Integration of a Reconstituted Cell-free Protein-synthesis System on a Glass Microchip

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Recently, a cell-free protein synthesis system reconstituted solely from essential elements of the Escherichia coli translation system, termed protein synthesis using recombinant elements (PURE), has been widely used in synthetic biology to analyze fundamental life systems. Here, the system was integrated on a glass microchip system to construct a simple protein synthesis system. GFP template DNAs were immobilized on Sepharose microbeads by streptavidin-biotin binding. The beads were introduced into a Y-shaped microchannel in a glass microchip with a 10-μm height dam structure, and a PURE system reaction mixture was flowed through the microchannel. The recovered solutions had a higher fluorescent intensity than that of the reaction mixture before its introduction into the microchannel, thus verifying that GFP synthesis had been achieved. The microchip with DNA immobilized microbeads is reusable. This is advantageous over a conventional in vitro protein synthesis protocol requiring the preparation and addition of template DNA or mRNA into the reaction mixtures in aspect of simpleness and rapidness.

Keywords Cell-free protein synthesis system, PURE system, continuous flow, microchip

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Introduction

The preparation of proteins from the genetic molecules DNA and RNA is one of the essential approaches to analyze their roles in living systems. Recent advances in gene synthesis techniques have enabled us to obtain the genetic molecules directly from sequence information, even when lacking access to the cells or their genetic molecules. Accordingly, a method to rapidly synthesize multiple proteins in parallel from their genetic information is expected to yield important insights in the field of molecular biology.

A cell-free protein synthesis system can meet the challenge of preparing multiple proteins. Such systems require only the addition of a template DNA or mRNA into the reaction mixtures and incubation for several hours to yield the proteins; they are therefore well suited for parallel synthesizes of multiple proteins. Furthermore, these methods can be used to synthesize cytotoxic or unstable proteins that could never be obtained by methods using living cells. They also offer simplified purification processes because it is not necessary to lyse the cells in order to purify the synthesized proteins.

One reconstituted cell-free protein synthesis system, the PURE (Protein synthesis Using Recombinant Elements) system, is unique because instead of using the normally applied cell-extract (S30) solution it uses purified factors and enzymes that recapitulate the protein synthesis apparatus in E. coli cells. This fully reconstituted system is highly controlled because all of its components are defined, thus providing a simple purification of products and the construction of a cell-mimetic reaction environment to analyze biochemical reactions in cells.

Here, we applied a microchip technique, which is a useful tool for exploiting the small-scale property, to the protein synthesis by the PURE system. The miniaturization of chemistry or biology using a microchip, called lab-on-a-chip technology or a micro total analysis system (μ-TAS), has generated substantial interest due to the reduced reagent consumption, space requirements and analysis times required for these systems, as well as their ease of operation. Exploiting these advantages, many researchers have developed novel methods to use microdevices for chemical and biochemical analysis and synthesis. The integration of the PURE system and high throughput parallel synthesis and analysis using could also be realized by applying this type of system.

A significant impediment to miniaturizing protein synthesis in this manner has been the adsorption or absorption of biomolecules to silicone rubber, polydimethylsiloxane (PDMS), which is a component of these devices. Minimizing the scale increases the surface area-to-volume ratio, thus amplifying interfacial phenomena at the PDMS surface. Therefore, methods to modify the surfaces of PDMS microchips have been developed to prevent these problems. Although a hydrophilic coating can prevent adsorption, the surface becomes more likely to absorb many biomolecules. On the other hand, although a hydrophobic coating will prevent absorption, it can make the surface more likely to adsorb many biomolecules. Alternative
solutions, such as the lipid-coated microdroplet array based method\textsuperscript{17} or a water in-oil droplet reactor,\textsuperscript{18} are quite effective. Also, they can carry out the PURE reaction system on a single molecule level, which is useful for molecular analysis. However, these droplet-based methods confine the PURE ingredients in a small space, limiting the amount of protein that can be recovered, and the operations are still complicated. Here, glass is generally known to have a property of little adsorption or absorption. Therefore, one strategy is to use a glass microchip to prevent the adsorption of proteins. Another strategy is to utilize DNA immobilized microbeads to synthesize proteins by simply flowing PURE ingredients continuously through microchannels on a microchip.

The aim of this report is to construct a simple and easy protein synthesis system based on these two strategies. First, protein synthesis using a PURE system with DNA immobilized on microbeads in a microtube was confirmed. PURE ingredients were then introduced into a microchip with DNA immobilized on microbeads, and synthesis of the prototypical protein, Green Fluorescent Protein (GFP) was verified.

**Experimental**

**Surface modification of beads**

A forward DNA primer that is biotinylated at the 5’ end (5’-biotin-GGGCCTAATACGACTCACTATAG-3’) was used for PCR amplification of the gene encoding Strep-tag II fusion GFP. The DNA template was prepared according to a previous report.\textsuperscript{4} Purified GFP templates were stirred with Streptavidin-coated beads (34 μm, Streptavidin Sepharose High Performance, GE Healthcare) in buffer containing 20 mM Hepes-KOH, pH 7.6 and 100 mM KCl for 1 h at room temperature on a tube mixer (TWIN3-28N, SCINICS, Japan). GFP template-immobilized beads thus obtained were filtered using ULTRAFREE-MC centrifugal filter devices (PVDF 0.45 μm, Merck-Millipore), washed, and resuspended in water and stored at 4°C until used.

**Protein synthesis reaction using the PURE system in a microtube**

Batch reactions using DNA-immobilized beads in the PURE system in a microtube were carried out using a Thermomixer comfort (Eppendorf) with mild shaking. Methods for the preparation and assembly of the PURE system components were described previously.\textsuperscript{19} After the protein synthesis reaction, aliquots (5 μL) were withdrawn and diluted to 100 μL with an HT buffer (50 mM Hepes-KOH, pH 7.6, 100 mM KCl, 10 mM MgCl\textsubscript{2}) and the fluorescence intensities were measured using a Wallac 1420 Multilabel counter (PerkinElmer).

**Microchip preparation**

A glass microchip with a dam structure was fabricated using a 2-step photolithographic wet-etching method.\textsuperscript{20} The fabrication scheme is briefly summarized here. A borosilicate glass (Tempax) substrate was coated with Cr, Au, and photoresist layers. The deep microchannel pattern was transferred by irradiating UV light through a photomask. The deep microchannel pattern was etched using an HF solution after development of the resist, Au, and Cr layers. The microchannel pattern was transferred by UV irradiation through a photomask. The deep and shallow microchannel patterns were then etched using an HF solution. After stripping the resist, Au, and Cr layers, the fabricated substrate was thermally bonded with a cover glass plate.

**Results and Discussion**

**Design and principle**

Figure 1a illustrates the concept of the PURE system using a glass microchip and microbeads. Sepharose-based microbeads are packed in a microchannel in front of a dam structure, as
that are adsorbed to the microchannel walls would block non-volume ratio, we carried out following experiments. Although and PDMS used for the chamber material. (a) Schematic illustration of the experimental protocol. (b) Relative fluorescence intensities of synthesized GFP on glass and PDMS, where the intensity of the synthesized GFP in a cuvette tube and that before the reaction were assigned a value of 100 and 0%, respectively. The values are represented as mean ± S.D.; n = 3.

shown in Fig. 1b. A Y-shaped channel is used to select packed beads with a length of 10 mm in this design, as in a previously developed DNA analysis system that used a microchip and microbeads.22 GFP template DNA was immobilized on the microbeads. After washing the channel and beads by introducing the buffer for the PURE system containing amino acids and nucleotides to perform blocking treatment of these materials and equilibrate the channel, PURE system reaction mixtures were introduced by a syringe pump, and the recovered eluate was collected in a microtube for fluorescent measurement as shown in Fig. 1c. Methods for the preparation and assembly of the PURE system components were described previously.19

In this system, most of the DNA molecules are tightly bound to the beads by interactions between biotin and streptavidin, which prevent adsorption of DNA to microchannel walls. Also, the nucleotides and proteins in the buffer for the PURE system that are adsorbed to the microchannel walls would block non-specific binding of DNA and synthesized proteins.

A PDMS-based microchip is usually used for the lab-on-a-chip technology or μ-TAS. However, as stated in Introduction, PDMS has adsorption and absorption problems,11,12 which are fatal for small amount of protein analysis. To confirm the difference when the PURE system reaction mixtures were clipped with glass or a PDMS sheet to increase the surface area-to-volume ratio, we carried out following experiments. Although microtubes are usually made of polypropylene, this is not a common material for a microchip. Because we here aim to create a protein synthesis system on a microchip to treat small amounts of samples as well as considering future atomization and parallelization, just glass and PDMS were compared.

As shown in Fig. 2a, PURE system reaction mixtures including template DNA for GFP (10 μL) were placed on glass and PDMS substrates washed with buffer before use, and the droplets were covered with cover plates to be confined in a 100-μm height space using spacers in order to produce a similar condition in the microchannel maintained in this report (100-μm height). After 1 h of incubation at 37°C, 100% humidity for the reaction, 4 μL of reaction mixtures were recovered and the fluorescent intensities were measured. As shown in Fig. 2b, the yield of GFP on glass, judged based on the fluorescent intensities, were about two-fold higher than that on PDMS, suggesting that the adsorption or absorption of biomolecules to PDMS were larger than that on glass. This difference is significant for the detection of small amount of proteins. Therefore, we chose glass as the microchip material for more efficient and stable protein synthesis. This result is probably because the blocking by the buffer was well carried out on a glass surface compared with that on a PDMS surface. Accordingly, glass can provide a surface with little non-adsorption property. Although about 30% decrease of the yield compared with that using microtubes is not good, the adsorption of molecules in a small space cannot be prevented perfectly. Additionally, the residual GFP on the glass and PDMS substrates were not confirmed because the sample amount was small.

The microchip with beads packed into a microchannel is shown in Fig. 3. The width and depth of the microchannel were 300 and 100 μm, respectively. The depth of the dam structure was 10 μm.

**Optimization of template GFP DNA concentration**

The DNA templates immobilized on single micro-beads were quantified. The molar ratios of template DNA on micro-beads changed from $1 \times 10^7$ to $1 \times 10^9$ DNA/bead during the immobilization experiment. After immobilization, the flow-through fractions were recovered from centrifugal filter devices, and then the residual, free DNA was analyzed by acrylamide gel electrophoresis.

As shown in Fig. 4a, no bands were observed at $1 \times 10^7$ DNA/bead, while bands were seen at the same position as free DNA at other concentrations, indicating that DNA templates were saturated at $2 \times 10^7$ DNA/bead in the immobilization step. Based on this result, the immobilization of DNA was carried out under saturating conditions so as to avoid nonspecific binding of other molecules to the micro-beads.
We then utilized the DNA-immobilized beads for GFP synthesis reactions carried out in the PURE system (Fig. 4b). Fluorescence intensities from the synthesized GFP increased proportionally to the time, thus reaching their optimal value when 2000 beads/μL were added to the reaction mixture. This value indicates that 33 – 66 nM DNA is optimum for beads-based GFP synthesis, which is one order of magnitude higher than that used for batch reactions with free DNA templates in the PURE system.23 The addition of excess beads decreased the GFP synthesis (Fig. 4b). Although we have no confirmed explanation for these observations, several reasons, such as the reduced flexibility of the DNA molecules, non-specific binding of the PURE components or the synthesized GFP, and that the reaction mixtures did not have sufficient reactant due to the low amount of PURE system mixtures for 4000 beads/μL, can be considered. The results mentioned here do not indicate the advantage of the beads-based protein synthesis in the PURE system because it requires a greater quantity of DNA molecules (33 – 66 nM) to reach maximum GFP synthesis compared to the use of free DNA (4 nM). However, the results demonstrate that DNA templates on immobilized beads can be used to synthesize proteins in the PURE system, which can be utilized for the construction of a microchip device containing a DNA template for protein synthesis using the PURE system.

**Investigation of the effect of bead number**

Fluorescence measurements under each condition are shown in Fig. 5. The fluorescence intensity at time = 0 corresponded to the background, which was that of the PURE system reaction mixtures before protein synthesis. Note that this background intensity was not due to the biomolecules present in the PURE mixtures. This was confirmed by the following verification experiment. The fluorescent intensity of three types of tubes (empty, containing water, and containing PURE mixtures before protein synthesis) were measured and any differences among these were under the measurement error. This result indicates that the background intensity was due to the cuvette tubes. The time required to pass through the beads-packed area was assumed to be nearly equal to the reaction time, and the fluorescence increased proportionally to the time, thus
demonstrating GFP synthesis by the PURE system on a microchip. Moreover, the same experiment could be carried out several times on the same microchip by thoroughly washing the microchannel with pure water in between the experiments. Therefore, reusability was demonstrated. Since we used DNA immobilized beads preserved for a few months in a refrigerator, long-term usability was also confirmed.

Although the determination of the GFP concentration was difficult in this range, because the absolute amounts of products were small for general analysis methods, such as gel electrophoresis, the background was due to the cuvette tubes, as described above. This background did not fluctuate very much compared to the signals (difference of plot values), as shown in the plot at the left-most of Fig. 5. This means that the linearity of the amount of GFP product against the reaction time was obvious. For improving the synthesized protein amount, the reaction volume, beads size, or peripheral tools, such as tubes and connectors, must be optimized. Also, the purification system of the products should be incorporated in the system for practical applications.

Conclusions

The present study describes a PURE system on a glass microchip using continuous flow to construct a simple and easy protein synthesis system. Protein synthesis using a PURE system with DNA immobilized on microbeads in a microtube was carried out by immobilizing about 1 – 2 × 10^7 DNA beads, and then quantifying the synthesized GFP by fluorescence measurements. PURE ingredients were next introduced into a microchip with DNA immobilized on microbeads. GFP synthesis in this system was also verified by fluorescence measurements. The reaction volume (beads-packed area) was about 0.3 μL. This experimental setup can be considered as the first step of a future fully automated protein synthesis system using small sample volumes.

The microchip with DNA immobilized microbeads is reusable. By washing the microchannel with pure water, the same microchip can be used for further GFP synthesis. Thus, once DNA is immobilized on the beads and the beads are packed into the microchip, protein expression can be performed without preparing any template, just like using cells for protein expression. This can be advantageous over a conventional in vitro protein synthesis protocol that requires the preparation and addition of template DNA or mRNA into the reaction mixtures.

It is well known that glass is chemically stable compared with PDMS, and is therefore a suitable microchip material. Consequently, glass-based microfluidic systems are expected to become standard methods for the protein synthesis system. To immobilize DNA, we here utilized beads, but further miniaturization will be realized by immobilizing and patterning DNA on micro or nano channel surfaces. By utilizing glass for microfluidics-based systems, the repeatability and productivity can be increased compared with that of PDMS microchips. Moreover, by incorporating glass valves and pumps on a microchip, parallel synthesis might also be achieved in the future, with a corresponding increase in the product yield.

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References