulation of synthesized macromolecules (mRNA, proteins) inside vesicles that occurs under the experimental conditions in the present study.

On the other hand, the expression levels inside and outside the vesicles were almost the same at 24 h after sample preparation (Figure 2b). This result confirms that the compartmentalized vesicle affords the internal environment for the effective synthesis of rsGFP. Although the detailed mechanism for attaining such high efficiency is still unclear, two possible factors can be considered: 1) biochemical substrates for the transcription/translation may be more concentrated inside the vesicle than the outside and 2) the efficiency of the enzymatic reactions may be enhanced in μm-sized vesicular environments. In relation to the former effect, we have recently found that the density of the number of the DNA molecules is enhanced, at least one order higher, in the intravesicular space relative to the external solution. Such an enhancement of the DNA concentration could be attributable to the local increase of osmotic pressure due to the numerous counter ions, which accompany the DNA molecule, being situated in a narrow space between lipid layers.[10] As for the second possible factor, we propose an enhancement of the surface effect in μm-sized vesicular system due to the larger surface/volume ratio compared with a centimeter-sized test tube. If enzymatic reactions are promoted on the periphery of the membrane, the rate of gene expression can be enhanced. Further studies are necessary to clarify the mechanism of the high efficiency of gene expression in vesicular environments.

To investigate the ability of the membrane to act as a barrier to external chemicals, proteinase K was added to the solution on the glass slide. Over several hours, proteinase K gradually diffused into the solution. The fluorescence intensity outside of the vesicles became weaker with the diffusion of proteinase K (Figure 3b). As a control experiment, we monitored the change in the fluorescence intensity inside and outside the vesicles by adding a buffer solution without proteinase K. The results confirmed that the fluorescence intensity outside the vesicle, as well as that inside, remained almost constant, this indicates that the lipid bilayer acts as a barrier to the penetration of proteinase K into cell-sized vesicles.

Over the past few decades, there have been several studies on biochemical reactions in small vesicles of less than 1 μm in diameter.[11] However, only a few studies have examined the use of giant vesicles containing biochemical processes.[12] Recently, a few research groups have studied protein synthesis inside vesicles. Oberholzer et al. used non-native RNA(poly(U)) as a template for synthesis of the polypeptide poly(Phe),[4] in which the translation reaction in the vesicles was measured by radioactivity. Yu et al. reported an experimental trial to form giant vesicles (eggPC/cholesterol/DSPE-PEG5000) in a solution undergoing gene expression.[5] They confirmed this protein synthesis by flow cytometry and fluorescence microscopy. However, the difference in fluorescence intensity between the inside and outside of individual vesicles has not been observed, at least in a reliable manner.

In the present study, using direct fluorescence microscopic observation, we obtained evidence of rsGFP protein synthesis inside a single cell-sized vesicle formed by the natural swelling of phospholipids, following encapsulation of the gene-expression system. Our data show that transcription and translation reactions of the GFP expression vector are more efficient within a cell-sized vesicle than in the solution outside the vesicle. In addition, we found that the GFP protein expressed inside the vesicle is protected from proteolysis by proteinase K added to the surrounding solution. Thus, we accomplished a series of processes (encapsulation of DNA, transcription, and translation within cell-sized vesicles) that might be indispensable for the formation of proto-cells.

From a theoretical point of view, some studies have predicted specific properties of an enclosed system with finite small volume, implying the importance of such properties in biology.[13] Further studies are necessary to obtain deeper insight into the physico-chemical properties of compartmentalized

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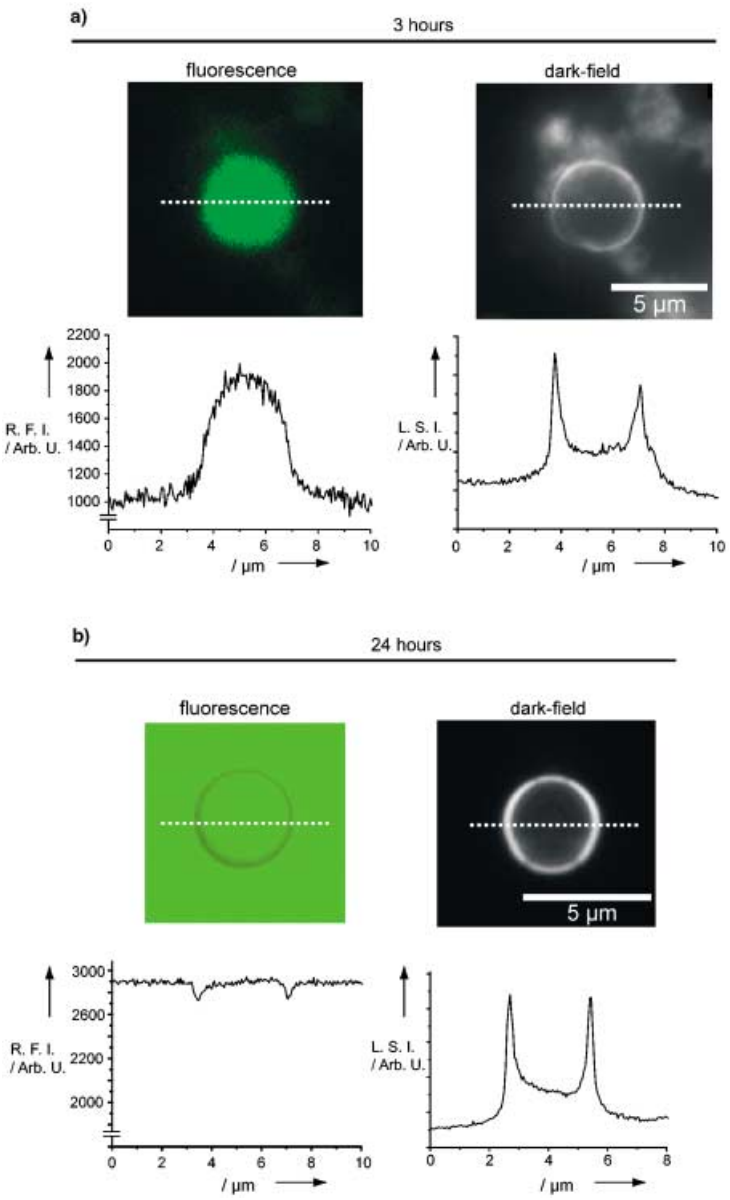

Figure 2. Images of a cell-sized vesicle observed 3 h and 24 h after the hydration of lipid films. Top: fluorescence and dark-field microscopic images of the vesicle. Bottom: cross-section profiles of the respective microscopic images. R.F.I. = relative fluorescence intensity, L.S.I. = light-scattering intensity. The R.F.I. minus the background fluorescence level is given, where the intensity in the solution without DNA is taken as the background level.

systems at a micrometer scale and to understand the biological significance of cell-sized systems.

Experimental Section

Giant-vesicle preparation: A solution of dioleoyl phosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC; Alexis Biochemicals; 10 mM) and dioleoyl phosphatidylglycerol (1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol, DOPG; Sigma; 1 mM) in methanol/chloroform (25 µL, 1:2 *v*/*v*; absolute solvents) in a glass test tube was left to dry overnight under vacuum to obtain lamellar lipid films. The films were hydrated with cell-free transcription/translation solution (25 µL) at 37 °C; this is generally called the "natural swelling method". For cell-free gene expression, we adopted a PROTEINscript-PRO Kit (Ambion) with E. coli S30 extract and T7 RNA polymerase for coupled transcription/translation, as originally developed by Zubay.[14] Plasmid pQBI T7 (5115 bp; Nippon Gene) encoding a T7 promoter and the rsGFP gene ($\lambda_{abs} = 473$ nm / $\lambda_{em} = 509$ nm) was dissolved in the transcription/translation mixture. The final mixture was composed of E. coli S30 extract, 0.3 × Master Mix (MM), amino acid (0.1 mM; L-methionine from Nakalai Tesque, otherwise from Ambion), plasmid DNA (20 ng µL^{-1}), and T7 RNA polymerase. Distilled water (Otsuka Pharmaceutical Factory) was used as nuclease-free water throughout the experiments. The reaction solution was prepared on ice to completely inhibit gene expression, according to the protocol of this kit as supplied by the manufacturer, except that the final concentration of MM was reduced (for better vesicle formation). Reagents were used in the experiments without further purification and treated with caution in RNase-free conditions. The samples containing hydrated lipid films were incubated at 37 °C.

Fluorescence microscopy: A cover glass was fixed at the center of a glass slide with double-sided adhesive tape (thickness ca. 0.5 mm). A suspension (30 µL) of vesicles containing the transcription/translation reaction solution was injected from one side of the space between the glass slide and the cover glass. These microscope experiments were carried out several times for each experimental condition. A fluorescence microscope (E600, Nikon) with a 100 W high-pressure Hg lamp and a dark-field condenser were used to observe the vesicles. A standard filter set (41001, CHROMA; ex 460 – 500 nm, dichroic mirror 505 nm, em 510 – 560 nm) was used to monitor the fluorescence of rsGFP. The images were captured with a digital camera (C4743-95, Hamamatsu Photonics) and recorded and analyzed with an image processor (Aqua Cosmos, Hamamatsu Photonics).

Fluorescence quenching experiment: Proteinase K solution (10 µL, 200 µg mL^{-1}) was injected from the other side of the apparatus mentioned above (after 24 h incubation). The two solutions were mixed gently at the center of the glass slide.

Confocal laser-scanning microscopy: A fluorescent lipophilic tracer of 4-Di-10-ASP (ex 492 nm, em 612 nm; Molecular Probes) was adopted to stain phospholipid membranes in a specific manner. A solution of DOPC (10 mM), DOPG (1 mM), and 4-Di-10-ASP (1 µM) in methanol/chloroform (25 µL, 1:2 *v*/*v*; absolute solvents) in a glass test tube was allowed to dry overnight under vacuum to obtain lamellar lipid films, which in turn were hydrated with the transcription/translation solution (25 µL) for as long as three hours at 37 °C. An aliquot (10 µL) of the solution thus prepared was placed on a glass slide and sealed by a cover glass with 30 – 60 µm depth. The sample was immediately observed with a confocal laser-scanning microscope (LSM inverted 510 equipped with a Plan Apo 63 × objective lens, Carl Zeiss). An argon laser (488 nm) was employed to excite the GFP and 4-Di-10-ASP. The emitted fluorescence was split into two different beams, 505 – 515 nm for GFP and 560 – 615 nm for 4-Di-10-ASP; this allowed observation of the expressed protein and membrane, respectively. The GFP concentration was estimated from an analytical curve of LSM images of pure rsGFP solution (see Supporting Information).

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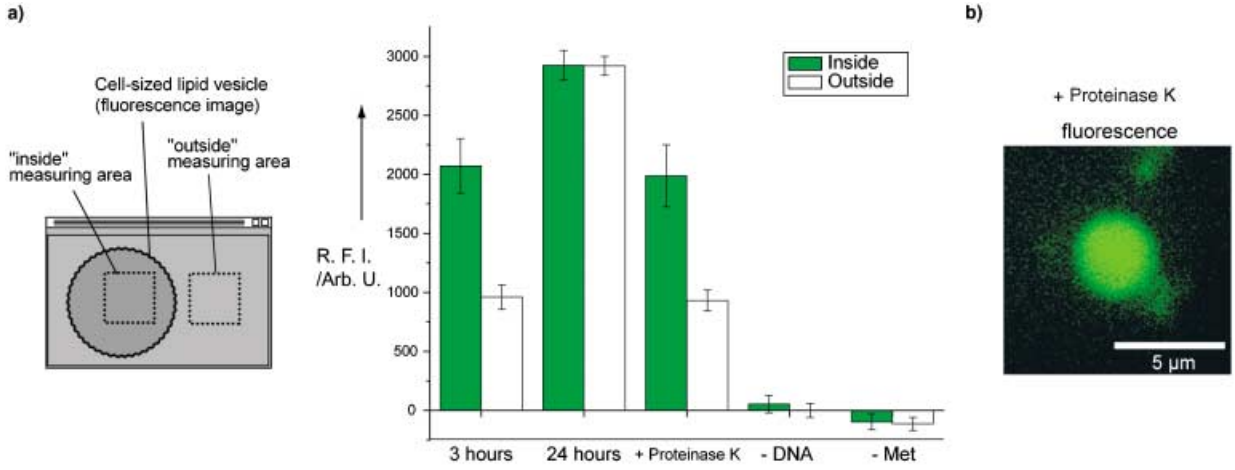

Figure 3. *a) Relative fluorescence intensities emitted from inside and outside individual giant vesicles containing expressed rsGFP protein. The effects of the addition of proteinase K as well as two negative control experiments (DNA-free, methionine-free) are also shown. The area used to measure the fluorescence intensity is* $3 \times 3\,\mu m^2$ *(*$> 10^4$ *pixels). The fluorescence intensity is expressed as the mean value* $\pm$ *SD (number of vesicles measured* n = *20 – 30). The relative fluorescence intensity is represented as in Figure 2. b) Fluorescence image of rsGFP on a vesicle 60 min after the addition of proteinase K to the external solution. The vesicle was incubated for 24 h (as in Figure 2b) before the addition of the proteinase.*

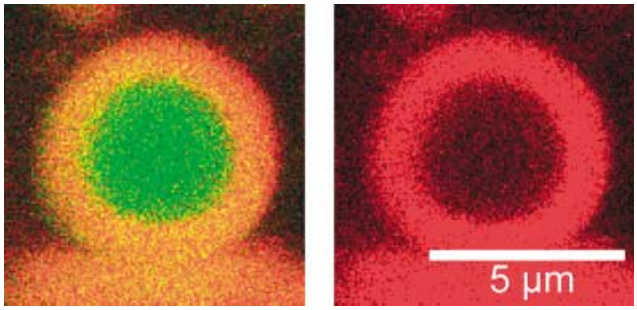

Figure 4. *Cross-sectional images of the giant vesicle observed by confocal laser scanning microscopy. The vesicle was formed through hydration for 3 h at 37 °C, similar to the conditions given in Figure 2a. Left: image on rsGFP (green), indicating the expression within the vesicular space. Right: image on the hydrophobic fluorochrome, 4-Di-10-ASP, indicating the spherical vesicular shape.*

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