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Immobilization of multi-enzyme microreactors inside microfluidic devices

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Abstract

A simple method to fabricate enzyme-containing microscopic hydrogel structures in microfluidic devices for the potential use in micro total analysis systems (μ-TAS) is described. Poly(ethylene glycol)-based hydrogel microstructures were prepared inside microchannels by photolithography and enzymes conjugated to a pH sensitive fluorophore (SNAFL-1) were incorporated into these hydrogel microstructures. Because of the ratiometric pH-dependent nature of SNAFL fluorescence, hydrogel microstructures exhibited a different emission intensity ratio with pH and this intensity ratio changed almost linearly between pH 7 and 12. When alkaline phosphatase-containing microreactors were exposed to *p*-nitrophenylphosphate (pNPP) as a substrate, phosphoric acid was produced inside the microstructure by enzymatic-catalyzed hydrolysis of the substrate and subsequently decreased the microenvironment pH. Because of the relatively rapid mass transport of analyte through the hydrogel, enzyme-catalyzed reaction was easily detected by change in emission intensity ratio before and after exposure to substrates. Enzyme-catalyzed reactions were quite fast and reached 90% of maximum value within 10 min. Data were analyzed using a modified Michaelis–Menten equation and apparent Michaelis constants could be obtained. This system was also successfully applied to urea hydrolysis by urease.

Keywords: μ-TAS; Hydrogel; Microfluidic device; Enzyme reaction; Fluorescence

1. Introduction

Miniaturization of analytic devices in micro total analysis systems (μ-TAS) represents a natural extension of microfabrication technology to chemistry and biology with applications in high throughput screening and in portable analytical measurement devices. The recent developments in μ-TAS have allowed the miniaturization and integration of (bio)chemical instruments into a microchip-like format ("lab-on-a-chip"). The miniaturization of chemical reactors to the micrometer scale creates various advantages over benchtop instruments, including smaller dead volume and sample consumption, shorter analysis time, low cost, greater sensitivity and the capability performing on simultaneous reactions [1–6].

These microanalysis devices can be classified into two categories based on the complexity of the fluidics involved. One category is microarray-based microdevices where substances such as DNA and protein are immobilized on the chip. The second category is microfluidics-based microdevices where substances are transported, reacted and separated on chip. Microfluidic devices offer potential analytical advantages over planar array microchip such as enhanced mass and heat transfer, lower sample volumes, and ease of integration with miniaturized sample preparation modules [7,8].

Several investigators have explored the role that enzymes could play in lab-on-a-chip technologies and have investigated enzyme-substrate reactions within microfabricated channel networks using electrokinetic or pressure-driven flow

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[9–12]. A conventional type of microfluidic device for enzyme assay consists of several reagent wells each connected by channels to a main channel where the reaction and analysis regions are located [13–15]. Recently, however, a few groups have tried to immobilize enzymes inside microchannels for assay development [16–18]. Ismagilov et al. also investigated enzyme reaction using fluid–fluid diffusional contacts using microfluidic arrays [19].

Hydrogels have been used in biology and medicine for many years because of their properties of hydrophilicity, biocompatibility, permeability and mechanical strength [20]. Highly cross-linked hydrogel networks are capable of encapsulating protein or mammalian cells and have been used for numerous applications such as biosensors, cell encapsulation and drug delivery [21–25]. Previously, we developed PEG hydrogel microbead or microarray containing enzymes for use as optical sensors and could detect up to micromolar concentration of analytes [26,27]. PEG hydrogels provided a protective environment for the immobilized enzyme and inhibited degradation and fouling. Since Beebe et al. proposed fabrication of hydrogel microstructures inside microchannels for the use as microactuators [28], several groups have tried to make hydrogels containing DNA or mammalian cells inside microchannels for the DNA hybridization or potential drug screening systems [28–31]. In this study, we fabricated enzyme-containing poly(ethylene glycol) (PEG) hydrogel microstructures in microfluidic channels and performed enzyme assays using carboxy seminaphthofluorescein (SNAFL-1) conjugated to alkaline phosphatase and urease in PEG hydrogel microstructures for the potential use of this systems as biosensors or microreactors.

2. Materials and methods

2.1. Chemicals and materials

Alkaline phosphatase (AP) from *E. coli*, urease, *p*-nitrophenylphosphate (pNPP) and urea as substrates for the enzymes, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocynate (TRITC) were purchased from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) diacrylate (PEG-DA, MW 575) and perfluorooctane was obtained from Aldrich Chemical Co. (Milwaukee, WI). 2-Hydroxy-2-methyl-1-phenyl-1-propanone (Ciba, Tarrytown, NY) was used as a photoinitiator. 5-(and-6)-carboxy SNAFL-1 was purchased from Molecular Probes (Eugene, OR). Poly(dimethyl siloxane) (PDMS) elastomer was purchased as Dow Corning Sylgard 184 (Midland, MI), which is composed of a prepolymer and curing agent. 3-(Trichlorosilyl)propyl methacrylate (TPM) was purchased from Fluka Chemicals (Milwaukee, WI). The photomasks for making hydrogel patterns were purchased from Advanced Reproductions (Andover, MA). Phosphate buffered saline (PBS, 0.1 M pH 7.4) consisted of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate, and 0.15 M NaCl in 18 MΩ cm deionized water (Milli-Q Ultrapure, Millipore)

2.2. Preparation of SNAFL labeled enzyme solution

Using an established protocol, SNAFL-enzyme conjugates were prepared by reacting 1 mg SNAFL-1 succinimidyl ester dissolved in 100 μL of DMSO with the enzyme dissolved in a 100 mM PBS solution (pH 8.2). Unreacted dye was separated by overnight dialysis [32]. The final enzyme concentration was approximately 2 mg/mL.

2.3. Fabrication of microfluidic device

Microchannels in PDMS were obtained by curing a 10:1 mixture of PDMS prepolymer and curing agent against a Si master which has a negative pattern of the desired microchannel defined with SU-850 negative photoresist (Microlithography Chemical Corp, Newton, MA). After cured for several hours at 60 °C, PDMS replica was removed from the master and oxidized in an oxygen plasma (Harrick Scientific Co., Ossining, NY) with glass slide for 1 min. Bringing the oxidized PDMS and glass slide into conformal contact resulted in an irreversible seal and thus formed an enclosed microchannel. These microchannels were treated with dilute TPM solution in perfluorooctane for 10 min immediately after sealing to enhance the adhesion of hydrogel microstructure inside the microchannels. To make inlet and outlet ports in the microfluidic device, several holes were punched through PDMS replica using 16-gauge needle. Polyethylene tubes were inserted into these holes and then connected to syringe pump (Harvard Apparatus, Holliston, MA) to complete the microfluidic device. These microfluidic devices were mounted on the stage of a microscope for real-time fluorescence detection and imaging. This experimental apparatus is illustrated in Fig. 1.

2.4. Fabrication of PEG hydrogel microstructures inside microchannels

PEG hydrogel microstructures were fabricated from PEG-DA (MW 575) as the base macromer. The gel precursor solution was prepared by dissolving 10 μL of photoinitiator per 1 mL of PEG-DA solution. When fluorescent hydrogel structures were to be prepared, 50 μL of SNAFL or SNAFL-labeled enzyme solution per milliliter of precursor solution was also added. The microchannels were filled with these precursor solutions and then exposed to 365 nm, 300 mW/cm^2 UV light (EFOS Ultracure 100ss Plus, UV spot lamp, Mississauga, Ontario) for 1 s through a photomask on the top of glass slide. After UV photopolymerization, only exposed polymer regions underwent free radical cross-linking and became insoluble in common PEG solvents such as water. Finally, by flushing the channel with PBS, desired hydrogel structures were obtained inside a microchannel. The final enzyme concentration within the gel was approximately 0.1 mg/mL.