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Cell-Free Expression of Soluble and Membrane Proteins in an Array Device for Drug Screening

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Enzymes and membrane protein receptors represent almost three-quarters of all current drug targets. As a result, it would be beneficial to have a platform to produce them in a high-throughput format for drug screening. We have developed a miniaturized fluid array device for cell-free protein synthesis, and the device was exploited to produce both soluble and membrane proteins. Two membrane-associated proteins, bacteriorhodopsin and ApoA lipoprotein, were coexpressed in an expression medium in the presence of lipids. Simultaneous expression of ApoA lipoprotein enhanced the solubility of bacteriorhodopsin and would facilitate functional studies. In addition, the device was employed to produce two enzymes, luciferase and β-lactamase, both of which were demonstrated to be compatible with enzyme inhibition assays. β-lactamase, a drug target associated with antibiotic resistance, was further used to show the capability of the device for drug screening. β-Lactamase was synthesized in the 96 units of the device and then assayed by a range of concentrations of four mock drug compounds without harvesting and purification. The inhibitory effects of these compounds on β-lactamase were measured in a parallel format, and the degree in their drug effectiveness agreed well with the data in the literature. This work demonstrated the feasibility of the use of the fluid array device and cell-free protein expression for drug screening, with advantages in less reagent consumption, shorter analysis time, and higher throughput.

There is an escalating need to discover new drug targets for various diseases that impact the quality of life of countless individuals. Once a target is validated, it is necessary to find drug candidates by screening a large library of compounds. For both target discovery and drug screening, novel platforms such as high-throughput screening (HTS) are required in the modern pharmaceutical era.[1−3]

Although cell-based HTS has become increasingly popular due to its ability to deliver phenotype information at the cellular level, there is still a considerable need to have innovative platforms that provide biological and chemical information at the molecular level.[4] Cell-based screening can result in "off target hits" because different portions of a cellular process other than the target could be affected by library compounds.[5] In addition, those compounds that cause cytotoxicity would result in reduced signal even though they do not inhibit the desired target.[5] Galarneau et al. showed a great example that both cell-free and cell-based assays were required to provide complementary and indispensible information for their study in protein−protein interactions.[4]

Cell-free protein synthesis (CFPS) is a platform technology that addresses the shortcomings mentioned above for cell-based assays. CFPS utilizes an efficient, often-coupled transcription and translation reaction to produce recombinant proteins.[6−12] It eliminates the time-consuming steps of conventional cell-based protein production, including transformation, cell culture maintenance, and expression optimization. The proteins synthesized could be used for functional and structural studies, drug screening, and other applications. Since there is no cellular control mechanism, CFPS will not suffer from cytotoxicity or other challenges associated with cell-based protein expression (e.g., inclusion body).[6] A large number of soluble proteins have been synthesized using CFPS, and many of them are enzymes and functionally active.[13]

More importantly, CFPS is an open system and, thus, can be modified by simply adding various components. This open nature has been used for producing membrane proteins. Lipids, liposome, and lipoprotein particles have been introduced into the cell-free

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expression media to enhance solubility of membrane proteins.[14−19] Several membrane proteins have been synthesized using either commercially available CFPS systems or ones created by individual laboratories.[14−19] CFPS simplified the process and reduced the time and labor required to produce membrane proteins. Many toxic effects attributed to overproduction of recombinant proteins in cell-based systems are eliminated by cell-free expression since viable host cells are no longer required.[14,18] As a result, CFPS has become an emerging alternative tool for the high level production of membrane proteins.[14]

Because CFPS is able to produce both soluble and membrane proteins, it could play a significant role in supporting the drug discovery pipeline. Membrane proteins, mainly G protein−coupled receptors, represent 45% of all drug targets, while enzymes account for 28% of them.[1] Thus, these two categories make up a total of 73% of all current drug targets.[1]

In this report, we describe the use of a miniaturized fluid array device for expression of both soluble and membrane proteins. The device was designed and optimized for high-throughput cell-free protein synthesis. The fabrication and characterization of the device have been previously reported.[20−22] We used the device for producing two membrane-associated proteins, bacteriorhodopsin and ApoA lipoprotein, in the presence of lipids. Coexpression of ApoA lipoprotein in the same CFPS addressed the solubility concern of bacteriorhodopsin.

In addition, the array device was also employed to produce two enzymes, luciferase and β-lactamase, both of which were demonstrated to be compatible with enzyme inhibition assays. Further, we used β-lactamase to show the adaptability of the device for drug screening. β-Lactamase is an enzyme produced by some bacteria to generate resistance to a β-lactam class of antibiotics such as penicillin and cephalosporins.[23] All of these antibiotics have a four-atom ring structure known as β-lactam. β-lactamase breaks the amide bond in β-lactam, deactivating its antibacterial properties and causing antibiotic resistance. One common practice is to combine antibiotics with a β-lactamase inhibitor that inactivates β-lactamase.[24] Since β-lactam-associated antibiotics constitute 50% of antibiotic consumption around the world,[25] it is important to have an efficient method to identify additional β-lactamase inhibitors. Therefore, we investigated the exploitation of CFPS to generate β-lactamase that functioned as a drug target, followed by employing four mock drug compounds to test their inhibitory effects on β-lactamase. The results demonstrated the feasibility of the use of the fluid array device for drug screening with advantages in reagent consumption, analysis time, and throughput.

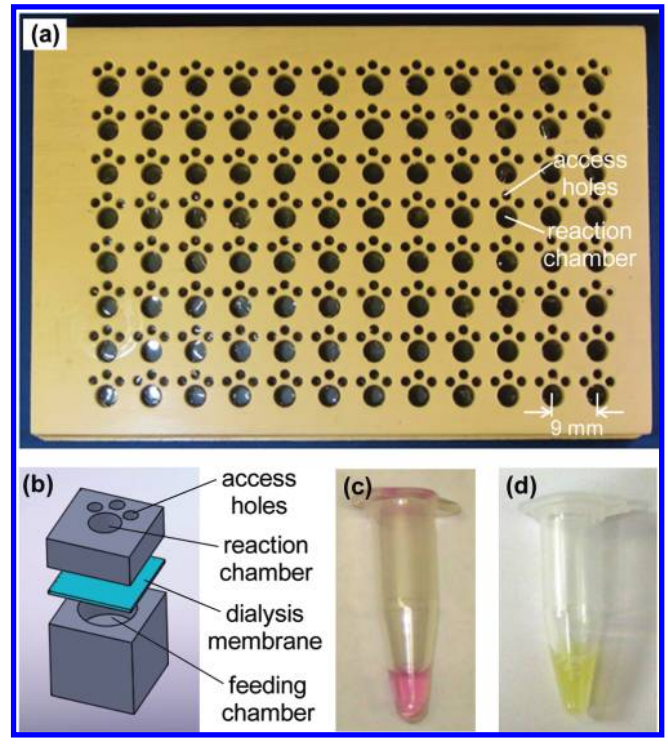

Figure 1. (a) Picture of a device in the format of a 96-well plate for protein expression. The middle layer (membrane) can be observed in the wells at the bottom left due to light reflection. (b) The cross-sectional view of one well unit, consisting of three access holes and one reaction chamber in the top layer, a dialysis membrane in the middle layer, and a feeding chamber in the bottom layer. (c) The expression product of bacteriorhodopsin and apolipoprotein coexpressed in the miniaturized fluid array device. (d) The expression product of bacteriorhodopsin and apolipoprotein coexpressed in a conventional microplate.

MATERIALS AND METHODS

Device Fabrication. The details of the device fabrication have been described elsewhere.[20−22] Briefly, two polypropylene sheets were machined to have desired wells with an appropriate size. A dialysis membrane with molecular cutoff of 8 KD was sandwiched between these two sheets by an adhesive. Figure 1a shows a picture of the assembled device in the format of a 96-well plate. The cross-sectional view of one unit is shown in Figure 1b. The top layer consists of a reaction chamber and three access holes for loading the feeding solution. The bottom layer contains a feeding chamber that encompasses both the access holes and reaction chamber. The dialysis membrane was used to retain newly synthesized proteins while allowing the nutrients (e.g., amino acids and ATP) in the feeding chamber to continuously replenish the reaction chamber. The membrane is sturdy and does not break if touched by a pipet tip by accident. The device was designed to be disposable; thus, each well was used once.

Protein Expression. Luciferase and β-lactamase were expressed using an RTS 100 wheat germ kit (Roche). The vector of

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luciferase (T7 control vector) was obtained from Promega while the β-lactamase vector was cloned in house using pIVEX-1.4 plasmid and verified by restriction enzyme digestion and gel electrophoresis.[22] The reaction and feeding solutions were prepared according to the manufacturer's instructions. The reaction solution was composed of 15 μL of wheat germ lysate, 15 μL of reaction mix, 4 μL of amino acids, 1 μL of methionine, and 15 μL of an individual DNA vector (2 μg). For negative controls, the DNA vector was replaced with the same volume of nuclease free water. The feeding solution was prepared by combining 900 μL of feeding mix, 80 μL of amino acids, and 20 μL of methionine. (All of these were provided in the kit and the concentration of each component was fixed by the manufacturer.)

Membrane proteins were expressed in the RTS 500 *E. coli* kit (Roche). The reaction solution was made by mixing 525 μL of *E. coli* lysate, 225 μL of reaction mix, 270 μL of amino acids without methionine, and 30 μL of methionine, 2 mg/mL 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids), 50 μM retinal (Sigma), and 5 μg/mL DNA vectors for bacteriorhodopsin and apolipoprotein. A stock solution of DMPC lipid (68 mg/mL) was prepared by adding DMPC in nuclease-free water, sonicating with a Vibra-Cell probe sonicator at a power of 6 W until the solution became clear, and then centrifuging it to retain the supernatant. The retinal solution (10 mM) was prepared in pure ethanol. DNA vectors for bacteriorhodopsin and apolipoprotein were prepared as reported previously.[16] The feeding solution was made by mixing 8.1 mL of feeding mix, 2.65 mL of amino acids without methionine, and 0.3 mL of methionine.

To carry out reactions in the device for either soluble or membrane proteins, 200 μL of the feeding solution was pipeted to the feeding chamber and 10 μL of the reaction solution was added to the reaction chamber. After sealing the device using a PCR tape (to prevent evaporation), the device was placed on an orbital shaker for four hours, rotating at a speed of 30 rpm. The synthesized proteins were then analyzed as discussed below.

Protein Assays. Luciferase was detected by injecting 30 μL of luciferase assay reagent (Promega) into the reaction chamber in the device, shaking the device for 2 s, and measuring luminescence over 10 s. All of these steps were carried out in a Mithras microplate reader (Berthold Technologies, Germany). β-Lactamase was measured using *m*-{[(phenylacetyl)glycyl]oxy}-benzoic acid (PBA, Calbiochem), a chromogenic substrate that changes color upon being enzymatically cleaved.[26] PBA (90 μL, 2 mM) was added into the expression product, followed by a 5 min incubation and absorbance measurement at 314 nm using a BioRad spectrophotometer.

Expression of the membrane proteins was indicated by color observation as discussed in the Results and Discussion. They were also verified by gel electrophoresis, followed by either Coomassie blue staining or Western blotting. For Coomassie blue staining, the sample aliquot was diluted at a ratio of 1:20 using 2× sodium dodecyl sulfate (SDS) sample loading buffer. A five μL aliquot of the resulting solution was heated at 99 °C for 10 min (to denature proteins) and then loaded onto a 15% SDS polyacrylamide gel. Electrophoresis was carried out with a Precision Plus protein standard ladder (BioRad) in a Mini-Protean III Cell system (BioRad). After electrophoresis at 150 V for 3 h, the gel was transferred to a container for Coomassie blue staining.

For the Western blotting, the samples were mixed in a ratio of 1:100 with the sample loading buffer. After the same electrophoresis procedure, the gel was transferred onto a nitrocellulose membrane under an electric current of 220 mA for 30 min. The membrane was then blocked with 5% fat free milk in PBS/Tween-20 buffer for 1 h, followed by overnight incubation in anti-His-peroxidase antibody (Roche) that was prepared in the blocking solution in a ratio of 1:50 000. After washing three times, the membrane was placed in a solution of 1:40 ECL Plus Western blotting reagents (GE Healthcare) for 5 min. The image of the membrane was obtained by exposing it onto a 9 × 10 in. Kodak film.

Enzyme Inhibition Assays. Proteins synthesized in the CFPS device were used directly for enzyme inhibition assays without harvesting and purification. Luciferase was chosen to be tested first using a known inhibitor *D*-luciferin 6′-methyl ether (LME, also known as 4,5-dihydro-2[6-methoxy-2-benzthiazolyl]-4-thiazole carboxylic acid).[27,28] LME solutions with a range of concentrations were prepared in nuclease-free water. To carry out the inhibition assay, 1 μL of a LME solution was added to the CFPS reaction product, followed by the luciferase assay as described above.

For the β-lactamase inhibition assay, three clinically used drugs, tazobactam (Sigma), potassium clavulanate (Sigma), and sulbactam (Astatech), as well as an additional compound with similar chemical structure, cefotaxime (Sigma), were used to study inhibition. To carry out the inhibition assay, a series of concentrations of each compound was prepared. Five microliters of each solution was added to the synthesized β-lactamase in the reaction chamber, followed by a 15 min incubation. The resulting mixtures were analyzed using the same protein assay procedure described above for β-lactamase. The degree of inhibition was calculated relative to the positive control (no inhibitors) and the negative control (no DNA vector for protein expression).

RESULTS AND DISCUSSION

Miniaturized Fluid Array Device. A picture of the miniaturized fluid array device is shown in Figure 1a. The device consists of 96 units, which are in agreement with a conventional 96-well microplate. The device was found to be compatible with commercially available microplate readers and reagent dispensing apparatuses; thus, it can be used for high-throughput applications such as drug screening. For each unit (Figure 1b), the top layer contains a reaction chamber for gene transcription and protein translation, the bottom layer contains a feeding chamber for replenishing nutrients (e.g., amino acids and ATP), and the middle is a dialysis membrane that connects these two chambers. As discussed in the literature,[6,12,29] the functions of the membrane are to (1) achieve continuous supply of additional nutrients; (2) retain proteins produced and large-molecule synthesis machinery; and (3) dilute the reaction byproducts (e.g., pyrophosphates) and reduce their effects on the reaction equilibrium. Compared to the commercially available CFPS systems (e.g., RTS 500 kit),[29] one major advantage of the fluid array device is lower reagent

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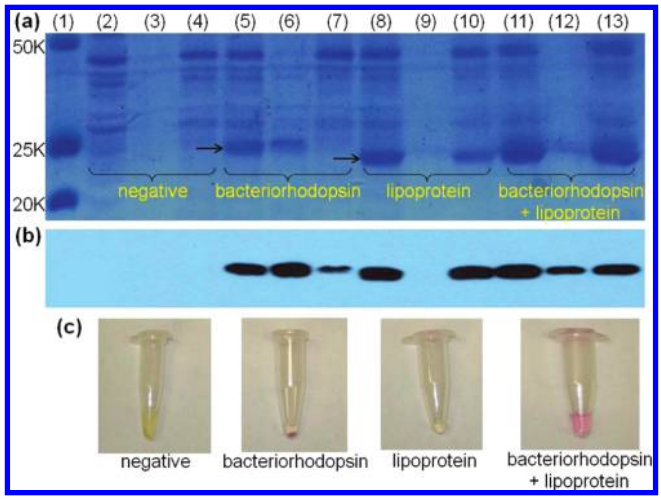

Figure 2. (a) Gel electrophoresis of membrane proteins. Lane 1 is a protein ladder. Lanes 2−4 are for the negative control. Lanes 5−7 are for expression of bacteriorhodopsin. Lanes 8−10 are for expression of apolipoprotein. Lanes 11−13 are for coexpression of bacteriorhodopsin and apolipoprotein. The first lane in each group is for the reaction mixture; the second lane is for the pellet, and the third lane is for the supernatant. The arrow in lane 5 indicates the position of bacteriorhodopsin while the arrow in lane 8 indicates the position of apolipoprotein. (b) Western blotting of membrane proteins with the same lane designation as in (a). (c) Pictures of tubes containing expressed protein products (after centrifugation). They are in the same order as in (a) from the left to the right.

consumption (10 μL versus 1 mL). The cost-savings would be substantial when a large number of arrays are required for high-throughput applications. The additional advantage of miniaturization is to enable a high-density array, which makes it possible to have high-throughput CFPS for applications such as drug screening.

An alternative way to run high-throughput CFPS is in a conventional 96-well or 384-well microplate. Without a membrane in the conventional microplate, we found the synthesis yield was much lower.[20−22] For the membrane protein to be discussed below, coexpression of bacteriorhodopsin and apoA lipoprotein in the fluid array resulted in a mixture with the characteristic purple color of bacteriorhodopsin as shown in Figure 1c, demonstrating the functional production of bacteriorhodopsin. Note that the protein expression product in the device was transferred to a transparent tube for the purpose of taking a picture. In contrast, when the same expression was carried out in a conventional microplate, the amount of proteins expressed was too low to have an effect on the color of the mixture as shown in Figure 1d. The yellow color of the mixture is very similar to the negative control that contains no DNA vectors.

Membrane Proteins. The fluid array device was used to produce two membrane-associated proteins, apoA lipoprotein and bacteriorhodopsin. ApoA lipoprotein can form nanoparticles due to the presence of lipid in the expression medium.[16] Its expression was confirmed by polyacrylamide gel electrophoresis (PAGE) as shown in Figure 2a (arrow in lane 8). Since the lipoprotein particle is soluble, it was in the supernatant after centrifugation (lane 10). Expression of bacteriorhodopsin in the fluid array was also confirmed by PAGE (arrow in lane 5). After centrifugation of the mixture, most bacteriorhodopsin molecules precipitated to form pellets since they were insoluble (lane 6).[15,16,30]

Bacteriorhodopsin and ApoA lipoprotein could be coexpressed in a single reaction; the lipoprotein's ability to form lipid bilayer patches from lipid micelles can be used to increase the solubility of other membrane-associated proteins such as bacteriorhodopsin.[15,16,30] The parallel expression of both proteins was confirmed by PAGE (lane 11). After centrifugation of the mixture, most bacteriorhodopsin molecules stayed in the supernatant due to the increased solubility in the presence of lipoprotein particles (lane 13).

Since there are many protein bands belonging to the components of the protein expression system, we carried out Western blotting to verify the presence of bacteriorhodopsin and nanolipoprotein in the samples. The blot in Figure 2b shows much cleaner protein bands for the proteins of interest. Note that Western blotting in Figure 2b is more sensitive than Coomassie blue staining in Figure 2a; hence, a minute amount of bacteriorhodopsin undetectable in lane 7 in Figure 2a was shown as a band in Figure 2b.

The effect of coexpressed lipoprotein on the solubility of bacteriorhodopsin is evident from the physical appearance of the protein product solutions at the end of expression, followed by centrifugation, as shown in Figure 2c. These proteins were expressed in the device, and the product mixtures were transferred to a tube, followed by centrifugation to separate soluble and insoluble materials for visualization. The negative control with no DNA vectors shows the color of the cell-free reaction mixture. The product mixture for expression of apoA lipoprotein is homogeneous since apoA lipoprotein is soluble. However, the product mixture for expression of bacteriorhodopsin contains a purple pellet and clear supernatant after centrifugation. This is in agreement of the result of gel electrophoresis in Figure 2a that bacteriorhodopsin is in the pellet. When two proteins were simultaneously coexpressed, the solubility of bacteriorhodopsin was increased in the presence of apoA lipoproteins. Importantly, the bacteriorhodopsin sample was purple, which indicates the formation of a functional protein in our device.

Soluble Proteins. As we have reported previously, several soluble proteins can be expressed in the miniaturized fluid array device, including luciferase, green fluorescent protein, β-glucoronidase, alkaline phosphatase, β-lactamase, and β-galactosidase.[20−22] In this work, we chose two soluble proteins, luciferase and β-lactamase, to demonstrate the enzyme inhibition assay. Luciferase gene has been extensively used as a reporter gene for visualizing biomolecular processes in living animals,[27,31] and luciferase-based luminescence assays have been increasingly used for high-throughput drug screening.[32] As a result, we selected luciferase to verify if the protein expressed in the CFPS device can be used for enzyme inhibition assays without harvesting and purification from the protein expression mixture that contains a number of cofactors, enzymes, and others. We first studied the kinetics of luciferase assay reactions in the presence of an inhibitor, luciferin 6′-methyl ether (LME). LME is a known luciferase assay inhibitor and has been used by others in the

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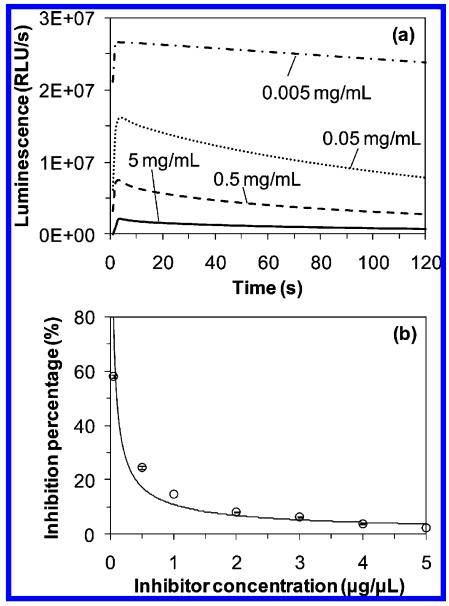

Figure 3. Inhibitory effects of luciferin 6′-methyl ether on the luciferase assay. (a) The luciferase assay reaction kinetics in the presence of various concentrations of inhibitors. (b) The inhibitory effects as a function of the inhibitor concentration. The decrease in percentage of the luminescence signal in the *y*-axis is relative to the positive control, in which no inhibitor was added. The lines are the best fit in the power relationship among the experimental data points. The error bars are invisible, as they are smaller than the data markers.

literature.[27,28] The inhibitory assay was performed using luciferase synthesized in the miniaturized fluid array device; the expression product was directly used for the assay without any treatment. Figure 3a shows the reaction kinetic curves we obtained. The resemblance of these curves with those in the literature using purified luciferase[33] indicates that the enzyme synthesized in the cell-free medium can be used for the enzyme inhibition assay without harvesting and purification. As a result, we can use CFPS products directly for the enzyme inhibition assay. Figure 3b shows the inhibitory effects on the luciferase assay as a function of the LME concentration.

In addition, we evaluated the specificity of each enzyme assay. For example, the UV absorbance-based enzyme assay of β-lactamase using *m*-{[(phenylacetyl)glycyl]oxy}benzoic acid (PBA) should be specific to β-lactamase. Figure 4 shows the assay kinetics, in which signal increased over a period of time when β-lactamase was cleaving the chromogenic substrate. It leveled off to form a plateau when the reactions reached equilibrium. However, when the same assay was applied to luciferase, there was negligible signal increase, except for the initial point that was due to the addition of a solution in a way similar to a negative control. These results suggest that the β-lactamase assay is specific. The data also indicate that reliable assay results for β-lactamase should be recorded at 5 min after the assay reagents were added into the expression products.

Drug Screening. To demonstrate the utility of the miniaturized fluid array for drug screening, β-lactamase was chosen as a mock drug target. β-Lactamase is an enzyme that hydrolyzes the amide bond of β-lactam antibiotics, causing the antimicrobial agents to lose their effectiveness. Three known β-lactamase

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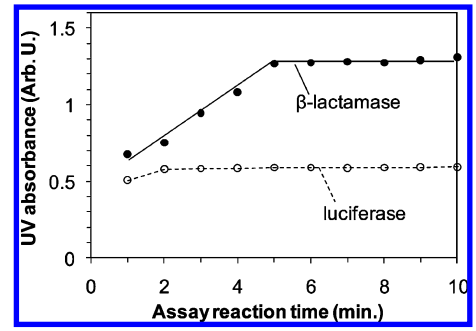

Figure 4. Enzyme assay kinetics of β-lactamase using a chromogenic agent, *m*-{[(phenylacetyl)glycyl]oxy}benzoic acid. The same assay was applied to luciferase, showing negligible increase over the background signal.

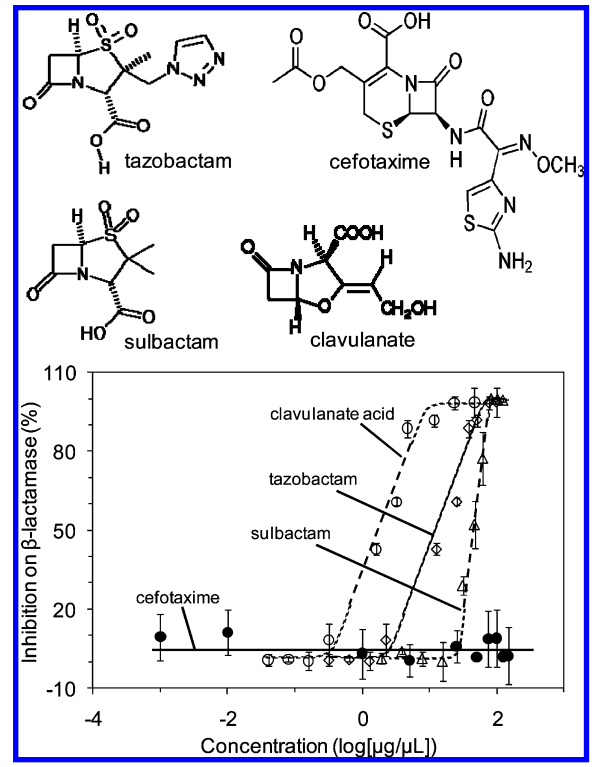

Figure 5. Inhibition of clavulanate acid (open cirles), tazobactam (diamonds), and sulbactam (triangles) on the enzymatic activities of β-lactamase. The concentrations of these inhibitors in the *x*-axis are in the log scale. Also shown is negligible and concentration-independent inhibition of cefotaxime (solid circles) on β-lactamase. Each data point represents an average obtained from three repeat experiments, and the error bars indicate one standard deviation. The chemical structures of all compounds tested are shown at the top; all of them have a β-lactam ring that consists of three carbon atoms and one nitrogen atom with a cyclic amide functional group.

inhibitors (clavulanate acid, tazobactam, and sulbactam) and one noninhibitory chemical (cefotaxime) were used as mock compounds. All of these compounds contain a β-lactam ring that can be hydrolyzed by β-lactamase, as shown in Figure 5. However, cefotaxime is a very large molecule, difficult to be hydrolyzed by β-lactamase. β-Lactamase was produced in each unit of the array device, and a series of varying concentrations of each compound was added into several wells to obtain a response curve. The relationship between the concentrations of each compound and the level of β-lactamase inhibition is also shown in Figure 5. The percentage of inhibition is calculated against the one without any

inhibitor (positive control). The results show that clavulanate acid, tazobactam, and sulbactam had inhibitory effects on the enzymatic activities of β-lactamase, while cetotaxime did not show any inhibitory effect. The I_{50} values (the concentration required to produce 50% inhibition[34]) of clavulanate acid, tazobactam, and sulbactam are 0.11, 0.87, and 24.8 μg/μL, respectively. As a result, clavulanate acid had the highest inhibitory effect among them, which agrees well with the previously published results.[35,36]

The result in Figure 5 suggests that protein expressed in a cell-free system can be used for drug screening just as those obtained from cell-based systems. The power of the fluid array device is evident from the fact that the inhibition-concentration curves of multiple compounds can be simultaneously obtained in one device through judicial experimental designs. Importantly, the device allowed us to determine not only if the compound is a drug candidate but also the effectiveness of the drug by comparing the various degrees of inhibition. An additional benefit of the use of the fluid array is that β-lactamase is in solution phase, so that its enzymatic activities are retained due to its appropriate three-dimensional configuration in an aqueous solution. As a result, it is superior over ELISA or protein chips in which proteins are attached to a solid surface and efforts must be made to minimize the loss of biological activities of the proteins.

CONCLUSION

A miniaturized fluid array device has been demonstrated capable of carrying out high-throughput cell-free protein synthesis. Compared to the conventional cell-free protein synthesis using commercially available RTS 500 kits,[29] the reagent consumption is 2 orders of magnitude less (10 μL of the reaction solution in our device versus 1 mL in the commercial kits). As a result, the cost-savings would be substantial when a large array is required for high-throughput applications (e.g., drug screening).

Cell-free expression of both soluble and membrane proteins has been achieved in the array device. A membrane protein synthesized in the device can be solubilized by coexpressing it with a lipoprotein in the presence of reagents necessary for correct folding, which would allow functional and structural studies of the membrane protein. Since membrane protein receptors represent the largest portion of drug targets, this capability could have great impact in searching for therapeutics.

Proteins synthesized in the device have been exploited for enzyme inhibition assays without protein harvesting and purification. Therefore, laborious and time-consuming procedures such as magnetic bead-based separation and chromatography-based purification can be eliminated. Potential losses of proteins and possible structure changes in the separation procedures have been reduced. In addition, a solution array (proteins in solution) does not compromise the kinetics of binding[37] whereas proteins immobilized on a solid surface in conventional protein chips are likely compromised by the fact that (1) only a portion of protein structures are exposed to ligands, (2) spatial hindrance exists during interactions between immobilized proteins and ligands in a sample, and (3) protein tertiary structures may change during immobilization.

The array format of the device enabled simultaneous expression of proteins, screening for a variety of inhibitors, and comparison of the inhibition levels among the inhibitors studied. We showed the capability of the device for drug screening by the use of β-lactamase, a drug target associated with antibiotic resistance, and four mock drug compounds. The inhibitory effects of these compounds on β-lactamase were measured in parallel, and their degrees in drug effectiveness were compared.

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