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An Active Mixing Microfluidic System for the Colorimetric Analysis of Intracellular Enzymes

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Abstract

A highly effective active micro mixer utilizing magnetic force with a simple configuration is explored to facilitate the fast mixing of more than two fluid flows and to integrate a microfluidic system capable of disrupting cells and reacting with biomolecules. The mixing performance of the active micro mixer is evaluated within an aqueous-aqueous system including dyes for visual observation and within an organic-aqueous system where enzyme reaction occurs. In addition, this magnetic stirrer for microfluidic-based research is used to create complete analytical micro systems, which facilitates cell lysis, sample preparation, and a subsequent analysis of intracellular biomolecules. Upon loading a sample of a whole cell and lysis reagent into a mixing chamber, the integrated microfluidic device carries out cell disruption by rotation of a micro magnetic-disc. Intracellular lipase samples from lyzed cells can be efficiently mixed and reacted with its substrate. We developed a micro-mixing fluidic system for cell lysis and enzyme reaction in order to detect colorimetric products.

Keywords: Cell disruption, Active micro mixer, Microfluidic device, Enzyme assay

Introduction

To create complete analytical micro systems by integrating various functional modules into a single chip, microfluidic technology has made significant advances over the last few years[1-4]. The mixing of multiple fluids in micro channels or chambers is an essential component in integrated microfluidic system applications, which is involved in the sample preparation of chemical cell lysis and biological reactions[5-7].

Until now, many micro-mixer designs have facilitated the development of microfluidic systems for mixing fluids at the micro-scale. Several types of flow dividing and recombining structures have been developed for split-and-recombine type mixing including ramp-like[8], fork-like[9,10], and three-dimensional curved architecture designs[11]. These passive mixers are typically accomplished by driving fluids through channels with delicate and fixed geometries[12,13]. Thus, passive mixing often requires expensive equipment for a three-dimensional fabrication process[7,14]. Also, there is no control of the mixing process as it occurs while a fluid pass through a specific channel geometry[15]. Compared with passive mixers, active mixers can be achieved and activated on-demand for mixing at very low flow velocities within large fluid chambers. These mixers can improve the mixing performance by periodic perturbation or chaotic advection of the flow fields. The methods used in active mixers have been demonstrated using bubble-induced acoustic actuation[16,17], electroosmotic actuation[18], and electrokinetic pressure[19]. In another application, Yaralioglu *et al*. reported on an acoustic stirring created by an ultrasonic wave that resulted in the mixing of two liquids in a chamber below a diaphragm[20]. However, these micro mixers, which can have excellent mixing capabilities, are not easy to integrate with other in-plane microfludic components because they require external power sources and additional on-chip wiring, and they sometimes may damage the activity of biomolecules.

We therefore present a simple and effective micro mixer utilizing a magnetic field, based on the similar mixing priciple with a large-scale magnetic stirrer. The fundamental notion of a magnetic stirrer in macroscopic mixers can provide an efficient and convenient blending without creating a complex structure. This is especially true of fluid mixing operations done over decades in a laboratory. In addition, stirring type mixers create an appropriate mixing effect by providing more interfacial area rather than by inducing microscale turbulence. Magnetic actuation, applied externally, eliminates the need for tether wires, reduces the control complexity, and allows for effective mixing in a large reaction chamber for sequential flow analysis[21,22].

In this study, the chaotic mixing of a micro disc embedded in a micro chamber is explored by applying an external rotating magnetic field in order to facilitate the mixing within a range of seconds and to also work effectively in a channel and reaction chamber. This micro mixer, based on the concept of a conventional magnetic bar-stirrer, was applied to the rapid mixing of two aqueous solutions, as well as to the enzymatic reactions between an organic solution and aqueous solution, in order to demonstrate that micro device-based mixers can handle modern and specialized issues such as sample preparations for analysis, and the wide-spread usable mixing tasks of blending, emulsification, and reaction. In addition, for a successful performance of an integrated microfluidic system involved in the sample preparation step by the chemical cell disruption and an enzyme reaction, a novel integrated microfluidic system is developed, which facilitates cell lysis, sample driving and mixing, and biological reaction by utilizing a simple fabrication process and inexpensive materials. This fully-integrated micro device, therefore, could provide a significant contribution to the ongoing efforts to miniaturize bio-analysis system and could allow for the design and operation of analytical devices for high-throughput applications such as in the analysis of biomolecules and chemicals.

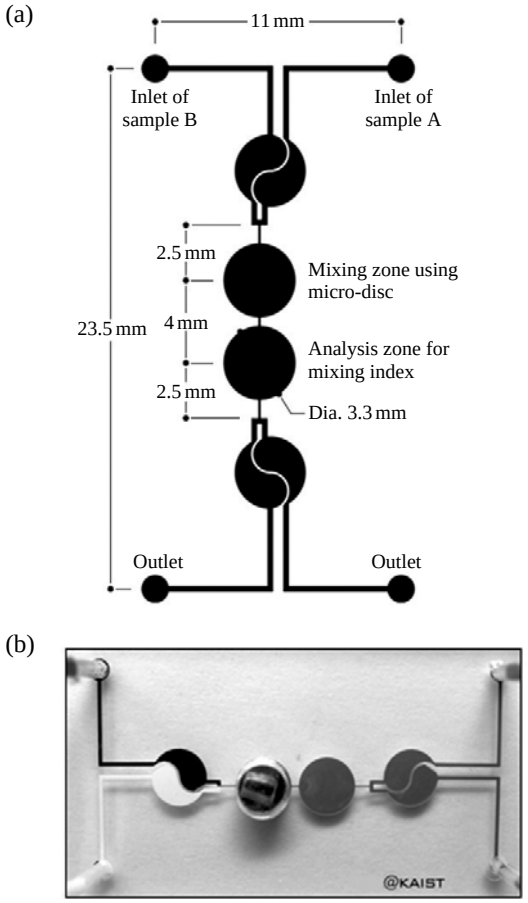

Figure 1. Schematic diagram of a micro-mixer in a microfluidic device. (a) Illustration of the layout and dimensions of the micro-mixer containing a magnetic disc. (b) Photograph of the micro-mixer system showing the mixing of white- and black-dyed solutions at 500 rpm.

Results and Discussion

Characterization of Micro-Magnetic Mixer

It is necessary to develop an on-chip microscale mixing system for the true integration of microfluidic and all other functional modules into one microsystem operating in a continuous-flow configuration. Thus, we developed a micro-mixer that allows for efficient mixing by using a magnet embedded in a mixing chamber (Figure 1). Figure 1a shows a schematic of the microfluidic device layout for the mixing of two aqueous solutions. The mixing performance was evaluated using black and white-dyed aqueous solutions. An optical microscope was used to monitor the fluid mixing and color change at the analysis zone of the outlet channel (Figure 1a). Figure 1b shows a photo image of this microfluidic device. The complete mixing was achieved in a short period of time (6 s) at a magnet rotation speed of 500 rpm. Thus, the active micro-magnetic mixer system developed in this study is suitable for the full mixing of fluids on a microfluidic chip. The procedures for the fabrication of a micro-mixer chip are depicted in Figure 2.

Cell Lysis in Micro-Mixer Channel

This micro-mixer achieved an efficient mixing effect in one second. Using this micro-magnet rotating disc mixer, a fully-integrated microfluidic sys-

tem, called a micro total analysis system (μ-TAS), was realized by cell lysis and subsequent lipase reaction. Two sample solutions, an intracellular sample and a lysis reagent, were injected into the cell lysis chamber from the respective inlets. The extent of cell lysis depended on the mixing homogeneity and mixing residence time. Table 1 shows the efficiency of cell lysis with increasing mixing time using a micro-magnet. To quantify the efficiency of cell disruption, the concentration of each collected lysis sample was assayed by a cell counting method. As the mixing time increased up to 20 min, the cell lysis efficiency for 500 rpm increased up to about 97%. We can suggest that a sufficient mixing power was offered to break the phase separation between the aqueous sample and organic solvent. Therefore, our active micro-mixer was carried out successfully for the disruption of the intracellular sample at 500 rpm for 20 min. After cell lysis, an elution buffer was introduced into the lysis chamber to allow the lysated sample solution to flow into the enzyme inlet port. The lysate sample solution was used to examine the lipase activity by colorimetric method.

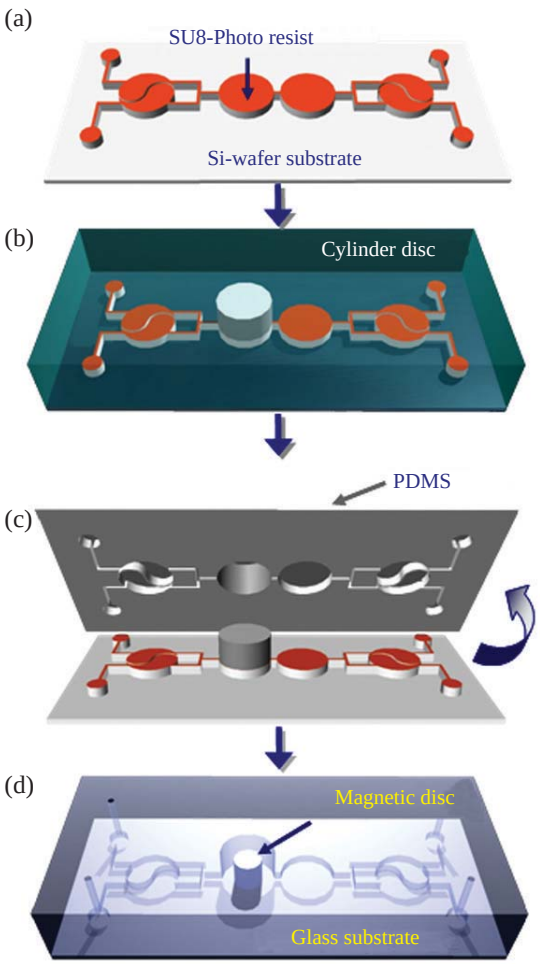

Figure 2. Process for the fabrication of a microfluidic micro-mixer system by PDMS molding.

Micro-Mixer for Enzyme Reaction

A colorimetric detection method is a powerful tool for analyzing biochemical processes such as enzyme-catalyzed reactions and the metabolic activities of living cells. Some of the advantages of colorimetric detection on a micro-chip is its simplicity and the ability to analyze small quantities of samples. We thus examined whether the micro-mixer developed in this study could be used for monitoring colorimetric enzyme-catalyzed reactions. Using this platform, the efficiency of an enzyme reaction can be evaluated by

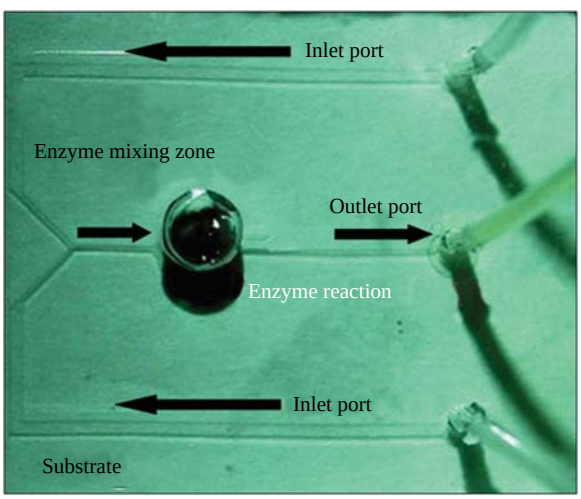

Figure 3. Image of microfluidic mixing for enzyme reaction. Mixing efficiency of the micro-magnet mixer.

Table 1. Cell lysis effect through the mixing time in a microfluidic chamber[§].

Mixing time (min)	5	10	15	20
Total cell number before cell lysis	2.62×10^7	2.95×10^7	2.51×10^7	3.49×10^7
Residual cell number after cell lysis	4.98×10^6	2.82×10^6	1.62×10^6	1.17×10^6
Cell lysis efficiency (%)	81.0 ± 2.75	90.4 ± 2.43	93.5 ± 2.13	96.7 ± 1.85

[§]The total cell number was counted three times using a heamacytometer on the slide glass under a light microscope.

altering the residence time in the mixing zone. Figure 3 shows a photograph of the micro-mixer device and a layout for an enzyme reaction. The efficiency of an enzyme reaction can be estimated by mixing the enzyme and substrate solutions, and subsequently monitoring the color intensities at the output channel. For quantitative analysis, the efficiency of an enzyme reaction was defined as

$$\textit{Efficiency of enzyme reaction} = (I\text{-}I_0)/I_0$$

where I is the color intensity after the enzyme reaction obtained at varying input flow rates, and I_0 is the color intensity obtained after a sufficient reaction time of 10 min. The effect of varying the input flow rate (thus, altering the residence time in the mixing chamber) on the efficiency of an enzyme reaction was examined at a magnet roation speed of 500 rpm. Two input solutions, lipase and substrate solutions, were introduced into the mixing zone using syringe pumps at flow rates of 0.25, 0.5, 1, 1.5, 2 and 2.5 mL/h. As the input flow rate increased from 0.25 to 2.5 mL/h, the efficiency of the enzyme reaction decreased monotonously (Figure 4). This was because of a longer residence time in the mixing chamber at a lower flow rate. It should also be noted that an enzyme reaction occurred with a successful mixing of organic and aqueous solutions in the micro-mixing chamber. Thus, the micro-mixer fluidic system developed in this study can be alternatively used for monitoring enzyme-catalyzed reactions of intracellular biomolecules on the micro-scale.

In this paper, we report on the development of a complete microfluidic chip for detecting lipase reactions by integrating a cell lysis chamber employing an active micro-mixer based on a rotating micro-magnet disc, a solvent lysis reagent, and a mixing chamber for studying enzyme reactions. Efficient cell lysis in a mixing chamber, specific and efficient lipase reaction, and colorimetric detection could be demonstrated. The micro-mixer developed in this study can be used for the efficient mixing of multiple fluids as well as for cell lysis as demonstrated above. Recently, we demonstrated that this micro-mixer chip can efficiently disrupt recombinant *Escherichi coli* cells for the analysis of intracellular proteins[23]. After lyzying the cells, the final collected cell lysate containing the lipase could be used for a colorimetric assay of an enzyme reaction. Also, the cell lysate containing the lipase could be specifically reacted with its substrate in the microchannels. The specific reaction of the intracellular lipase in a microfluidic system suggests that various nanobiosensor devices can be similarly manufactured for the detection of biotechnological interactions. These assays could be carried out in microfluidic channels, which will allow for high-throughput bioassays of multiple samples. Live cells expressing a specific antigen could be efficiently lysed *in situ* in a micro-mixer by mixing with the cell lysis reagent. Also, a recombinant protein can be used in a quality that is suitable for biomolecular interaction studies. This technology should be useful for developing lab-on-a-chip biosensors and diagnostic microsystems for various nanobiotechnological applications.

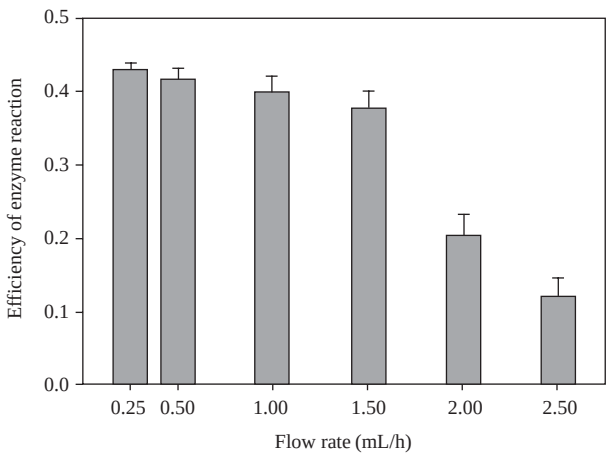

Figure 4. Efficiency of enzyme reaction with an increase of flow rate in the range of 0.25-2.5 mL/h in a microfluidic mixer.

Materials and Methods

Chemicals

Unless otherwise stated, all chemical reagents were purchased from Sigma (St. Louis, MO, USA). Poly (dimethylsiloxane) (PDMS) substrates were made using a Sylgard® 184 silicon elastomer kit (Dow Corning, Midland, MI, USA).

Construction of Expression Vectors for Lip1 Protein

E. coli BL21 (DE3) (Novagen, Darmstadt, Germany) was used as a host strain for general cloning works and gene expression studies. The plasmid used in this study is listed in our previous report[23]. Polymerase chain reaction (PCR) experiments were performed using a PCR Thermal Cycler MP TP 3000 (Takara Shuzo Co., Shiga, Japan) with a High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Berverly, MA, USA). DNA sequences of all clones

were confirmed by automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., IL, USA). Primers for the amplification of the *Bacillus* sp. TG43 lipase (Lip1) gene were designed based on the reported sequence (GenBank accession no. AF141874)[23]. The PCR product encoding Lip1 was cloned into the pET-22b (+) (Novagen, Darmstadt, Germany), yielding pET-Lip1.

Production of Recombinant Proteins in *E. coli*

A recombinant *E. coli* BL21 (DE3) strain harboring pET-Lip1 was cultivated in a Luria-Bertani medium (10 g/L bacto-tryptone, 5 g/L yeast extract and 5 g/L NaCl) supplemented with 100 μg/mL of ampicillin at 37˚C and 250 rpm. At an OD_{600} (DU® 650 Spectrophotometer, Beckman, Fullerton, CA, USA) of 0.4, cells were induced with 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for the production of a recombinant lipase. After induction, cells were further cultured for 4 h, and used for cell lysis experiment. A protein concentration was determined by Bradford's method using a bovine serum albumin (Sigma) as a standard.

Micro-Mixer Fabrication

Figure 1 shows the designed microfluidic mixer chip, in which a magnetic disc is embedded in the micro chamber. A magnet embedded inside the mixing chamber is activated using the rotating magnet in a commercial stirrer (SCINICS, Japan). The micromixer chip was fabricated by soft-lithography and replica molding methods using PDMS (Figure 2). First, a negative photoresist (SU-8, Microchem) master was made on a silicon wafer. The desired pattern was made on the wafer by soft-lithography. PDMS was prepared as a mixed solution of base and curing agent in a volume ratio of 10 to 1. Before pouring the PDMS solution onto the wafer, a cylinder-disc supporting material was placed in the micro cavity to keep the parts from being covered by the PDMS solution. After curing the PDMS at 70˚C for 2 h, the cylinder disc was replaced with an NdFeB magnet with a diameter of 1.5 mm and thickness of 1.2 mm (Donghwa Industry, Korea). After curing, the PDMS replica was peeled off from the master, plasma treated, and then bonded to a slide glass substrate (38 mm × 70 mm).

Cell Lysis using Chemical Reagent

A culture broth (3 mL) was centrifugated at 3,500 × *g* for 5 min at 4˚C, and the cell pellet was washed with 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by centrifugation at 12,000 × *g* for 5 min. The cell pellet was resuspended in 200 μL of TE buffer and then introduced into the sample inlet port at a flow rate of 0.5 mL/h for 1 min. Simultaneously, the lysis solution (2% xylene, 7.9% acetone, 0.1% toluene)[23] in TE buffer was added to the mixing chamber at a flow rate of 0.5 mL/h for 1 min. To lyse the cells, the cell solution and lysis solution were mixed in the mixing chamber at 500 rpm and 30˚C for 20 min.

Lipase Activity

The lipase activity was assayed by the colorimetric method using ρ-nitrophenyl decanoate as a substrate. The ρ-nitrophenyl decanoate was dissolved in acetonitrile at a concentration of 10 mM. Ethanol and 50 mM Tris-HCl (pH 8.0) were subsequently added to make a substrate solution having a volume ratio of 1 : 4 : 95 (10 mM ρ-nitrophenyl decanoate in acetonitrile : ethanol : Tris-HCl). After removing cell debris, cell lysates and the substrate solution were introduced from the inlet ports at a flow rate of 0.25-2.5 mL/h. After incubating the reaction mixture at room temperature, the activity was assayed by detecting the yellow-colored product, ρ-nitrophenol. The lipase activity assay was used to examine the extent of cell lysis at different flow rates in the micro channels.

Acknowledgements

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