ANALYTICAL SCIENCES FEBRUARY 2009, VOL. 25

Rapid Communications

Microchip-based Homogeneous Immunoassay Using a Cloned Enzyme Donor

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We have realized a cloned enzyme donor immunoassay (CEDIA) on a microchip in 96 s. CEDIA is a homogeneous immunoassay, based on the bacterial enzyme β-galactosidase, which was genetically engineered into two inactive fragments: an enzyme donor and an enzyme acceptor. A model analyte was theophylline, and the detectable concentration range was from 0 to 40 μg mL^{-1}. Our CEDIA using a microfluidic device was very simple and rapid, unlike microchip-based heterogeneous immunoassays and CEDIA on a well-type microchip.

(Received December 15, 2008; Accepted January 8, 2009; Published February 10, 2009)

Many types of microchips have been developed for chemical and biological analyses. They have various advantages, such as reduced sample and reagent consumption, high reaction efficiency, short analysis time, simple operation, and easy integration and automation.

Immunoassay is one application that makes use of these advantages. Many types of immunoassays on a microchip have been applied practically in various fields, including point-of-care testing in clinical diagnosis and on-site environmental analyses.[1-9] Immunoassays are classified into homogeneous and heterogeneous types, and they are done in homogenous and heterogeneous systems, respectively. The main features of each assay are that heterogeneous immunoassays require the physical separation of antibody-antigen complexes prior to detection; homogenous immunoassays do not require an additional physical separation step, and are generally simpler to perform. Most types of immunoassays belong to heterogeneous assays, which are generally very sensitive. A representative heterogeneous immunoassay on a microchip is a sandwich assay, which consists of a capture step in which an antigen is captured by a primary antibody, and a subsequent sandwich step in which the captured antigen binds to a labeled second antibody. The sandwich immunoassay on a microchip takes more than 10 min because of its several steps, such as flowing, washing and reflowing, and it requires a microchip pretreatment by the immobilization of a primary antibody. On the other hand, homogeneous immunoassays are less sensitive than heterogeneous assays. However, homogeneous assays are rapid because they consist of simple operations, and they are appropriate for the analysis of small molecules, unlike the sandwich assay, where an antigen must possess two or more epitopes. Diffusion immunoassay (DIA) on a microchip has been proposed, which is based on the accumulation of antigen-antibody complexes in the middle of a microchannel after the mixing of antigens and antibodies.[10] A fluorescence polarization immunoassay (FPIA) on a microchip has been reported, which is based on measuring fluorescence polarization after competitive binding of an analyte and a tracer to an antibody.[11,12] Both DIA and FPIA on a microchip, which are carried out in homogeneous systems, use simple operations and are very rapid because they do not require washing, reflowing or immobilizing.

The cloned enzyme donor immunoassay (CEDIA) has become widely used to analyze drug concentration in blood for clinical diagnosis.[13–15] CEDIA has been commercialized by Microgenics Corporation (Fremont, CA), among others, and has been applied to the quantitative analysis of various molecules, such as drugs that have a narrow therapeutic concentration range, which include theophylline, phenytoin, carbamazepine *etc*. CEDIA is a homogeneous immunoassay that allows the sensitive detection of small molecules without separation steps, the same as FPIA. The scheme of CEDIA is shown in Fig. 1. It is based on the bacterial enzyme β-galactosidase, which has been genetically engineered into two inactive fragments: an enzyme donor (ED) and an enzyme acceptor (EA). The complementation of ED and EA forms an active enzyme. The covalent attachment of an analyte or a ligand to ED does not affect the ability of ED to associate with EA. Analyte-ED conjugate binding to an antibody does not associate with EA, and form an enzyme. An analyte present in a sample competes for binding to a limited number of antibody sites, making an analyte-ED conjugate available for enzyme formation. The amount of active enzyme formed is directly proportional to the analyte concentration in a sample. During the assay, the level of an enzyme with β-galactosidase activity can be determined spectrophotometrically by the rate of hydrolysis of a chromogenic substrate, chlorophenol red-β-galactopyranoside (CPRG), in a commercial CEDIA kit. In clinical diagnosis, technicians have to conduct a quantitative analysis of the drug level in blood by a conventional CEDIA, and more than 10 min is required.

CEDIA on a microchip has been developed as an ImmunoChip format, which is a well-type microchip, and antiepileptic drugs,

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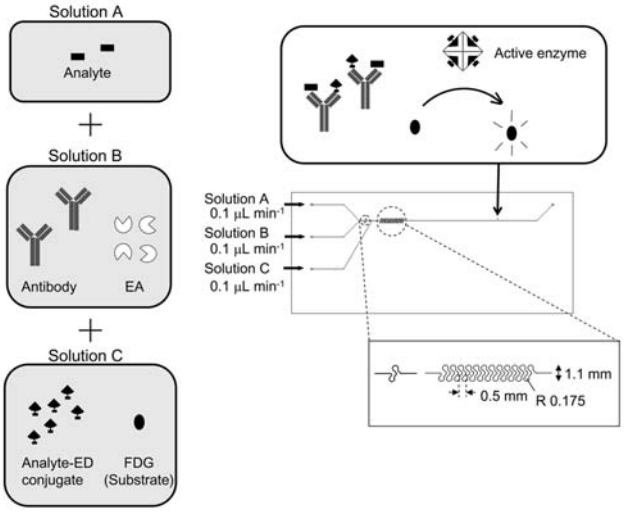

Fig. 1 Schematic illustration of the microchip-based CEDIA. Solution A is mixed with Solution B at 37˚C. Then Solution C is added to the solution and incubated at 37˚C. Subsequently, the fluorescence intensity is measured.

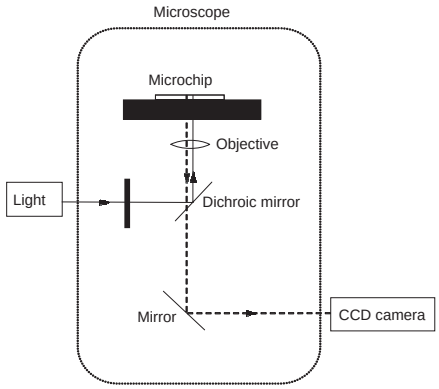

Fig. 2 Schematic diagram of the fluorescence detection system.

such as phenytoin and carbamazepine, were analyzed quantitatively.[16,17] In the assay, 6-*O*-*β*-galactopyranosyl-luciferin, which is a bioluminogenic substrate with high sensitivity, was used in place of CPRG because it was difficult to detect the chromogenic substrate in the small volume on a microchip due to the low sensitivity of CPRG. The assay could be performed with 25 μL of a serum sample and less than 1 μL of reagents in 5 min.

Paper reports on a different type of microchip-based CEDIA. We used a microfluidic device that could be easily connected with other microchannels and microchips. Fluorescein di(*β*-D-galactopyranoside) (FDG), which is a fluorogenic substrate with high sensitivity, was used in place of CPRG because of the low sensitivity of CPRG in our assay. Nonfluorescent FDG is sequentially hydrolyzed by the enzyme, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein.[18] Our model analyte was theophylline, a kind of bronchodilator, which is used for the treatment of apneic attacks in premature babies and asthma attacks in adults and children. It is essential to monitor the concentration of theophylline in blood in any treatment for premature babies, who are the most sensitive among patient users. Theophylline should be monitored especially in the initial period of use, and in the case of use with other drugs, because the metabolism of theophylline differs among individuals and it is easily affected by other drugs. Furthermore, monitoring the theophylline concentration is very important because of its narrow therapeutic concentration range, which is typically between 10 and 20 μg mL^{-1}.

The microchip used in this work was fabricated on a Pyrex glass substrate using standard photolithographic and wet chemical etching techniques. The microchip had been designed and manufactured by IMT (Institute of Microchemical Technology, Kanagawa, Japan) for our previous work (Fig. 1).[12] The microchip was 30 mm long and 70 mm wide, and its channels were 100 μm wide and 40 μm deep. The main channel had one short zigzag-like structure after the confluence of two solution channels, and the microchip had a long zigzag-like structure after the third solution channel entered the main channel; this design was to promote mixing. The microchip was examined using an optical microscope that was linked to a visible-light source for the excitation light and a CCD camera (Fig. 2). Ocular lenses had ×10 magnification, and objective lenses had a numerical aperture of 0.75 and ×10 magnification. A dichroic mirror included a filter for the excitation light (465 – 495 nm wavelength) and a filter for the emission light (515 – 555 nm wavelength) (Nikon). The microchip was surrounded by a holder that could adjust the microchip temperature. The flow rates of solutions used were controlled through 500 μL syringes and syringe pumps. Fused-silica capillary tubes were used for connecting the syringes and the microchip.

The CEDIA theophylline II kit of Microgenics included Reagents 1 and 2. Reagent 1 was the combination of an EA powder and an antibody solution, and Reagent 2 was the combination of a powder of theophylline-ED conjugate and CPRG, and 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer. Reagent 2 of the CEDIA kit, but without CPRG, was kindly provided by Microgenics. The concentrations of the theophylline solution were 0, 10, 20 and 40 μg mL^{-1}. Each theophylline solution was prepared by adding theophylline to phosphate buffered saline. Solution A was prepared by mixing 21.0 μL of each theophylline solution with 445.0 μL of MES buffer. Solution B was Reagent 1. Solution C was prepared by mixing 13.5 μL of 0.1 M FDG in DMSO with 486.5 μL of Reagent 2 without CPRG. Solutions A, B and C were introduced through different inlets, each at flow rates of 0.1 μL min^{-1} by syringe pumps (Fig. 2). Solutions A and B were mixed in the main channel, followed by Solution C. As a blank, DMSO was used in place of FDG in DMSO in Solution C. Fluorescence was detected about 60 mm from the confluence point of the three solutions. The fluorescence intensity was obtained as average values of the intensity in CCD images with 20000 pixels. These samples were run in triplicate. The temperature of the microchip was controlled at 37˚C by a temperature-controller (IOK-40, IMT, Japan).

A standard curve plotting the fluorescence intensity against the theophylline concentration is shown in Fig. 3. The fluorescence intensity was proportional to the theophylline concentration, which is typical for CEDIA. The therapeutic range of the theophylline concentration in serum was from 10 to 20 μg mL^{-1}. The obtained standard curve suggests that determining the theophylline concentration is feasible near the therapeutic range using our system.

Nonfluorescent FDG is sequentially hydrolyzed by the enzyme, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein. The K_M for FDG conversion to FMG has been determined to be approximately 18 μM, although much higher values (>600 μM) have also been reported.[19] The turnover rate for hydrolysis of FDG to FMG (1.9 μmol min^{-1} mg^{-1}) is much slower than for the conversion of FMG to

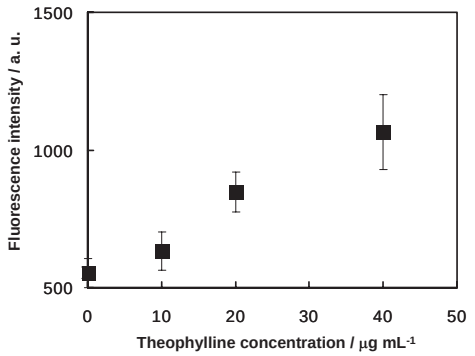

Fig. 3 Standard curve plotting fluorescence intensity against the theophylline concentration.

fluorescein (22.7 μmol min^{-1} mg^{-1}).[20] Low levels of β-galactosidase activity are readily detectable with FDG, due to the superior spectral characteristics of fluorescein (maximum extinction coefficient of 90000 cm^{-1} M^{-1}, fluorescence quantum yield of 0.92). Because the rate-limiting state in the hydrolysis of FDG is the conversion of FDG to FMG, few FMG would exist in the reaction system. These characteristics of FDG contribute to the realization of quantitative analysis in our nanoliter-order-sized microfluidic system.

This microchip was optimized for complete mixing and the diffusion of theophylline, fluorescence-labeled theophylline and antibody solutions.[12] The flow rates after the confluence of two solutions and three solutions were 0.2 μL min^{-1} (50 mm min^{-1}) and 0.3 μL min^{-1} (75 mm min^{-1}) in the main channel of this microchip, respectively. We obtained the linear velocities from the flow volume rate by assuming that the cross section of the channel was a rectangle (100 × 40 μm). It took 6 s from the first to the second confluence, and 48 s from the second confluence to the detection point. Further, it took 83, 83 and 96 s from the inlets of Solutions A, B and C to the detection point, respectively. In a previous study, theophylline and fluorescence-labeled theophylline were completely mixed and diffused between the first and second confluences, and the solution and antibody were completely mixed and diffused between the second confluence and the detection point.[12] In this study, theophylline in Solution A, and EA and antibody in Solution B would not be completely mixed in the channel between the first and second confluences, because the antibody is a large molecule. Theophylline, which is a small molecule, would be completely diffused in the channel between the first and second confluences. Therefore, almost all theophylline molecules would be bound to antibody molecules if the binding sites of the antibody molecules are not saturated. On the other hand, the antibody and theophylline-antibody complex would be completely mixed and diffused in the channel between the second confluence and the detection point, just as seen in the previous study. Since ED is a small polypeptide,[13] and theophylline is a small molecule, the theophylline-ED conjugate, which would be a small molecule, and the theophylline-ED-conjugate-antibody would also be completely mixed and diffused. The diffused theophylline-ED-conjugate would be complemented with EA to form an active enzyme, which would convert FDG to fluorescein by hydrolysis. Fluorescein, which is a small molecule, would be completely diffused within the channel in about 6 s;[12] that is, the conversion of FDG to fluorescein would take place continuously, and fluorescein would be diffused rapidly within the channel.

The conventional CEDIA consists of two-step procedures. A serum sample is mixed with a solution containing EA and an antibody, and incubated for 5 min. Then, the solution is mixed with a solution containing an analyte-ED conjugate and CPRG, and incubated for 5 min. In contrast, Yang *et al.*[17] developed a one-step CEDIA as an ImmunoChip format, which was simple and done in 5 min. Their simple and rapid assay was due to both miniaturization of the reaction system and mixing and the incubation of the antigen, EA, antibody, analyte-ED conjugate and substrate in the one-step CEDIA. We realized a simpler CEDIA on a microfluidic device in 96 s despite the two-step procedures. Our simpler and more rapid assay was due to the nanoliter-order-sized reaction system in the 100 μm-wide and 40 μm-deep microchannel.

We developed a microchip-based CEDIA, which was simple and rapid. The quantitative analysis of theophylline was performed within a short analysis time (96 s) with reduced sample sizes. The microchip-based CEDIA should find frequent use for point-of-care testing in the clinical field.

Acknowledgements

We thank Dr. Rueyming Loor of Microgenics Corporation for providing reagents and Mr. Kenji Uchiyama of Institute of Microchemical Technology, Co., Ltd., (IMT) and Mr. Yoshikuni Kikutani of Kanagawa Academy of Science and Technology for valuable discussions concerning chip fabrication. Microchip devices were fabricated at IMT. This work was partially supported by a grant from JSPS (17310087).

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