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Multi-step Reactions on Microchip Platform Using Nitrocellulose Membrane Reactor

Sung-Soo Park[1], Hwang-Soo Joo[1], Seung Il Cho[2], Min-Su Kim[2], Yong-Kweon Kim[2], and Byung-Gee Kim[1]*

[1] School of Chemical Engineering, Seoul National University, Seoul 151-744, Korea
[2] School of Electrical Engineering and Computer Science, Seoul National University, Seoul 151-744, Korea

Abstract A straightforward and effective method is presented for immobilizing enzymes on a microchip platform without chemically modifying a micro-channel or technically microfabricating a column reactor and fluid channel network. The proposed method consists of three steps: the reconstitution of a nitrocellulose (NC) membrane on a plane substrate without a channel network, enzyme immobilization on the NC membrane, and the assembly of another substrate with a fabricated channel network. As a result, enzymes can be stably and efficiently immobilized on a microchip. To evaluate the proposed method, two kinds of enzymatic reaction are applied: a sequential two-step reaction by one enzyme, alkaline phosphatase, and a coupled reaction by two enzymes, glucose oxidase and peroxidase, for a glucose assay.

Keywords: microchip electrophoresis, nitrocellulose membrane, immobilized enzyme, enzymatic assay

INTRODUCTION

An enzyme assay is an important and versatile tool in clinical diagnosis and chemical analysis. Microchip systems have also adopted many kinds of enzymatic reaction due to their minimal consumption of the enzyme and substrate, fast reaction speed, and highly resolved separation [1,2]. For such microchip applications, stable and effective enzyme immobilization has long been a focus of study [2, 3] and can be basically divided into two categories: chemical modification of the microchip surface [4-6] and technical fabrication that guides an enzyme solution to a specific location [7-11]. Chemical modification involves surface patterning for selective immobilization in the desired area, yet also includes loss of enzyme activity. Thus, adsorption and indirect immobilization are often preferred to covalent bonding. In contrast, technical fabrication is better for a specific application, yet it is difficult to extend to other applications.

Accordingly, the current report examines a very simple and efficient method for immobilizing various enzymes within microchip devices. A reconstituted nitrocellulose (NC) membrane, commonly used as an immobilization matrix, is coated on the surface of the chip substrate. An enzyme can then be easily immobilized by hydrophobic interaction on the NC membrane. Next, a PDMS substrate, on which a channel network is fabricated, is assembled. To examine the feasibility of this microchip device, two kinds of multistep reaction, a sequential two-step reaction by one enzyme, alkaline phosphatase, and coupled reaction by two enzymes, glucose oxidase and peroxidase, are carried out and analyzed.

***Corresponding author**
Tel: +82-2-880-6774 Fax: +82-2-883-6020
e-mail: byungkim@snu.ac.kr

MATERIALS AND METHODS

Materials and Reagents

The fluorescein diphosphate (FDP) and boric acid were both purchased from Sigma (St. Louis, MO, USA). The octadecyltrichlorosilane was from Aldrich (Milwaukee, WI, USA), and the fluorescein and dichlorodihydrofluorescein diacetate (H_2DCFDA) were from Molecular Probes (Eugene, OR, USA). The HPLC grade acetone, methanol, propanol, and chloroform were from Duksan Reagents (Seoul, Korea). The hydrogen peroxide and extra pure sodium hydroxide were from Oriental Chemical Industries (Seoul, Korea). The glucose was purchased from Shinyo Pure Chemicals Co. (Osaka, Japan) and the alkaline phosphatase, glucose oxidase, horseradish peroxidase, and esterase were from Sigma. The H_2DCFDA was hydrolyzed to dichlorodihydrofluorescein (H_2DCF) using esterase. Stock solutions of 15 mM FDP, 15 mM fluorescein, and 15 mM H_2DCF were prepared in methanol. Fresh diluted sample solutions were prepared daily in 20 mM boric acid and adjusted to the experimental pH with 1 M NaOH using deionized water (Millipore, Bedford, MA, USA). All solutions were filtered using a nitrocellulose membrane filter with a 0.45 μm pore size (Millipore) and degassed using an ultrasonic cleaner (Branson Ultrasonic Co., Danbury, CT, USA).

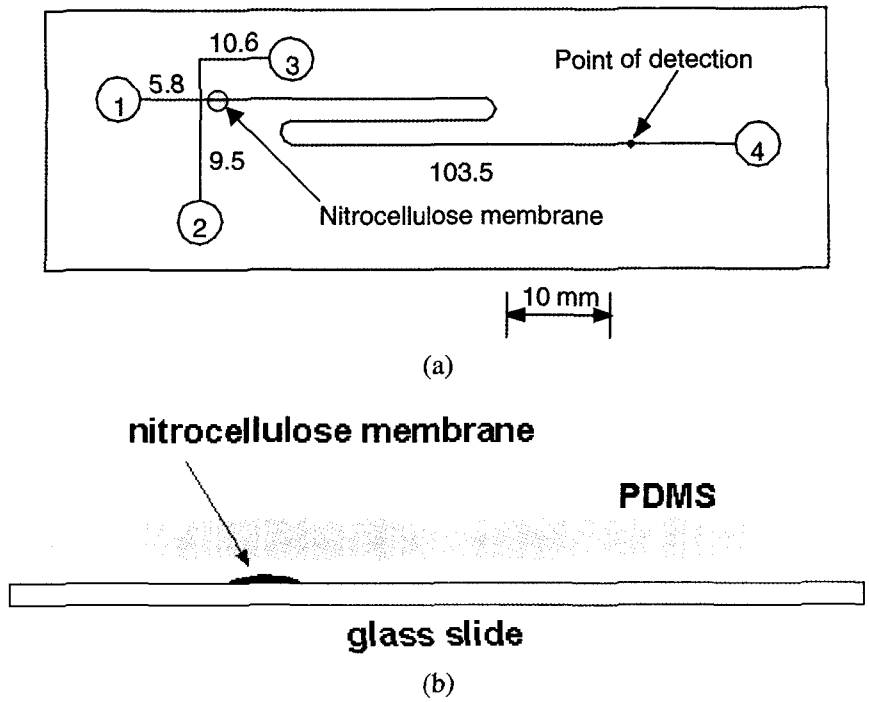

Fig. 1. (a) Schematic layout and dimensions of microchip. (b) Side view of microchip.

Nitrocellulose Membrane and Enzyme Immobilization

The preparation of the reconstituted NC membrane and the enzyme immobilization on the chip substrate have been described elsewhere [12]. Briefly speaking, the NC membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was dissolved in an acetone: propanol (1 : 1) solution at 2.5 mg/mL. The NC solution, 0.5~1.5 μL, was spotted on a slide glass and the NC membrane reconstituted by air-drying. 0.5 μL~1.5 μL of the prepared enzyme solution (10 mg/mL) was then spotted on the NC membrane, incubated for 1 hr at room temperature, and washed out. Thereafter, the PDMS channel and the slide glass were assembled manually.

Fabrication of Microchip

The micro-channels were fabricated using PDMS (Sylgard 184 silicone elastomer, Dow Corning Co. Midland, MI, USA) molding technology [13,14]. A thick photoresist, N-PMER (PMER N-CA3000PM, TOK, Kanagawa, Japan) was used as the PDMS replication mold. A photolithographic process was used to generate the channel mold for the PDMS channels without any wet etching procedures. A replicated PDMS channel was bonded to a microscope slide glass substrate (75 mm × 25 mm). Due to the good adhesive properties of PDMS, a closed PDMS channel system was readily formed on the slide glass by mere adhesion without applying any external force. A schematic diagram of the channel arrangement and their geometrical dimensions is shown in Fig. 1. Dimensions are given in millimeters. Reservoirs are labeled (1) sample, (2) buffer anode, (3) sample waste, and (4) buffer cathode. The channel depth was 37 μm and width at a half-depth was 60 μm. The effective separation channel length was 93.5 mm and the distance to the detection point from the buffer waste was 10 mm. The center of reconstituted nitrocellulose membrane was positioned at 3.7 mm from the injection T cross.

Instrumentation

The high voltage power supply (CZE1000R, Spellman, Hauppauge, NY, USA) with a voltage range of 0~30 kV was used to apply voltage to platinum electrodes (Aldrich) in each reservoir. The microchip electrophoresis system was assembled in-house. A laser beam of 488 nm, emitted from an argon ion laser (Melles Griot, Irvine, CA, USA), was impinged and focused using a 10× microscope objective (Edmund Optics, Barrington, NJ, USA) on the microchip at a 45° angle from the microchip surface and at a 90° angle from the separation channel. The fluorescent light emitted by the sample was collected by another 10× microscope objective through a 500 nm long-pass filter (Melles Griot), and detected using an integrated photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). The data acquisition and analysis were performed using a PCI-MIO-16E-4 DAQ board (National Instruments, Austin, TX, USA) with LabVIEW 6.0 (National Instruments).

Microchip Electrophoresis

The running buffer was prepared by dissolving 20 mM

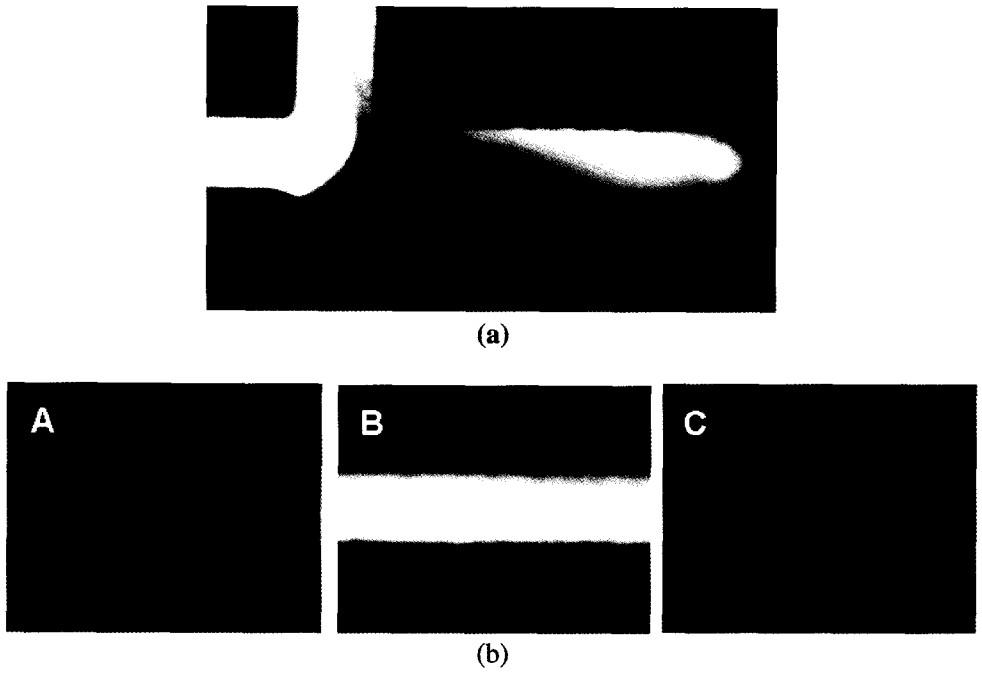

Fig. 2. (a) Image of gated injection. (b) FDP hydrolysis on NC membrane on microchip.

boric acid and adjusting the pH with 1 M NaOH. The microchip electrophoresis was performed under a gated sample-loading mode and separation mode, as described elsewhere [15,16]. That is, electrokinetic injection was performed by floating the potential in the buffer reservoir for 1~10 s, while maintaining a fixed potential in the other reservoirs. A snap shot of one experiment loading 1 μM fluorescein was taken using a confocal fluorescence microscope (Fig. 2 (a)). The voltages applied to the buffer anode, sample, sample waste, and buffer cathode reservoirs were 5, 4, 2, and 0 kV, respectively. Immediately after the injection, all the potentials were switched off, called the "zero potential mode" [11]. The injected sample then reacted with the immobilized enzyme and reapplying the potentials separated the reaction products. The electric field strength applied to the separation channel during the separation process was 441 V/cm. The gated injection and FDP hydrolysis on the NC membrane were observed using a laser-scanning confocal fluorescence microscope (Carl Zeiss, Germany)

RESULTS AND DISCUSSION

Nitrocellulose Membrane at Microchip

Since the NC membrane was positioned between the glass and the PDMS substrate (Fig. 1 (b)), its thickness and shape could be controlled to prevent solution leakage. As such, no band broadenings or shifts were exhibited in the electropherogram of 10 μM fluorescein when using a 3 mm~5 mm diameter NC membrane [1 μL of 2.5 mg/mL NC with acetone: propanol mixture (1 : 1 vol. ratio)] (data not shown), indicating that the reconstituted NC membrane in the microchannel exerted no effect on the electrophoretic separation. In addition, a real time reaction was observed on the NC membrane when using a confocal fluorescence microscope. After alkaline phosphatase was immobilized on the NC membrane, 10 μM FDP (20 mM boric acid pH 9.0) was injected for 5 s, then all the potentials were floated. The fluorescence at the center of the NC membrane was observed using a confocal microscope (Fig. 2 (b)). Photos A and B were taken 10 s and 50 s after injection, respectively. At 60 s after injection, all the potentials were reapplied. After another 5 s, photo C was taken. These results then confirmed that the diffusion of the substrate and product into the NC membrane outside the channel was negligible.

Hydrolysis of FDP

The proposed microchip system was evaluated using the hydrolysis reaction of FDP by alkaline phosphatase. 1 μL of 10 mg/mL alkaline phosphatase solution was spotted on the reconstituted NC membrane (5 mm diameter) and incubated for 1 h, then a PDMS substrate with a channel structure was assembled by positioning the center of the NC membrane 3.7 mm from the cross T-section (Fig. 1). Since the enzyme substrates were electrokinetically injected, the injection time of the substrates was determined from the EOF velocity and length of the NC membrane. As the length of the NC membrane used in the current study was 5 mm, and the closest end of the NC spot was positioned 1.2 mm from the cross T-section, the injected length of the substrate had to be at least 6.5 mm. The applied EOF was 1.5 mm/s, measured

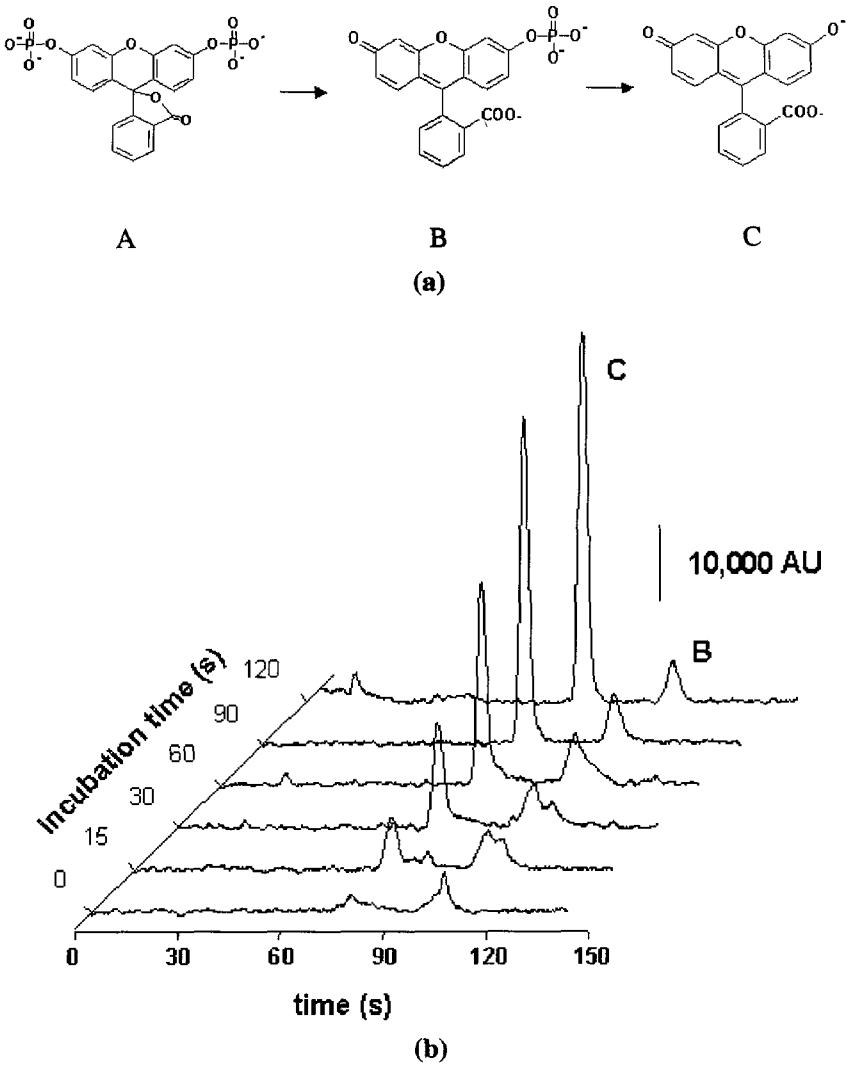

Fig. 3. Analysis of FDP hydrolysis by alkaline phosphatase. (a) Reaction scheme. (b) Electropherograms of FDP hydrolysis.

using a 5% DMSO solution as a non-fluorescent neutral marker and separation buffer (20 mM borate pH 9.0) containing 1 μM fluorescein (data not shown). Taking this into account, the substrate was injected for 5 s to supply a sufficient amount for the NC membrane reactor. Thereafter, the duration of the enzyme reaction was regulated using the zero potential mode. In a continuous flow, the substrate was naturally hydrolyzed to FMP and fluorescein without applying the zero potential mode (Fig. 3). As the incubation time increases, the quantity of the final product, *i.e.* fluorescein, increased dramatically.

Glucose Assay

Glucose was assayed using a two-enzyme system. Glucose oxidase oxidizes β-D-glucose into D-gluconolactone and H_2O_2. H_2DCF was used as the substrate for peroxidase to detect hydrogen peroxide formation (Fig. 4 (a)). Although, the optimum pH for a two-enzyme system is 5.0~6.0, the fluorescent intensity of dichlorofluorescein (DCF) is highest at pH 9.0. Thus, in the current study, different pHs were used: pH 6.0 for the sample buffer and pH 8.0 for the separation buffer. The sample solutions, which had different concentrations of glucose and 50 μM H_2DCF, were injected for 10 s and incubated for 30 s. Then, electropherograms were collected. As seen in Figs. 4 (b) and (c), the substrate H_2DCF and product DCF were well separated and a standard curve monitoring the glucose level could be plotted (r^2 = 0.98).

CONCLUSION

A very simple procedure for immobilizing enzymes in a microchip system was developed. As such, an NC membrane was reconstituted on a microchip surface by spotting and air-drying, then real enzyme reactions were performed for validation. Observations using a confocal

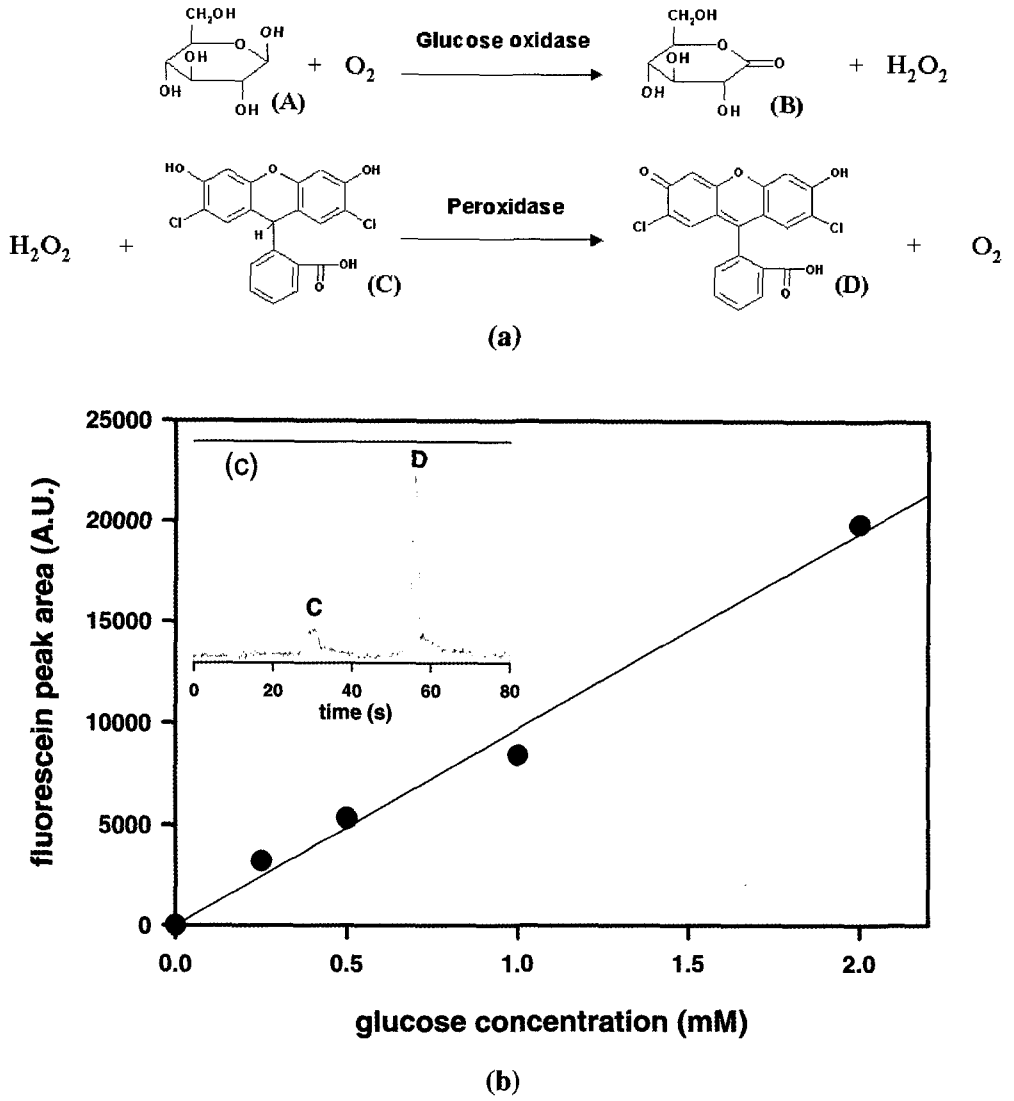

Fig. 4. (a) Reaction scheme for glucose assay. (b) Standard curve from microchip analysis. (c) Electropherogram of 1 mM glucose analysis.

fluorescence microscope confirmed an effective enzyme reaction on the NC membrane and negligible diffusion into the NC membrane outside the channel. Two kinds of enzyme reaction, *i.e.* a sequential two-step reaction by alkaline phosphatase, and coupled reaction by glucose oxidase and peroxidase, were carried out and analyzed. This kind of immobilization technique combined with a separation tool on a chip can be widely used for enzyme diagnosis, and constructing multiple enzyme reactions for studying enzyme reaction networks.

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NOMENCLATURE

NC	: nitrocellulose
PDMS	: polydimethylsiloxane
FMP	: fluorescein monophosphate
FDP	: fluorescein diphosphate
H_2DCFDA	: dichlorodihydrofluorescein diacetate
H_2DCF	: dichlorodihydrofluorescein
DCF	: dichlorofluorescein

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