Protein synthesis in a device with nanoporous membranes and microchannels

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Cell-free protein synthesis (CFPS) is an alternative approach to cell-based recombinant protein production. It involves *in vitro* transcription and translation in a cell-free medium. In this work, we implemented CFPS in a plastic array device. Each unit in the array consisted of an inner well and an outer well. Two synthesis steps, gene transcription and protein translation, took place in the inner well, in which a cell-free medium was used to provide ribosomes and additional components necessary for protein synthesis. The outer well was concentric to the inner well and it functioned as a nutrient reservoir. A nanoporous membrane was sandwiched between the inner and outer wells for retaining the synthesized proteins and removing the reaction byproducts. A microfluidic channel was employed to connect these two wells for supplying fresh nutrients for longer reaction time and higher expression yield. Synthesis of luciferase was shown to last 8 times longer and yield 10 times more proteins than in a conventional container. The device also enables more than 2 orders of magnitude reduction in reagent consumption compared to a bench-top instrument. The effects of the membrane pore size and microfluidic channel on the protein production yield were also studied. The array device has potential to become a platform for parallel protein expression for proteomics applications, matching high-throughput gene discovery.

Introduction

Biological synthesis (expression) of proteins is needed for studying the functions of the corresponding genes. Gene transcription and protein translation are typically carried out in host cells such as *E. coli*. However, some proteins are not expressed well in host cells due to their insolubility, degradation, and cytotoxicity.[1,2] As an alternative approach, cell-free protein synthesis (CFPS) has been developed that involves a series of biological reactions and uses a cell lysate to provide ribosomes and additional components essential for the expression of protein-encoding sequence.[1–7] The lack of cellular control mechanisms in the CFPS enables it to overcome the limitations in solubility and cytotoxicity.[1,2] CFPS has been demonstrated for various applications, including in situ immobilization of expressed proteins onto solid surfaces,[8,9] synthesis of drug transporters,[10] polypeptide display,[11] gene expression,[12] toxin detection,[13] and high-throughput screening.[14,15]

Compared to cell-based protein expression, CFPS has lower expression yield due to the fact that no nutrients are supplied as in cell culture. The depletion of the reactants (nutrients) and inhibition from the reaction byproducts lead to a shorter reaction time, significantly reducing protein expression yield. To address this limitation, instruments that continuously supply the reactants and remove the byproducts have been developed by Spirin *et al.*[16] and several others.[4,17] Their commercial counterpart (*e.g.*, RTS) also exists,[18] consuming 1 mL of reactants and 10 mL of nutrient solution. However, the implementation of above systems requires specific setup and excessive amounts of reagents. Therefore, high-throughput protein synthesis could be cost-prohibitive.

To address the issue, CFPS has been implemented in miniaturized devices or micro-well arrays. A couple of research groups applied microfluidics technology to protein synthesis.[19–23] In the work by Nojima *et al.*, two reactants flowed from two inlets and mixed through a Y-shaped channel structure.[21] The key drawback of these efforts is the use of excessive accessories (*e.g.*, external pumps and valves) and the lack of integration, making the devices difficult to be implemented in a high-throughput format. CFPS has also been demonstrated in microplate format.[24,25] Angenendt *et al.* accomplished protein synthesis in a microfabricated nL wells[24] and we demonstrated expression of three proteins in micro-wells and used the response pattern of an array for identification of two toxin stimulants.[13] One of the major downsides of these devices is that they do not consist of any fluid manipulation. As a result, nutrients cannot be refurbished and inhibitory byproducts cannot be removed, significantly reducing protein expression yield. We recently reported the implementation of CFPS in device with a large number of microfluidic channels and there are no excessive accessories due to the use of passive pumping.[26] However, lack of physical separation barrier between the nutrients and reactants preclude prolonged protein expression.

In this report, we describe the demonstration of protein synthesis in an array device with two orders of magnitude reduction in the volume of both reactants and nutrient solution. The significant drop in the reagent consumption lowers the cost of protein synthesis, especially when a large number of proteins are investigated. The design of the device enabled the placement of both reactant and nutrient solutions. A nanoporous membrane and a microfluidic channel were employed to supply

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the nutrients, remove the reaction byproducts, and retain the proteins synthesized while providing flow manipulation without pumping and valve accessories.

Experimental section

Reagents and materials

RTS 100 wheat germ kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). T7 luciferase DNA vector, luciferase assay reagent and nuclease-free water were purchased from Promega Corporation (Madison, WI). Polycarbonate sheets with thickness of 2.5 mm were acquired from McMaster-Carr (Los Angeles, CA). Polycarbonate nanoporous membrane with a pore size of 10 nm was from GE Osmonics Labstore (Minnetonka, MN) and those with 15-nm and 100-nm pores were from Whatman Inc (Clifton, NJ), while 2-nm cellulose membrane (a dialysis membrane with 8 KD cutoff) was from Spectrum Labs (Rancho Dominguez, CA).

Device fabrication

The device design is shown in Fig. 1a and composed of a porous membrane, which is sandwiched between two polycarbonate sheets containing concentric wells. The top part of the device was created by drilling through-holes in a 2.5-mm-thick polycarbonate sheet using a CNC-milling machine (Flashcut CNC, Menlo Park, CA). The diameter of an inner well is 3 mm, surrounded by a 1-mm-thick wall, while the diameter of an outer well is 7 mm. The distance between the hole centers is 9 mm, according to the microplate standards defined by the Society for Biomolecular Screening (SBS) and accepted by the American National Standards Institute (ANSI). The middle part is one of three polycarbonate membranes, including a pore size of 10 nm, 15 nm and 100 nm, or a 2-nm-pore cellulose membrane. The bottom part was fabricated by milling an array of shallow wells with a diameter of 7 mm in a polycarbonate sheet. The well depth ranges from 100 μm to 2 mm, though 500 μm were used for most experiments. Two methods were used to assemble three parts together. For cellulose membranes, a disk of membrane was first glued to the bottom of each inner well using a thin layer of poly(dimethylsiloxane). Using the same glue, the bottom and top layers were then placed in contact after alignment (which was straightforward since the well sizes are in mm). The assembly was placed in an oven at 100 °C for 2 h for glue curing. For polycarbonate membranes, three parts are aligned and then clamped together between two glass plates. The assembly was then put in an oven at 160 °C for 0.5 h. Before use, the device was rinsed with nuclease-free water, followed by sterilization under UV exposure for 30 min.

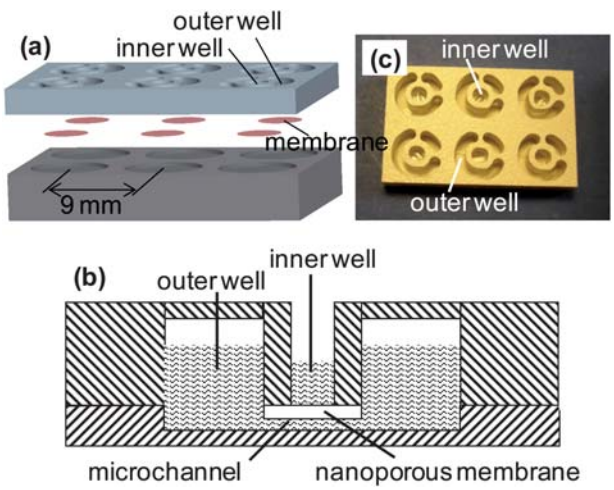

Fig. 1 The layout of an array device for protein expression. (a) Three components including inner wells and outer wells (top), membranes (middle) and microfluidic channels (bottom). (b) The cross-sectional view of one unit showing inner well, outer well, membrane and microfluidic channel. (c) Photograph of an assembly device. The membrane at the bottom of some inner wells can be observed due to the reflection of light.

Luciferase synthesis

Luciferase synthesis was carried out by using RTS 100 wheat germ expression kit according to the manufacturer's instructions. Briefly, the reaction solution was prepared by mixing 15 μL of wheat germ extract, 15 μL of reaction mix (provided in the kit), 4 μL of amino acids (without methionine), 1 μL of methionine and 15 μL of nuclease-free water containing 1 μg of luciferase vector. For each inner well, 8 μL of the reaction solution was used. The feeding solution was prepared by combining 900 μL of feeding mix (provided in the kit), 80 μL of amino acid (without methionine) and 20 μL of methionine. In each outer well, 80 μL of the feeding solution was introduced. The solution level in the outer well was ~2 mm higher than in the inner well. A biocompatible PCR tape (Corning, NY) was used to seal the device to prevent evaporation. For comparison, 8 μL of the reaction solution without or with 80 μL of the feeding solution was pipetted into a microcentrifuge tube respectively. Both the device and the tube were placed on a shaker and the reactions took place at ambient temperature for 0.5, 1, 1.5 2, 4, 6, and 10 h. The amount of luciferase synthesized was determined by mixing the expression product with luciferase assay reagents (Promega), followed by luminescence detection in a luminometer (Berthold, Germany).

Results and discussion

Device fabrication

To develop a high-throughput protein synthesis system in a cost-effective way, we have exploited polycarbonate substrate to fabricate the device. Polycarbonate is one of widely used plastic materials for fabricating microfluidic devices due to its flexibility in the micromachining process, such as injection molding, hot-embossing, micromilling and laser ablation. The layout of the integrated array device is shown in Fig. 1. It consists of three components (Fig. 1a). The top part consists of an array of inner and outer wells. The bottom part contains microfluidic channels. The depth of the microfluidic channel ranges from micrometers to a few millimeter. The middle part is nanoporous membranes, which are sandwiched between the two polycarbonate sheets containing multiple concentric wells as illustrated in Fig. 1b. A picture of an assembled device is in Fig. 1c. The integration of porous membranes and a microfluidic channel was to realize continuous supply of nutrients and selective removal of small-molecule byproducts, enhancing the synthesis yield. One of the mechanisms for supplying nutrients and removing byproducts is diffusion exchange.[27] The pore size of membrane is critical to the

exchange of molecules through the membrane. The multiple well array format allows simultaneous production of a group of proteins for proteomics application.[28,29]

Luciferase synthesis

The synthesis of luciferase was carried out in the device using a protocol similar to what has been described previously.[13,30] The reactants, including the DNA expression vector, T7 RNA polymerase, and cell-free expression medium, were placed in the inner well. The expression vector consisted of the coding sequence of luciferase and the necessary regulatory elements such as T7 promoter, ribosome binding site, start codon, stop codon, and T7 terminator. The outer well was filled with the nutrient solution containing amino acids, adenosine triphosphate (ATP), guanosine triphosphate (GTP), and other components in the cell-free expression medium. When the reactions proceeded, the concentration of the reactants in the inner well decreased. As a result, the same solutes in the outer well would transport into the inner well *via* diffusion resulting from the concentration gradient. Similarly, the reaction byproducts (*e.g.*, hydrolysis products of triphosphates) would diffuse out of the inner well due to the difference in their concentrations between two solutions separated by the membrane. The protein products were retained in the inner well due to the partition of the nanoporous membrane. In addition to diffusion, a supplementary flow to drive nutrients from the outer well into the inner well existed, resulting from a hydrostatic pressure due to the difference in the solution level between the two wells.

Fig. 2 shows the synthesis yield of luciferase as a function of the synthesis time. The amount of luciferase synthesized was determined by a luminescence assay. When CFPS was implemented in a microcentrifuge tube, the synthesis of luciferase stopped after 0.5 h and the yield was very low. In contrast, when it was in the device, luciferase was continuously synthesized up to 4 h. No further increase in the synthesis yield after 4 h was due to the fact that the reactions reached equilibrium. The yield of luciferase production in the miniaturized device was about 10 fold higher than in the microcentrifuge tube. When the same amount of the nutrient solution used in the outer well was added into the reactants, and the combined mixture was used in the tube, the synthesis yield increased only 20%. These results indicate that continuous feeding of the nutrients and removal of the byproducts through a membrane in the device are necessary to achieve high yield of protein synthesis.

It should be noted that the volume of reactants in the inner well is 8 μL while the volume of nutrients is 80 μL. These volumes are more than 2 orders of magnitude less than the volumes used in the CFPS instruments (*e.g.*, RTS500 kits),[18] in which 1 mL of the reaction solution and 10 mL of the feeding solution are used respectively. The decrease in the reagent volume will significantly reduce the reagent consumption when CFPS is used for high-throughput assays in an array device with 96 or 384 wells.[24]

Microchannel dimension

The dimension of the microchannel between the inner and outer wells is expected to play a role in the protein expression yield. We fabricated a variety of the microchannel dimension by changing the depth of the outer well. The resultant microchannel depth ranged from 100 μm to 2 mm. The effect of the microfluidic channel dimension on the luciferase synthesis yield is shown in Fig. 3. The result suggests that there is a hindrance when the depth is 100 μm. However, there was no significant difference when the depth was at other dimensions (changed from 500 μm to 2 mm). We can infer from the result that the membrane might partially touch the channel bottom due to membrane sagging when the depth was 100 μm (minus 25 μm, the thickness of the membrane). When the depth was larger than 200 μm, the channel dimension did not play a significant role because the diffusion through nanopores in the membrane (rather than the microchannel) should be the rate-limiting step.

Pore size of nanoporous membranes

In the previous CFPS work,[1–4,30] dialysis membranes were employed for separating the reactant and nutrient solutions. We

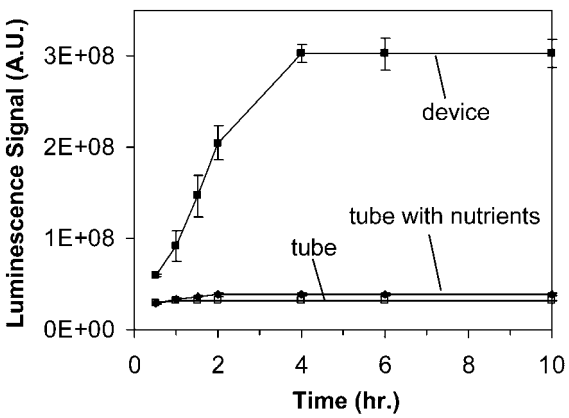

Fig. 2 The synthesis yield of luciferase as a function of the reaction time. The luminescence signal in Y axis indicates the amount of luciferase synthesized. Three curves represent the results from 8 μL reactants in a microcentrifuge tube, a mixture of 8 μL reactants and 80 μL nutrients in a microcentrifuge tube, and 8 μL reactants in the inner well and 80 μL nutrients in the outer well of a microfluidic device, in which the pore size of the membrane is 2 nm and the microchannel depth is 500 μm. The error bars indicate the standard deviation obtained from three repeat experiments.

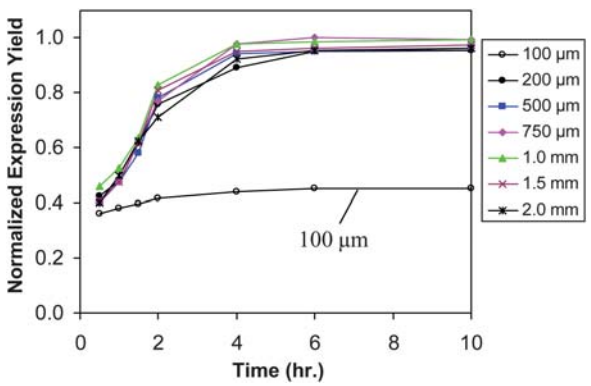

Fig. 3 The effects of the microchannel depth on the protein synthesis yield. The pore size of the membrane used is 2 nm. The expression yield (luminescence signal) is normalized against the experiment showing the highest luminescence signal.

investigated the use of nanoporous membranes for CFPS since it is possible to laminate a nanoporous membrane thermally with other plastic layers as practiced in industry. Dialysis membranes are required to be stored in a solution, making it difficult to handle during the manufacturing process. In addition, nanoporous membranes have been used for dialysis[31] and interconnects.[32] Membranes with different pore sizes (2, 10, 15 and 100 nm) were investigated. The images of the membranes with a pore size of 10 nm and 100 nm are shown in Fig. 4; they were obtained using a field emission scanning electron microscope (SEM). Also shown are the SEM pictures of the membranes after heating in an oven at 170 °C for 2 h. No noticeable change in the pore size was observed after thermal treatment, indicating the lamination process at a high temperature did not have a detrimental effect.

The effects of the pore size on the protein synthesis yield are shown in Fig. 5. When the 100-nm membrane was used, no significant difference existed between the device and a microcentrifuge tube. This result indicates that reagents can flow between the inner and outer wells and proteins synthesized cannot be retained. When the pore size decreased from 100 nm to 15 nm, the protein synthesis yield improved considerably. The synthesis yield further improved when the pore size decreased to 10 nm, and then to 2 nm. It is understandable because the molecular cutoff of 2-nm membrane is 8 KDa and that of a 10-nm membrane is about 100 KDa while the molecular weight of luciferase is 61 KDa. A fraction of luciferase molecules likely passed through the 10-nm membrane, but very few luciferase molecules transported through the 2-nm membrane. The results indicate that the pore size of the membrane is critical for the protein synthesis yield. The pores should be large enough to allow small molecules (*e.g.*, amino acids and ATP) to pass, but small enough to prevent large molecules (*e.g.*, proteins and ribosomes) from transporting.

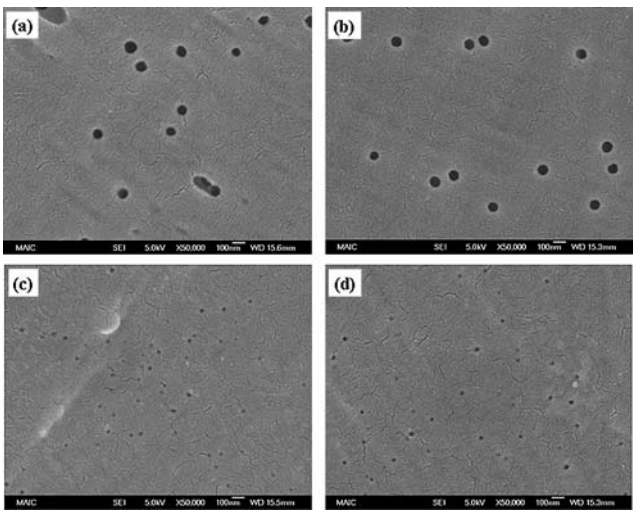

Fig. 4 Micrographs of polycarbonate membranes with a pore size of 100 nm (a) and 10 nm (c). No effects of thermal treatment at 170 °C on these membranes can be observed in the corresponding pictures (b) and (d). The length of the dimension bars in all images is 100 nm.

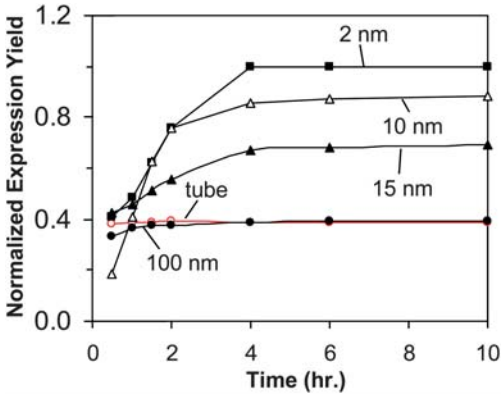

Fig. 5 The effects of the pore size of nanoporous membranes on the protein synthesis yield. The microchannel depth is fixed at 500 μm. The expression yield (luminescence signal) is normalized against the 2-nm-pore membrane. The temporal profile of the luciferase synthesis in a microcentrifuge tube is also provided for comparison.

Conclusion

An array device has been developed and demonstrated for cell-free protein synthesis. The integration of nanoporous membranes and microchannels with the device allowed continuous feeding of nutrients and removal of byproducts, increasing the synthesis duration 8 times and the synthesis yield 10 times than in a conventional reaction container. Although the device has only six wells in the current form, the well spacing conforms to the conventional 96-well microplate, allowing expansion of the array with minimal additional effort. In addition, this arrangement ensures the compatibility of the device with a variety of commercial fluid-dispensing systems and plate readers. The array device in this work has potential to become a platform for parallel protein expression for proteomics applications, matching high-throughput gene discovery.

Compared with the system developed by Spirin et. al[2,16,33] and Roche,[18] the operation of device reduced the amounts of reagents due to smaller dimension. In addition, high-throughput protein synthesis can be easily implemented using the device.

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