Facile Fabrication of an Enzyme Microreactor Using Magnetic Microbeads

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ABSTRACT

Microreactor technology has received great attention from many research groups and companies because of its advantages over conventional macroscale reactors, e.g., safety, lower reagent consumption, lower waste emission, shorter analysis time, and better control on mass and heat transfer. In this paper, we present a simple method to fabricate an enzyme microreactor using soft lithography and magnetic microbeads. For proof of concept, we performed an enzymatic reaction by flowing non-fluorescent resazurin through diaphorase (DI) (E.C. 1.6.99) covalently attached to magnetic microbeads packed in a PDMS microfluidic chip to convert it to fluorescent resorufin in the presence of nicotinamide adenine dinucleotide phosphate (NADH). We investigated the effects of flow rate, and pH on the reaction yield to find the optimal condition. At a flow rate of 41.2 μL/h, a stable state for the enzyme reaction in the microfluidic format was achieved within 50 s.

Keywords: microfluidics, enzyme, microreactor, magnetic microbeads, diaphorase

1 INTRODUCTION

Microreactors have been proven to be a powerful tool in chemistry and biotechnology [1-3] and are particularly useful for studies using reagents that are expensive or of limit amount, such as enzymes and antibodies. The kinetics of enzymes can be studied in both homogeneous and heterogeneous reactions in the microreactor. However, many researchers prefer heterogeneous reactions because they allow the reuse of enzymes, avoid secondary reactions like autoproteolysis, simplify product purification, and improve the enzyme reaction rate [4-8]. There are two schemes of enzyme immobilization, depending on the location of immobilization. The first is onto the walls of the microchannel [9-11], and the second is onto a solid support, such as monolith, sol-gel, controlled pore glass, and silica and polystyrene beads, inside the microchannel [12-20]. Immobilization of enzyme onto the microchannel walls provides limited loading capacity due to the small surface-to-volume ratio, while immobilization onto solid supports usually requires tedious, time-consuming, multiple-step reactions. Additional difficulty comes from the packing of the solid support into the microchannel, which requires special structures, such as weirs and frits, to hold the derivatized support in the microchannel. This makes the design and fabrication of microreactors more costly and complicated. In our previous work, we studied the affinity binding of peptides to teicoplanin (a glycopeptide antibiotic) using magnetic microbeads (2.7 mm in diameter) derivatized with teicoplanin. Teicoplanin-modified microbeads (used as the affinity column) and underivatized microbeads (used as the control column) were simultaneously packed in a few minutes into given sections of a microchannel with the aid of a holding magnetic (2 mm in diameter) imbedded into the PDMS [21]. The length and density of the two columns could be easily controlled and with good reproducibility. The extent of interaction between the peptide and the two types of beads in either microchannel resulted in differences in migration time of the peptide, and this difference was used to obtain a value for the binding constant of the peptide to teicoplanin.

Herein, we demonstrate a facile method, employing an embedded magnet, of fabricating an enzyme-catalyzed microfluidic reactor. In these studies, diaphorase (DI) (a flavin-bound enzyme) is used to convert resazurin (non-fluorescent) to resorufin (fluorescent) in the presence of nicotinamide adenine dinucleotide phosphate (NADH). (Figure 1). DI was immobilized onto carboxylic-terminated magnetic micro-beads and then packed into a given section of a microchannel. Optimal conditions for the microreaction were examined and are detailed in this paper.

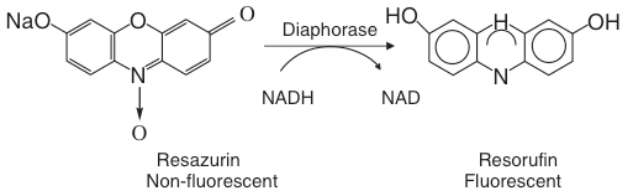


Figure 1. Enzymatic conversion of resazurin to resorufin

2 MATERIALS AND METHODS

2.1 Reagents

Resazurin sodium salt, bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate (NADH), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), and diaphorase (DI) (E.C. 1.6.99) from *clostridium kluyveri* were purchased from Sigma-Aldrich Co. (St. Louis, MO). Zinc chloride, 2-(*N*-morpholino)-ethanesulfonic acid (MES), and potassium

chloride were purchase form Fisher Scientific, Inc. Negative type photoresist SU-8 2025 and its developer were obtained form Microchem (Newton, MA). Poly(dimethylsiloxane) (PDMS) oligomer and its cross-linking agent Sylgard 184 were purchased from Dow Corning (Midland, MI). Carboxylic group terminated Dynal® magnetic beads (2.7 μm in diameter) were purchased from Invitrogen (Carlsbad, CA).

Zinc chloride was used as activator. A stock solution of zinc chloride was prepared in 5 mM HCl. 0.20M Tris solution was used as the running buffer. A stock solution of NADH and resazurin was resolved in the running buffer just before use. A stock solution of potassium chloride was daily prepared in the running buffer containing 0.02% BSA. The substrate solution was prepared by mixing the stock solutions at different ratios.

2.2 Instrumentation for data acquisition

A fluorescence microscope (Nikon Eclipse TE-2000-U, Nikon Co., Kyoto, Japan) with a 100 W mercury lamp and a charge-coupled device camera was used to monitor the diaphorase-catalyzed reaction in real time. Red fluorescence was observed with Nikon filter Y-2E/C (ex/em 540-580 and 600-660 nm).

2.3 Fabrication of the enzyme microreactor

The fabrication of the enzyme microreactors includes three steps: (1) fabrication of a hydrophilic polydimethylsiloxane (PDMS) microfluidic chip, (2) derivation of magnetic microbeads with diaphorase, and (3) incorporation of the DI-modified magnetic microbeads into the PDMS microfluidic chip.

The microchip was prepared by conventional soft photolithography techniques. The simple straight pattern was designed using AutoCAD software (San Rafael, CA) and printed as a high-resolution (10,000 DPI) photomask (CAD/Art Services, Inc., OR). Negative-type photoresist was spin-coated onto a 3 in. silicon wafer at 1200 rpm for 30 s. The channel is 150 μm wide and 25 μm high at the top. The mold was spin-coated with degassed PDMS pre-polymer solution at 1300 rpm for 150 s and then kept at 70 ^{o}C for 15 min. A rare earth magnet (2 mm in diameter) was placed above the channel where the magnetic micro-beads were initially packed. Degassed PDMS pre-polymer solution was poured onto the assembly and baked for 2 h at 70 ^{o}C. The assembly was peeled off from the mold. Holes (3 mm in diameter) were punched to serve as the sample and buffer reservoirs. The PDMS assembly was irreversibly sealed to a glass slide after treating with oxygen plasma. The channel surface was coated with hydrophilic polymers using the method described by Wu [22].

The carboxylic acid-terminated magnetic microbeads were derivatized by incubating the mixture of 100 μL of the bead solution, 60 μL of diaphorase in MES, 30 μL of EDC, and 10 μL of MES buffer for 6 h at room temperature with slight shaking. The solution was then discarded and beads were suspended in Tris (50 mM, pH 7.4) buffer for 15 min in order to quench non-reacted carboxylic acid groups. The beads were then washed with PBS (100 mM phosphate, pH 7.4, 150mM NaCl, 0.1% BSA) (5) and resuspended in PBS (1 mL) (100 mM, pH 7.4, 150 mM NaCl) buffer.

The packing of DI-derivatizaed magnetic microbeads was performed using a peristaltic pump. The microchannel was rinsed with redistilled water and the running buffer for 3 min, followed by introduction of 5 μL of the suspension of the DI-derivatized beads at 2.1 μL/h. At this flow rate, the magnetic microbeads were retained by the holding magnet. This step was continued until the formation of a microbead column with a total length of 1.2 mm. The microreactor was rinsed with the running buffer before use (Figure 2).

2.4 Data acquisition and analysis

To perform the enzymatic reaction, the substrate solution was pumped through the microreactor packed with DI-derivatized microbeads. Each run was repeated twice. Etween two runs, the microreactor was rinsed with the running buffer, PBS buffer, and the running buffer for 3, 5, and 5 min, respectively. The progress of each enzymatic reaction was monitored in real time with a CCD camera mounted on a fluorescence microscope and saved as an MPG video file. To extract the fluorescence intensity of the product, the video clip was exported to a sequence of 8-bit TIFF images with Apple QuickTime Player (version 7.5.5), and the average intensity within a square area (10 X 10 pixels) in the center of the microchannel was extracted using ImageJ (Version 1.42g). The fluorescence trace was plot versus the elapsed time for further analysis.

3 RESULTS AND DISCUSSIONS

3.1 Nonspecific adsorption of microbeads

A rare earth magnet (2 mm in diameter) was used to retain the magnetic microbeads in a buffer suspension pumped into the microchannel and it worked up to a flow rate of 2.1 μL/h. There is no obvious nonspecific adsorption of the magnetic beads to the walls of the microchannel outside the section covered by the holding magnet because the hydrophilic surfaces of the microbeads and coated microchannel suppressed interactions [22, 23].

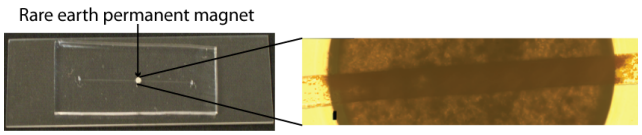


Figure 2. A microreactor with magnetic beads packed in the PDMS microchannel. The rare earth (neodymium-iron-boron, NdFeB) magnet was embedded in the chip on top of the microchannel during fabrication.

3.2 Amount of DI loaded into the microchip

To estimate the total amount of the enzyme loaded into the microchip, DI was cleaved from the magnetic microbeads bu incubating the microbeads with 100 mM NaOH solution for 2 h at room temperature. The concentration of DI was determined to be 0.4 μg/μL by the absorption OD value at 280 nm on a UV/Vis spectrophotometer. According to the concentration and volume of the bead suspension, the amount of DI loaded into the microchip was estimated to be 2.0 μg.

3.3 Optimization of the reaction yield

For comparison with the continuous-flow microreactor, a mixture of 8.0 μg DI, 1.5 mM NADH, 30 mM zinc chloride, 1.2 mM resazurin, and 2.6 mM potassium chloride at pH 8.5 was allowed to react in an Eppendorf tube for 15 min, and the corresponding conversion of resazurin to resorufin was used as a batch reaction standard. In the following microreactor experiments, the concentration of resazurin was kept at 1.2 mM, and the conversion of resazurin was compared to that of the batch reaction standard. The ratio of the conversion of resazurin in the two reactors was defined as the normalized conversion of resazurin in the microreactor:

$$\text{Normalized Conversion } (\%) = \frac{F_M}{F_B} \times 100 \qquad (1)$$

where F_M and F_B are the fluorescence intensity values of resorufin in the microreactor and batch reactor, respectively. The conversion of resazurin in the microfluidic reactor depends on the flow rate, solution pH, and the concentration of NADH, activator ($ZnCl_2$), and salt. Figure 3 shows the fluorescence microscopic graphs and the corresponding curve exported from the video taken at a flow rate of 41.2μL/h. In less than 50s a stable state for the enzyme reaction in a microfluidic format at a flow rate of 41.2 μL/h was achieved. Figure 4A shows the effect of flow rate on the conversion of resazurin. The maximum of conversion was obtained at a flow rate of 4.1μL/h. The conversion of resazurin in a microfluidic reactor decreased with increasing flow rate. We attribute this to the difference in time the substrate is in contact with the DI-derivatized micro-beads. As the flow rate increases contact time decreases. The conversion of resazurin in the reactor exceeded its conversion in a batch reactor at a flow rate of less than 41.2μL/h. This could be explained by the effect of the ratio of enzyme to substrate. For a given plug of substrate, the enzyme-to-substrate ratio is much higher in a microfluidic reactor than in the batch reactor. A flow rate of 41.2μL/h was used in subsequent experiments.

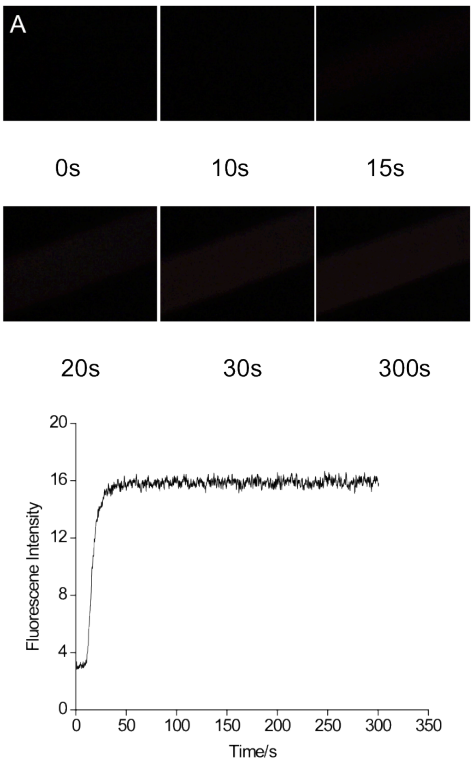
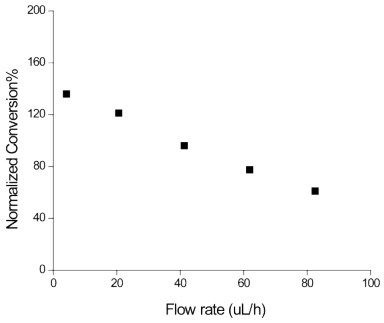


Figure 3. (A) Fluorescence micrograph of the microreactor. (B) The corresponding intensity vs. time plot of resorufin at a flow rate of 41.2 μL/h.

A



B

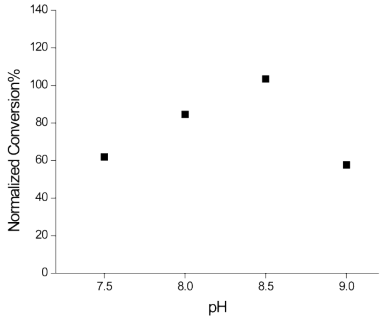


Figure 4. Effect of flow rate (A) and pH (B) on the conversion of resazurin in the microreactor.

The pH of the solution is an important parameter in most enzyme-catalyzed reactions. Figure 4B shows the influence of pH on the activity of DI. As can be seen, the activity of DI is very sensitive to pH. The maximum activity of DI was obtained at a pH 8.50. At pH 7.5 and 9.0, the activity of DI decreased by almost 50%. These observations agreed well with the behavior of DI in solution.

4 CONCLUSIONS

In the present work, an enzyme-catalyzed microfluidic reactor was prepared using diaphorase-modified magnetic micro-beads. The enzyme modified beads could be easily packed into micro channels with well defined spatial resolution and reproducibility under the help of a holding magnet imbedded into PDMS. Optimal conditions for the enzyme reaction were obtained and the conversion of resazurin was more than twice that when compared to a batch assay standard. This work has great potential in the development of microscale enzyme assays. The amounts of enzyme and substrate required is orders less than that used in typical enzyme assay protocols which should benefit those working with expensive reagents. Future work is focused on examining other enzyme systems and in developing multi-step enzyme reactions.

5 ACKNOWLEDGEMENTS

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REFERENCES

[1] Miyazaki, M., Maeda, H., *Trends Biotechnol.* 2006, *24*, 463-470.

[2] Heo, J., Thomas, K. J., Seong, G. H., Crooks, R. M., *Anal. Chem.* 2003, *75*, 22-26.

[3] Shiddiky, M. J. A., Rahman, M. A., Park, J.-S., Shim, Y.-B., *Electrophoresis* 2006, *27*, 2951-2959.

[4] Thomsen, M., Nidetzky, B., *Engineering in Life Sciences* 2008, *8*, 40-48.

[5] Mao, H., Yang, T., Cremer, P., *Anal. Chem* 2002, *74*, 379-385.

[6] Yakovleva, J., Davidsson, R., Lobanova, A., Bengtsson, M.*, et al.*, *Anal. Chem* 2002, *74*, 2994-3004.

[7] Kerby, M., Legge, R., Tripathi, A., *Anal. Chem* 2006, *78*, 8273-8280.

[8] Koh, W., Pishko, M., *Analytical and Bioanalytical Chemistry* 2006, *385*, 1389-1397.

[9] Thomsen, M. S., Nidetzky, B., *Eng. Life Sci.* 2008, *8*, 40-48.

[10] Mao, H., Yang, T., Cremer, P. S., *Anal. Chem.* 2002, *74*, 379-385.

[11] Yakovleva, J., Davidsson, R., Lobanova, A., Bengtsson, M.*, et al.*, *Anal. Chem.* 2002, *74*, 2994-3004.

[12] Kerby, M. B., Legge, R. S., Tripathi, A., *Anal. Chem.* 2006, *78*, 8273-8380.

[13] Koh, W.-G., Pishko, M. V., *Anal. Bioanal. Chem.* 2006, *385*, 1389-1397.

[14] Aman Russom, Tooke, N., Andersson, H., Steme, G., *Anal. Chem* 2005, *77*, 7505-7511.

[15] Wang, S.-C., Chen, H.-P., Lai, Y.-W., Chau, L.-K.*, et al.*, *Biomicrofluidics* 2007, *1*, 034104.

[16] Seong, G. H., Heo, J., Crooks, R. M., *Anal. Chem.* 2003, *75*, 3161-3167.

[17] Peterson, D. S., Rohr, T., Svec, F., Frechet, J. M. J., *Anal. Chem.* 2002, *74*, 4081-4088.

[18] Ma, J., Liang, Z., Qiao, X., Deng, Q.*, et al.*, *Anal. Chem.* 2008, *80*, 2949-2956.

[19] Luckarift, H. R., Ku, B. S., Dordick, J. S., Spain, J. C., *Biotechnol. Bioeng.* 2007, *98*, 701-705.

[20] Logan, T. C., Clark, D. S., Stachowiak, T. B., Svec, F., Frechet, J. M. J., *Anal. Chem.* 2007, *79*, 6592-6598.

[21] Liu, X., Gomez, F. A., *Electrophoresis* 2009, *in press*.

[22] Wu, D., Qin, J., Lin, B., *Lab chip* 2007, *7*, 1490-1496.

[23] *Dynabeads® M-270 Immunoassays (Carboxyl) Mnuals, Invitrogen*.