Cell-Free Protein Synthesis in Microfluidic Array Devices

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We report the development of a microfluidic array device for continuous-exchange, cell-free protein synthesis. The advantages of protein expression in the microfluidic array include (1) the potential to achieve high-throughput protein expression, matching the throughput of gene discovery; (2) more than 2 orders of magnitude reduction in reagent consumption, decreasing the cost of protein synthesis; and (3) the possibility to integrate with detection for rapid protein analysis, eliminating the need to harvest proteins. The device consists of an array of units, and each unit can be used for production of an individual protein. The unit comprises a tray chamber for in vitro protein expression and a well chamber as a nutrient reservoir. The tray is nested in the well, and they are separated by a dialysis membrane and connected through a microfluidic connection that provides a means to supply nutrients and remove the reaction byproducts. The device is demonstrated by synthesis of green fluorescent protein, chloramphenicol acetyltransferase, and luciferase. Protein expression in the device lasts 5−10 times longer and the production yield is 13−22 times higher than in a microcentrifuge tube. In addition, we studied the effects of the operation temperature and hydrostatic flow on the protein production yield.

Introduction

Biological synthesis of a protein includes the steps of gene transcription and protein translation, which are typically carried out in host cells such as *Escherichia coli*. Although it is prevalent among academic and industrial labs, recombinant protein production based on *E. coli* has inherent limitations including formation of insoluble protein aggregates (inclusion bodies), degradation of proteins by intracellular proteases, low or no expression for the genes whose products are toxic to the host cell, and lack of post-translational modification (*1*−*3*). To address some of these challenges, a few eukaryotic systems have been developed using *Saccharomyces cerevisiae*, insect, or mammalian cells. In addition, cell-free protein synthesis, a process called in vitro transcription and translation (IVT), has been developed (*4*−*12*). In IVT systems, a DNA template consisting of a coding sequence is transcribed into messenger RNA; either eukaryotic or prokaryotic lysate is then exploited to provide ribosomes and additional components necessary for protein translation. The expression system may also be reconstituted from purified recombinant elements (*13*, *14*). IVT has been demonstrated for various applications, including in situ immobilization of expressed proteins onto solid surfaces (*15*, *16*), synthesis of drug transporters (*17*), polypeptide display (*18*), gene expression (*19*), and high-throughput screening (*20*).

High-throughput protein production in an array format is desirable for genomics and proteomics. Completion of mapping the human genome has prompted strong interest in identifying the functions of newly discovered genes and the proteins encoded therein. To match high-throughput gene discovery, methods to produce a large number of proteins in parallel are needed. However, the common method of obtaining proteins using recombinant technology based on *E. coli* is difficult to implement in a high-throughput format. It comes to an understanding that the strategies for addressing the needs of high-throughput protein analysis include parallelization, miniaturization, and automation (*3*). A platform of using a microfluidic array for cell-free protein synthesis will support the strategy.

Microfluidics technology has been used to construct miniaturized analytical instruments called "lab-on-a-chip" devices. The principles of microfabrication and microfluidics, as well as their current and potential applications, have been reviewed in the literature (*21*, *22*). Common analytical assays, including polymerase chain reaction, protein analysis, DNA separation, and cell manipulation, have been implemented in centimeter-scale chips with micron-scale elements. The size reduction of an instrument has many advantages including faster analysis speed, less sample and reagent consumption, and the ability to operate in a high-throughput format.

IVT has been implemented in miniaturized devices or a microwell format (*23*−*28*). However, one of the major problems with the microplate is its short reaction time because of the fast depletion of reactants and inhibition from the reaction products, thus significantly reducing protein expression yield. To address the similar problem in the macro-scale, Spirin and co-workers invented the continuous-exchange cell-free (CECF) system, in which the reaction byproducts are continuously removed and the nutrients are refurbished (*2*, *29*, *30*). The technique has been commercialized by Roche Applied Science (*31*); the instrument with reagent kits is called Rapid Translation Systems (RTS). Borrowing the concept, we have developed a nested well array for CECF protein synthesis.

In this paper, we describe device fabrication, protein synthesis in the device, and comparison with the macro-scale results. The device is made from plastic as previously mentioned (*32*), but the details will be discussed here including a study on the biocompatibility of the materials used to fabricate devices. The function of the device is demonstrated by synthesizing green

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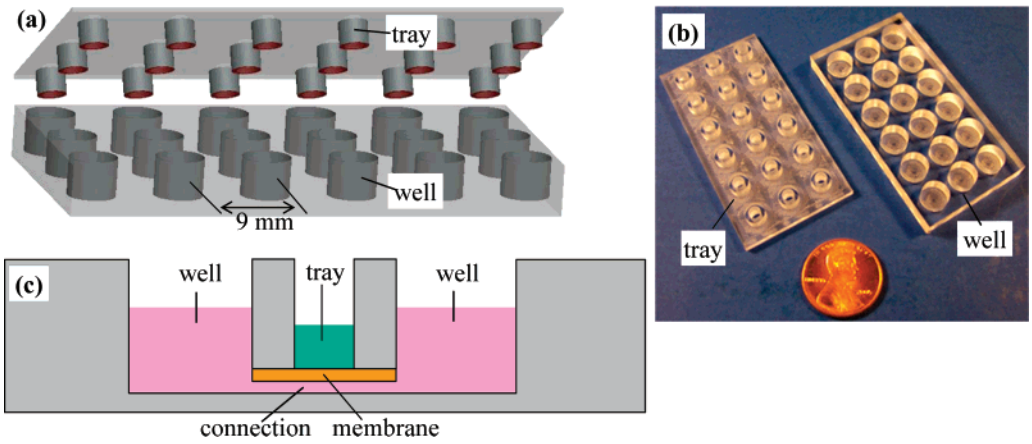

Figure 1. Illustrations of an array device for protein synthesis. The units are laid out according to the standards of 96-well plates (i.e., 9 mm pitch). The tray chamber is for IVT reactions; the well chamber is concentric with the corresponding tray chamber and functions as a nutrients reservoir. (a) Three-dimensional view; (b) picture of both parts; (c) cross-sectional view of a tray nested in a well, showing the dialysis membrane and fluidic connection.

fluorescent protein (GFP), chloramphenicol acetyl-transferase (CAT), and luciferase. We compared the production yield of IVT between the nested well device and a microcentrifuge tube. The effects of miniaturization on the reaction conditions (temperature and time) and the product yield are investigated. We also studied the effect of hydrostatic flow on the protein production yield.

Materials and Methods

Reagents and Materials. RTS 500 *E. coli* kit, RTS 100 wheat germ kit, and the expression vectors containing the gene of GFP or CAT were obtained from Roche Diagnostics GmbH (Mannheim, Germany). T7 luciferase DNA vector, luciferase assay reagent, DNA marker, nuclease-free water, phMGFP vector, and PCR master mix were acquired from Promega Corporation (Madison, WI). DNA primers were synthesized by Integrated DNA technologies (Coralville, IA). Agarose and ethidium bromide were purchased from Fisher Scientific (Atlanta, GA). Acrylic sheets with thickness of 5.5 mm were from Lucite International (Cordova, TN). The dialysis membrane with the molecular weight cutoff of 8 KDa was obtained from Spectrum Labs (Rancho Dominguez, CA). Epoxies 353ND-T and 302-3M were purchased from Epoxy Technologies (Billerica, MA), while Krazye Glue, Super Glue, and Vetbond were from Elmer's Products (Columbus, OH), Loctite (Avon, OH), and 3M Health Care (St. Paul, MN), respectively.

Device Fabrication. The device design is shown in Figure 1a and consists of two parts. The upper part, referred to as tray, is fabricated by milling through-holes and a common flange in a piece of 5.5-mm-thick acrylic using a CNC-mill (Flashcut CNC, Menlo Park, CA). The inside diameter of the holes is 3 mm, surrounded by a 1 mm thick wall, creating a structure with an outside diameter of 5 mm. The distance between the hole centers is 9 mm, following the microplate standards defined by the Society for Biomolecular Screening (SBS) and accepted by the American National Standards Institute. The bottom part, referred to as well, is created by milling 4 mm deep wells into a piece of 5.5-mm-thick acrylic. The well chambers are 7 mm in diameter; each well is concentric with the corresponding tray chamber when they are assembled. A picture of both parts in a 3×6 array is in Figure 1b.

A dialysis membrane is incorporated in the device to connect the two chambers as illustrated in Figure 1c, providing a means to supply nutrients continuously and remove the reaction byproducts. The membrane is glued to the bottom of each tray chamber using adhesives. A variety of glues are tested, and their biocompatibilities are studied as discussed below. The glue of the choice (epoxy 353ND-T) is cured at 80 °C in an oven for 20 min after the application. To eliminate possible inhibition of enzymatic reactions, both tray and well plates are rinsed with nuclease-free water and then sterilized by exposure to UV light for 30 min.

Biocompatibility Study. Polymerase chain reaction (PCR) is exploited to study the biocompatibility of the adhesives that are used to glue the dialysis membrane to the tray (*33*, *34*). A small piece of a glue sample is made by curing the adhesive according to the manufacturer's instruction. The sample is immersed in 50 μL of a PCR solution in a PCR tube. The PCR mixture is prepared by following the kit's procedures. DNA template is phMGFP vector. The sequences of the primers are 5′-CAA CAG TCT CGA ACT TAA GC-3′ and 5′-AAA ACC TCC CAC ATC TCC-3′. The solution is then placed in a PTC-100 programmable thermal cycler (MJ Research, Waltham, MA). There are 25 cycles of DNA amplification, and each cycle consists of 45 s denaturation (94 °C), 45 s annealing (57 °C), and 1 min extension (72 °C). The amount of PCR products is quantified by gel electrophoresis. Agarose gel is prepared by dissolving agarose in a Tris-boric acid-EDTA (TBE) buffer (pH 8.0) and casting it into a slab. The gel slab is then immersed in a TBE buffer containing ethidium bromide. The PCR products are added to the sample wells in the gel, followed by electrophoresis. After electrophoresis, the gel is rinsed to remove the excess ethidium bromide and then documented using a UV transilluminator.

Protein Synthesis. GFP and CAT are expressed using a RTS 500 kit. Reaction and feeding solutions are prepared as recommended by the manufacturer. In brief, the reaction solution contains 0.525 mL *E. coli* lysate, 0.225 mL reaction mix, 0.27 mL amino acid without methionine, and 30 μL methionine. The feeding solution consists of 8.1 mL feeding mix, 2.65 mL amino acid without methionine, and 0.3 mL methionine. To run the protein synthesis in the device, the tray is filled with 8 μL of the reaction solution containing GFP vector or CAT vector while 80 μL of the feeding solution is added to each well. A biocompatible PCR tape (Corning, NY) is used to seal the tray to prevent evaporation. For comparison, 8 μL of reaction solution is dispensed into a microcentrifuge tube and incubated for the same time period as for the device, though no feeding solution is used. Both the device and the tube are placed on a shaker with a speed of 100 rpm and incubated at room

temperature for 2, 4, 6, 10, 14, 20, and 24 h. At the end of GFP expression, the tubes and the device are stored at 4 °C overnight for appropriate folding. This step is not required for CAT. Western blotting is used to quantify the synthesized GFP and CAT. Gel images are scanned by a flatbed scanning and quantified using ImageJ from the National Institute of Health (http://rsb.info.nih.gov/ij). GFP production is also confirmed by a microplate reader (Tecan Safire, Research Triangle Park, NC). The production of luciferase is carried out using a RTS 100 wheat germ expression kit. The amount of luciferase synthesized is determined by luminescent luciferase assay. The detailed protocol for luciferase synthesis and assay has been described previously (*32*).

Results and Discussion

Device Design. The devices are made from plastic, since plastics offer advantages including low material and manufacturing cost, disposability to avoid possible contamination, and biocompatibility (*35*, *36*). Polymethylmethacrylate (PMMA) is chosen for this work since it is widely exploited as the material for fabricating microfluidic devices as a result of its excellent optical properties, low fluorescence background, and compatibility with chemical or biological reagents. The design of the array device for protein expression is illustrated in Figure 1. The device consists of an array of units; each unit is for expression of one protein (e.g., GFP). Both gene transcription and protein translation take place in the tray chamber (reaction chamber), while the well chamber (feeding chamber) functions as a nutrient reservoir. The well chambers contain amino acids, adenosine triphosphate (ATP), guanosine triphosphate (GTP), and buffer. ATP is critical to activate amino acid substrates, and GTP is the energy source required for the function of ribosomes. The tray chamber contains cell-free expression system with other reagents as in the well chamber.

As illustrated by Spirin et. al (*2*, *29*, *30*) and Roche's RTS (*31*), continuous supply of nutrients and selective removal of small molecule byproducts are critical to achieving high protein expression yield. We obtain these desirable features by incorporating a dialysis membrane and a fluidic connection as shown in Figure 1c. The dialysis membrane has the molecular weight cutoff of 8 KDa, allowing continuous feeding of small-molecule nutrients (e.g., ATP and GTP). It also lets small molecular byproducts (e.g., hydrolysis products of triphosphates) diffuse away while retaining the synthesized proteins in the tray.

The connection between the tray and well chambers is a hollow circle, though it looks like a channel in the cross-sectional view in Figure 1c. The depth of the connection ranges from hundreds of micrometers to a few millimeters. One of the mechanisms for supplying nutrients from the well chambers to the tray chamber and for removing byproducts from the tray chamber to the well chambers is diffusion, the transport of species resulting from the difference in the concentration of solutes between two solutions separated by the membrane. The rate of diffusion is determined by the diffusion coefficient and the concentration gradient of solutes. In addition, the flow to supply fresh solution from the well to the tray is augmented by a hydrostatic pressure, which is caused by the difference in the solution level between the tray and well. When the well has slightly higher solution level than the tray, the pressure difference resulting from the height difference will drive solution from the well into the tray.

Incorporation of the dialysis membrane and fluidic connection enables continuous supply of nutrients and selective removal of small molecule byproducts. The nutrient-feeding solution will avoid fast depletion of the energy source, and the accumulation of small molecular byproducts is reduced. This device design allows protein synthesis to continue for up to 20 h with higher protein yield as discussed later.

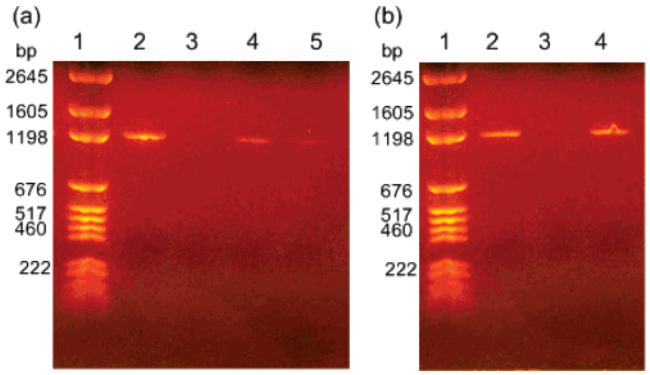

Figure 2. Biocompatibility study of adhesives using PCR. A small piece of a dried adhesive is placed in the PCR reactions. The PCR products are analyzed by agarose gel electrophoresis and stained by ethidium bromide. The electrophoresis images are collected while being illuminated by a UV light. (a) Cyanoacrylate glues. Lane 1: DNA marker; lanes 2 to 5: positive control, Krazy Glue, Vetbond, and Super Glue. (b) Epoxies. Lane 1: DNA marker; lanes 2 to 4: positive control, 302-3M, and 353ND-T.

Using the dimension discussed in Materials and Methods, the volume of a tray chamber is 39 μL. We typically fill in the tray with 8 μL of an IVT solution; this volume is more than 2 orders of magnitude less than 1 mL of a reaction solution in RTS 500. Similarly, the volume of a well chamber is 95 μL when the depth of the connection is 1 mm after a tray is placed inside. We typically fill in the well with 80 μL of a feeding solution; this volume is again more than 2 orders of magnitude less than 10 mL of a feeding solution in RTS 500. The decrease in the volume of both reaction mix and feeding resolution will significantly reduce reagent consumption when using IVT for high-throughput assays (*24*).

Although Figure 1 shows a 3 × 6 device, an array of a higher number (e.g., 8 × 12) can be implemented as is readily appreciated by those in the field. As explained above, such an array device has the following advantages: (1) less reagent consumption due to miniaturization; (2) higher protein expression yield due to incorporation of a mechanism for fluid manipulation; and (3) a potential to implement protein synthesis in a high-throughput format.

Biocompatibility. Protein synthesis involves a series of complex biochemical processes; hence the materials used to fabricate the array device must be biocompatible. Since acrylic is used in contact lenses and bone cement, it is likely to be biocompatible. Our experimental results indicate that is the case after sterilization, but protein expression cannot be realized if either tray or well is not sterilized.

The biocompatibility of adhesives used in the device fabrication was tested. We followed the literature to exploit polymerase chain reaction (PCR) to study the biocompatibility of the adhesives (*33*, *34*). PCR is a method of quickly replicating DNA segments, and the mechanism of DNA amplification closely replicates the transcription phase of protein expression. Much of the PCR process is also automated, making it easier to test than actually expressing the proteins. Using a DNA template with appropriate primers, a PCR product of 1190 base pairs was obtained and detected as shown in Figure 2. When a small piece of a dried adhesive is present in PCR, it may inhibit PCR, resulting in either less or no PCR product. The detection of the PCR product was achieved by gel electrophoresis, and the resultant gel images are in Figure 2a and b. The first lane is a

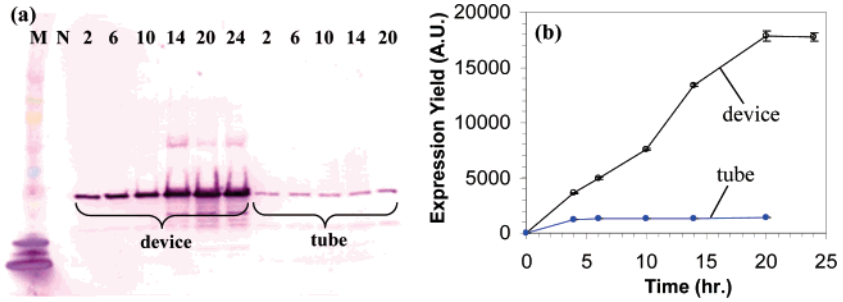

Figure 3. Comparison of in vitro GFP synthesis between a device and a microcentrifuge tube. (a) Western blot analysis of GFP expressed. The first lane is protein markers (M) and the second lane is the negative control (N); the lanes on the left are for GFP expressed in the device and the numbers on the top indicate the reaction time (hours); the lanes on the right are for GFP expressed in the tube and the reaction time is also indicated on the top. (b) The production yield of GFP as a function of the expression time. The data for the device are represented by the open circles, while those for the tube are by the closed circles. The error bars are obtained from three repeat experiments in different chambers in the same device.

DNA marker, which serves as a ruler to calibrate the molecular size of the product; the number of base pairs in each band appears to the left of each picture. The other lanes contained either a positive control, which had no adhesive in the reaction tube, or one of the adhesive-containing samples.

The brightness of the signal depends on the amount of the PCR product. As a result, a dimmer signal than the control indicates that the glue inhibits the reaction, suggesting that it should be avoided for device fabrication. To maximize the protein yields, it is imperative that the device itself has minimal, if any, negative effect on the transcription or translation process. It was found that all adhesives tested exhibit some amount of inhibition to the PCR results except 353ND-T.

Of the three cyanoacrylate glues tested in Figure 2a, Vetbond inhibited the reaction the least, indicating that it would be a choice for device fabrication, but the low viscosity of Vetbond made application of the glue problematic. Two types of epoxies were then examined. The results indicated that 353ND-T has a negligible inhibitory effect on PCR reaction, as shown in Figure 2b. In addition, 353ND-T is more viscous, allowing the formation of a film. Therefore, 353ND-T is the choice of the adhesive for the device fabrication.

Protein Synthesis. To demonstrate the function of the array device, GFP was first expressed using a commercial *E. coli* system with a GFP coding sequence. GFP is a widely used fluorescent molecule with known DNA sequence (*37*). The expression product was analyzed using Western blotting as described in Materials and Methods. Figure 3a shows the Western blot image, and the signal intensity is plotted in Figure 3b. The results indicated that GFP is continuously synthesized in the device for up to 20 h. To show the effects of the flow manipulation, we did the same reactions in a microcentrifuge tube and found that protein expression ceased after 4 h. This result clearly illustrates the importance of incorporation of the dialysis membrane and fluidic connection in the device. Continuous supply of nutrients and selective removal of small molecule byproducts enable protein synthesis to continue for up to 20 h in the device, which is 5 times longer than the expression time in the tube. The longer expression time results in higher expression yield. The amount of GFP synthesized in the device is about 13 times larger than in the tube.

The error bars in Figure 3b are obtained from three repeat experiments in different chambers in the same device. As a result, the data are an indication of the well-to-well variation in the array device. At 4 h, the relative standard deviation of the expression yield is 1.1%, which is slightly larger than 0.6% at the same time when the GFP expression yield in the microcentrifuge reaches the plateau.

Because GFP is naturally fluorescent, the expression product can also be quantified by fluorescence emission. The emission spectrum of synthesized GFP determined using a commercial microplate reader is shown in Figure 4a, as is the spectrum of the recombinant GFP purchased. The similar fluorescence spectrum also confirms our success of GFP expression in the device. It should be noted that the GFP synthesized must be incubated at 4 °C to let proteins fold correctly in order to have appropriate three-dimensional structures for fluorescence. Figure 4b shows the fluorescence intensity as a function of the incubation time. After 20 h incubation, the fluorescence intensity reached a plateau.

Similar expression has been achieved for two other proteins, CAT, an enzyme responsible for bacterial resistance to an antibiotic drug (chloramphenicol), and luciferase, an enzyme from firefly tails that catalyzes the production of light in the presence of luciferin and ATP. CAT is expressed in the same *E. coli* expression system; success of the protein expression is confirmed by Western blotting in Figure 5a. The signal intensity as a function of the reaction time is shown in Figure 5b. These results also suggest that longer expression time can be achieved in the device than in the tube. As a result, the production yield increased more than 22 fold in the device. This confirms that we achieved the desired fluid manipulation, both continuous feeding of the nutrient solution and removal of the byproducts, in the device. Synthesis of luciferase was carried out using a wheat germ expression system. Detection of the expression product was achieved by monitoring the intensity of luminescence after mixing the product with luciferin and ATP as reported previously (*32*).

Reaction Temperature. All of the protein expression experiments discussed above took place at ambient temperature. For commercial RTS instruments from Roche, the recommended operation temperature is 30 °C. As a result, we studied the effect of the reaction temperature on protein synthesis in microcentrifuge tubes. Temperature control was achieved by placing the tubes in a PCR machine. Figure 6a shows the Western blot image of GFP synthesized at room temperature and at 30 °C. The GFP expression yield at room temperature was about 97% of that at 30 °C, as shown in the quantitative illustration in Figure 6b. This result suggests that the GFP expression at room temperature is comparable to that at 30 °C and it is not necessary to integrate a heater in the array device.

The result in Figure 6a also confirms the success of GFP expression by comparing the molecular weight of the expression product with the GFP purchased. According to the protein markers, the molecular weight of GFP expressed is estimated at ~31 KDa, which is slight larger than the recombinant GFP

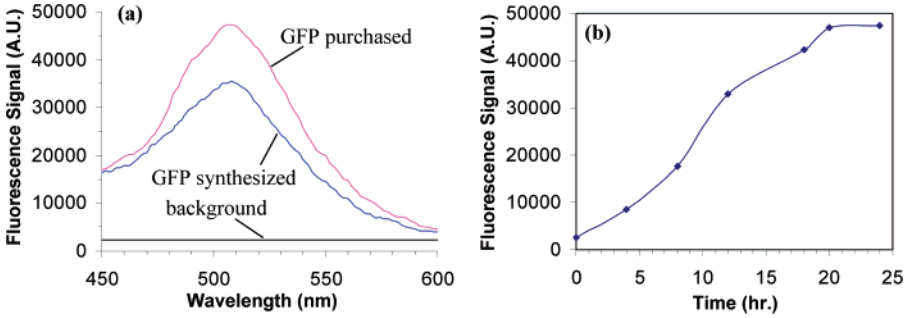

Figure 4. (a) Comparison of fluorescence emission spectra between synthesized and purchased GFP. The excitation wavelength is 488 nm. For GFP synthesized, 5 μL of the expression product is mixed with 35 μL of pH 7.4 PBS buffer in a 50 μL cuvette. The concentration of GFP purchased is 2 μg/mL for this spectrum. (b) The fluorescent signal as the function of the folding time.

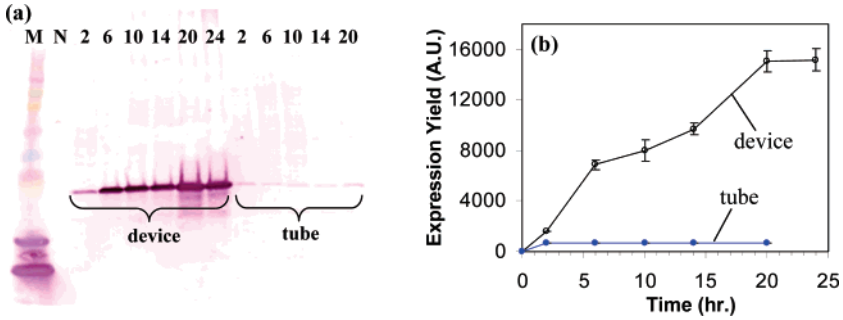

Figure 5. Comparison of in vitro CAT synthesis between a device and a microcentrifuge tube. (a) Western blot analysis of CAT expressed. The first lane is protein markers (M) and the second lane is the negative control (N); the lanes on the left are for CAT expressed in the device and the numbers on the top indicate the reaction time (hours); the lanes on the right are for CAT expressed in the tube and the reaction time (hours) is also indicated on the top. (b) The production yield of CAT as a function of the expression time. The data for the device are represented by the open circles while those for the tube are by the closed circles. The error bars are obtained from three repeat experiments in different chambers in the same device.

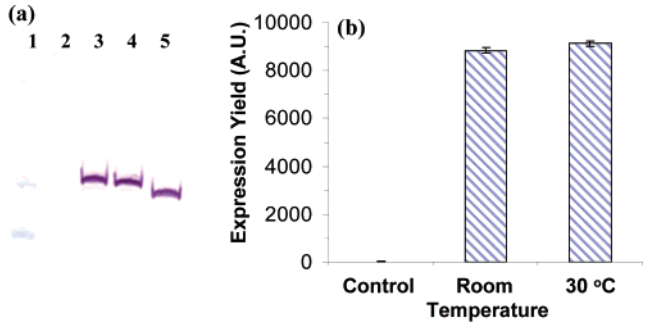

Figure 6. Comparison of in vitro GFP synthesis between at room temperature and at 30 °C. (a) Western blot analysis. Lane 1: prestained protein markers; 2: negative control; 3: GFP synthesized at room temperature; 4: GFP synthesized at 30 °C; 5: recombinant GFP (rGFP) purchased. (b) Quantitative comparison of the GFP expression yield between room temperature and 30 °C. The error bars are obtained from three repeat experiments.

purchased due to six histidines at the C-terminal of our synthesized GFP. The negative control in the experiment contains all reagents except for the expression vector.

Hydrostatic Flow. As mentioned above, one mechanism to supply nutrients from the well to the tray is the hydrostatic flow due to the difference in the solution level between the tray and well. We studied the effects of the fluid level difference on the protein expression yield. Figure 7 shows luciferase expression yield temporal profiles when the fluid level difference was changed from −0.5 to 2 mm. A negative value refers to the situation that the fluid level in the well is lower than in the tray, whereas a positive value refers to the situations that the fluid level in the well is higher than in the tray. The difference between the fluid levels was controlled by loading different amounts of fluids based on the calculated volumes. The value of the fluid level difference refers to the initial condition only;

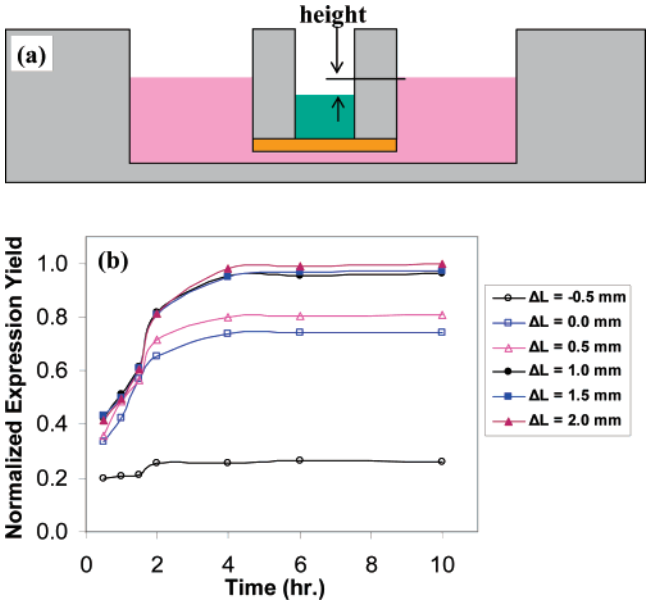

Figure 7. The effects of the fluid level difference (ΔL) on the protein expression yield. (a) Illustration of the fluid level difference between the tray and well chambers. (b) Luciferase expression yield as the function of the IVT time. The expression yield is normalized against the experiment showing the highest luminescence signal.

no disruption was made during protein expression. The fluidic connection depth was fixed at 500 μm. When the height was −0.5 mm, i.e., a reverse flow from the tray to the well existed, protein expression was significantly hindered as a result of depletion of the expression components in the tray. When the fluid level difference was near 0, the expression yield increased 183% and the normal temporal profile was observed. In this condition, the fluid flow should be primarily due to diffusion

and agitation. When the fluid level difference was increased from 0 to 2 mm, the expression yield further increases 38%, presumably due to additional hydrostatic flow. This result indicates that hydrostatic flow plays a role in the replenishing nutrients in the device.

Conclusion

An array of nested wells with microfluidic connections has been developed for cell-free protein expression. The array device was fabricated by milling a tray chamber from acrylic, attaching a dialysis membrane, and then nesting it in a well chamber. The device has been demonstrated for in vitro synthesis of GFP, CAT, and luciferase. The duration of protein expression was significantly extended due to continuous feeding of nutrients and removal of byproducts. The reactions in the wells with the feature of continuous-exchange lasted 5−10 times longer than the same reactions in a microcentrifuge tube. The production yield in the device was 13−22 times higher than that in the conventional reaction vessels (either microcentrifuge tubes or 96-well plates).

The array device has a potential to be used for high-throughput protein expression and functional analysis. It is highly desirable to identify the functions of newly discovered genes by studying the corresponding proteins. As a result, protein expression in an array format is needed to match high-throughput gene discovery. In addition, it is possible to integrate the array device with assay and detection elements so that the expressed proteins can be analyzed in the device. The integration gets rid of the need to harvest proteins before analysis, saving time by eliminating the laborious separation process. Examples may include fusing proteins with GFP for monitoring protein expression process (*38*) and coupling with an enzymatic inhibition assay for drug screening (*24*).

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