DOI: 10.1002/cjoc.201200725

Open Tubular Microreactor with Enzyme Functionalized Microfluidic Channel for Amperometric Detection of Glucose†

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A simple and efficient method using enzyme immobilized microfluidic channel as open tubular microreactor was designed for amperometric detection of glucose. The microreactor was composed of a polydimethylsilicone/glass hybrid device with three reservoirs, a cooling cave and a 6 cm capillary with a sampling fracture as microchannel. The microchannel was further modified by thermal polymerization, followed by covalently attaching with glucose oxidase. Through fracture sampling and electrochromatography separation, the production via enzymatic reaction was determinated by Pt electrode at the end of capillary. The linear range for the detection of glucose was 0.05—7.5 mmol•L^{-1} with detection limit of 23 μmol•L^{-1}. The inter- and intra-chip reproducibilities for determination of 2.5 mmol•L^{-1} glucose were 98.5% (n=5) and 96.0% (n=5), respectively. With the advantage of flexible assembly, rapid efficiency, good stability and low-cost, this microreactor provided a potential platform for establishing a portable enzyme-based chemical detection system in practical application.

Keywords microreactor, amperometric detection, glucose, enzymatic catalysis, microfluidic channel

Introduction

Micro-total analysis system has attracted considerable interest due to its low cost, short analysis time, high automation, good portability and disposability.[1-3] In order to enhance the ability in molecular recognition, microfluidic device has been closely combined with biological technology.[4-8] Based on a variety of biochemical reactions, such as immune response, DNA hybridization and enzymatic reaction, this kind of minimized equipments with specific recognition has been developed as different microreactors. These microreactors, especially enzyme microreactors,[9-13] play important roles for the applications in environmental safety, food analysis, drug screening, and clinic analysis.

The main development of the enzyme microreactor is to establish heterogeneous enzyme analysis system in chip. For constructing heterogeneous enzyme microreactor, different kinds of supporters including microbeads,[14-17] monolithic column[9,10] and micro-membrane[11,18,19] have been employed for the immobilization of enzymes in microchannels. For example, a direct molding method of prepolymer on the printed master has been used for the immobilization of microbeads on microchip surface.[14] An enzyme microreactor has been designed to permit the *in situ* packing of enzyme immobilized porous glass into a section of channel.[16] Although these microspheres can maintain the activity of enzymes, it is time-consuming to design the special microstructures of channels and chambers, or append extra plugs, barriers or magnetic components for fixing beads.[20,21] Micro-monolithic column based microreactor can overcome some disadvantages and increase the surface area for enzyme immobilization, however it is also disadvantageous since the easily polluted and clogged channels usually cost long time for elution and extra high-pressure pumps are required for liquid driving. Therefore, it is promising to employ the inner wall of microchip channels as supports for enzyme immobilization without using other instrument for machining.

Comparing with the poly(dimethylsiloxane) (PDMS), the silica capillary as microchannel shows highly uniform inner surface, good stability and reduced adsorption of analytes. In our previous report, the molecularly imprinted silica capillary as microchannel in a PDMS/glass hybrid chip was proposed for separation and amperometric detection of enantiomers.[22] Here, a simple microreactor with enzyme immobilized on the inner-wall of silica capillary as microchannel was designed for amperometric detection of glucose. The microdevice was composed of a 6-cm long silica capillary, two PDMS moulds with three reservoirs and a cooling cave

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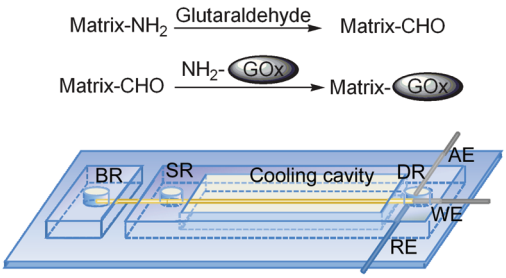
Received July 15, 2012; accepted August 19, 2012.
† Dedicated to the 80th Anniversary of Chinese Chemical Society.

(Scheme 1). The analytical performance of the microreactor was evaluated by using glucose oxidase as the model enzyme. Different from previous enzyme modified electrodes for the detection of glucose, this method could avoid sample pretreatment and the interference of other species coexisting in blood samples due to the rapid separation and need smaller amount of sample due to use of microreactor. This microreactor with simple preparation, rapid efficiency, good stability and low-cost can be used for a disposable analytical device and provides a promising application platform in actual testing of food safety.

Scheme 1 Schematic representation of PDMS/capillary/glass chip for constructing enzyme microreactor with three-electrode electrochemical detection system



BR: buffer reservoir, SR: sample reservoir, DR: detection reservoir, Matrix: poly(AM-EDMA), WE: working electrode, AE: auxiliary electrode, RE: reference electrode

Experimental

Materials

Fused-silica capillaries with inner diameters (i.d.) of 25 and 50 μm and outer diameter (o.d.) of 375 μm were purchased from Yongnian Optic Fiber Plant (Hebei, China). Sylgard 184 silicone elastomer and curing agent were purchased from Dow Corning (Midland, MI). 3-(Methacryloyloxy)propyltrimethoxysilane (*γ*-MPS), acrylamide (AM), and ethylene glycol dimethacrylate (EDMA) were all purchased from Alfa Aesar (Ward Hill, MA). 2,2'-Azobisisotyronitrile (AIBN) was obtained from Acros (Geel, Belgium). HPLC-grade acetonitrile (ACN) and glucose oxidase (GOx, EC 1.1.3.4, type X-S, lyophilized powder, 100—250 units/mg, from *Aspergillus niger*) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isooctane was supplied by Shanghai Chemical Reagent Co. (Shanghai, China). *D*-(+)-Glucose was bought from Sinopharm Chemical Reagent Co., Ltd. (China). Glucose stock solution was mutarotated overnight at room temperature prior to use. The electrophoresis buffer for microchip electrochromatographic separation was phosphate buffer saline (PBS, pH 7.4, 20 mmol•L^{-1}). Aqueous solutions were prepared using ⩾18 MΩ ultrapure water (Milli-Q, Millipore). All solutions were kept in a freezer to prevent deterioration. Glucose injection was purchased from local clinique (Jiangxi Kelun Pharmaceutical Co., Ltd and Shandong Hualu Pharmaceutical Co., Ltd).

Equipments

A laboratory-built high-voltage power supply automatically controlled by computer was used to supply separation voltage between 0 and 5000 V and sampling voltage between 0 and 1000 V, respectively. Amperometric experiments were performed on a CHI 650D electrochemical analyzer (Co., CHI, U.S.A.) with a home-made platinum (Pt) micro-disk working microelectrode with 250 μm diameter, a minimized Pt auxiliary electrode and a minimized Ag/AgCl reference electrode. A 40× multiple light microscope (Nanjing Optics Instruments Factory, Nanjing, China) was employed to monitor the position of the electrodes.

Scanning electron microscopic (SEM) images were obtained using a Hitachi S-4800 scanning electron microscope (Japan). Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 400 FTIR spectrometer (Madison, WI, USA). Solid state UV-vis spectra were characterized with a UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Kyoto, Japan). Ultrasonic disintegrator with a 2 mm-o.d. probe from Ningbo Scientz Biotechnology (Ningbo, China) was used to prepare the sampling fracture on the separation capillary.

Fabrication of poly(AM-EDMA)-coated capillary

A fused silica capillary was flushed with 1 mol•L^{-1} NaOH followed by water for at least 30 min each. Then the capillary was silanized by filling a mixture of *γ*-MPS and 0.06 mol•L^{-1} acetic acid (V : V=4 : 1), and the mixture was kept in the capillary for 1.5 h. The silanized capillary was then flushed with water and dried with a flow of nitrogen. The preparation conditions of polymer coated channel are shown in Table 1. The optimized mixture for prepolymerization was composed of AM (33.9 mg), EDMA (90 μL), and initiator (AIBN, 1.0 mg) in 1.5 mL of ACN and 70 μL of isooctane. After polymerization, the capillary was thoroughly flushed with water and PBS, respectively, to remove any unreacted reagent and solution. 2.5% glutaraldehyde solution was then introduced in channel for 2 h, followed with water and PBS. Glucose oxidase with a concentration of 10 mg•mL^{-1} in PBS was pumped into the channel for 0.5 h at room temperature, with an injection speed of 1 μm•min^{-1}. After washing with water and PBS, the capillary was sealed with soft plastic rubber and kept at 4 ℃ when not in use.

Fabrication of chip-based microreactor

Chip-based microreactor was assembled according to our previously reported procedure.[22,23] Briefly, a poly(dimethylsiloxane) matrix with an inner channel of 375 μm diameter, a cooling cavity and three reservoirs was obtained by pouring the mixture of Sylgard 184 silicone elastomer and curing agent (m : m=10 : 1) into a mold and then divided into two segments as the polymer retainers. The three reservoirs were sampling reservoir (SR), buffer reservoir (BR) and detection res-

ervoir (DR), respectively. One small scratch at the position of 0.8 cm from one end of the as-prepared capillary (6.0 cm length) was made before integration, the capillary was then inserted into the polymer retainer with the scratch placed in the SR center, at which the sampling fracture was formed by sonicating the small scratch in SR with a 2 mm o.d. ultrasonic probe. Consecutively, the WE was inserted in a guide channel exactly opposite to the end of the separation channel at an optimum distance of (15±5) μm. The RE and AE were inserted to DR to obtain an integrated three-electrode system for amperometric detection.

Electrochemical detection

Prior to detection, the SR, BR and DR were filled with PBS and the microchannel was washed with PBS by adopting a separation mode at a voltage of 800 V. Afterward, the fracture sampling mode was performed between the SR and BR by applying an injection voltage of 200 V for 2 s, and subsequently the separation voltage was applied between BR and DR with SR floating by automatically switching the high-voltage contacts. The results of electrophoretic analysis were recorded on a CHI 650D using the "amperometric current to time curve" mode at an applied potential. All experiments were carried out at room temperature.

Table 1 Condition optimization for preparation of poly(AM-EDMA)-coated capillary

Number	Diameter of capillary/μm	M/C[a]	Temperature/℃	Time/h	Resolution
1	50	1∶2	60	3	Ok
2	50	1∶1	60	3	Ok
3	50	2∶1	60	3	Blocked
4	50	2∶1	50	2	Blocked
5	50	3∶1	60	2	Blocked
6	25	1∶2	60	3	Dissatisfied
7	25	1∶1	60	3	Dissatisfied
8	25	2∶1	60	2	Blocked

[a] M/C: the molar ratio of monomer to cross-linker.

Results and Discussion

Optimization for preparation of microchannel

In order to increase the loading of enzyme in the inner channel, the synthesis conditions including the inner diameter of capillary, the molar ratios of functional monomer to cross-linker, polymerization temperature and time were optimized. As shown in Table 1, the polymerization in the capillary with the diameter of 25 μm was easily blocked. Thus, the capillary with a diameter of 50 μm was chosen as microchannel. Comparing capillary 1 with 2, since the high ratio of monomer to cross linker should obtain more amino group for immobilization of enzyme, the optimal molar ratio of monomer to cross linker was 1∶1 for the preparation of poly(AM-EDMA) coated microchannel.

Characterization of enzyme immobilization on microchannel wall

The SEM image of the bare fused-silica capillary displayed a slippery inner wall (Figure 1A). After the polymer was deposited on the inner wall of capillary, the morphology of the poly(AM-EDMA) coated capillary showed a rough surface (Figure 1B), significantly different from that of bare capillary, which increased the inner surface area and benefited to the immobilization of enzyme. Moreover, the resultant open tubular capillary was easily flushed with solution at a low pressure, which facilitated the elution and immobilization. After the enzyme was immobilized on the polymer coated microchannel, the size of the aggregation became large (Figure 1C), indicating the successful immobilization of GOx on the microchannel wall.

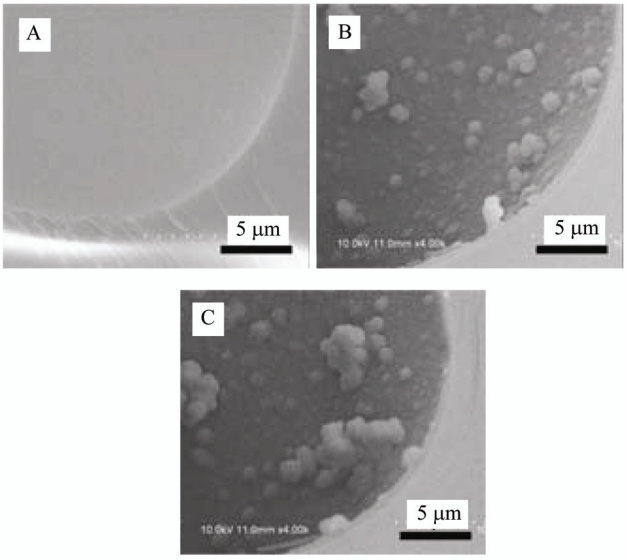


Figure 1 SEMs of (A) bare, (B) poly(AM-EDMA) immobilized and (C) poly(AM-EDMA)-glutaldehyde-GOx immobilized capillary.

To further characterize the immobilization progress, FT-IR and solid state UV spectra of the poly(AM-EDMA), poly(AM-EDMA)-glutaldehyde and poly(AM-EDMA)-glutaldahyde-GOx were compared in Figure 2. The FT-IR spectrum of poly(AM-EDMA) showed two absorption peaks at 3570 and 3440 cm^{-1} (curve a), which were corresponding to the characteristic absorption of primary amine group for N—H stretching vibration. After the glutaraldehyde was covalently linked to the polymer layer, the peak at 3570 cm^{-1} was significantly decreased (curve b). Meanwhile, the peaks at 2738 and 2853 cm^{-1} displayed the characteristic absorption of aldehyde group for =C—H stretching vibration, which validated the amidation reaction. After the GOx was immobilized, the peak at 3576 cm^{-1} reappeared and the peak at 2738 cm^{-1} disappear (curve c), proving that GOx was covalently linked to the polymer

layer.

The solid state UV spectrum showed the absorption of pure poly(AM-EDMA) at around 200 nm, in a far ultraviolet region (Figure 2B, curve a). After the glutaraldehyde was covalently linked to the polymer layer, a weak absorption peak appeared at 292 nm (Figure 2B, curve b), which was attributed to the n → π* electronic transition of aldehyde group. After the GOx was immobilized, the typical UV-visible absorption peaks of GOx were observed at 283, 371 and 452 nm (Figure 2B, curve c), proving the successful immobilization of the enzyme on the polymer film surface.

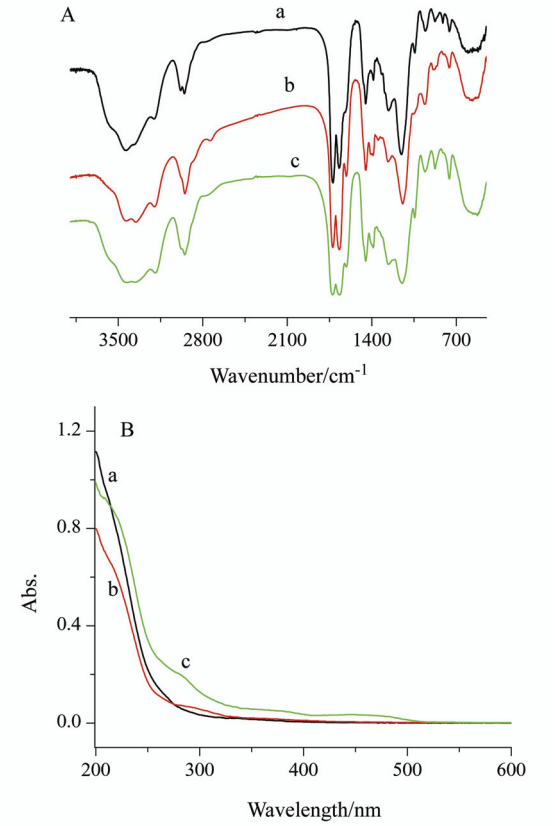


Figure 2 (A) FT-IR spectra and (B) solid state UV spectra of poly(AM-EDMA) (a), poly(AM-EDMA)-glutaldahyde (b), and poly(AM-EDMA)-glutaldahyde-GOx (c).

Optimization of detection potential and separation voltage

Due to easy miniaturization, high sensitivity, fast and convenient operation, amperometric detection was used to evaluate the performance of chip-base microreactor. In the enzyme immobilized microchannel, glucose was driven electrokinetically to the zone and reacted with GOx to form the enzymatic product of hydrogen peroxide, which was electroactive and electrochemically detectable at Pt working electrode. Figure 3 shows the current response, which dramatically increases with the detection potential from 0.5 to 0.7 V, and further slowly increases from 0.7 to 0.9 V. Considering the interference that high potential easily led to the oxidization of interfering substances at the electrode, 0.7 V was selected as detection potential.

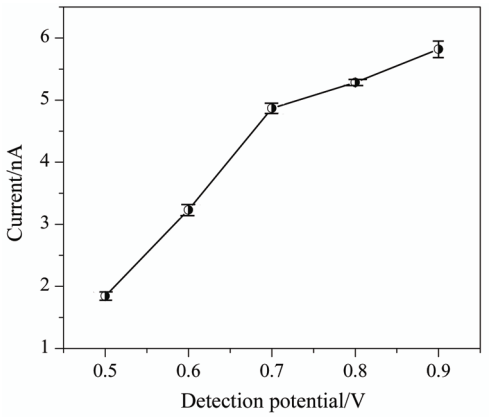


Figure 3 Effect of detection potential on peak current for 2.5 mmol•L^{-1} glucose using 20 mmol•L^{-1} pH 7.4 PBS as the running buffer at separation voltage of 1200 V (n=3).

Separation voltage greatly affected the migration time and shape of current signal. Figure 4 illustrates the effect of separation voltage on electrophoretic analysis of 2.5 mmol•L^{-1} glucose at separation voltages ranging from 600 to 1800 V on the microreactor at the detection potential of 0.7 V. With the enhancement of the separation voltage, the migration speed of enzymatic product in the channel increased, however, the peak current and area reduced, while the low separation voltage resulted in a longer migration time and serious tailing (Figures 4a and 4b). To balance the above two aspects, separation voltage at 1200 V was chosen as the optimum separation voltage and applied in the following experiments.

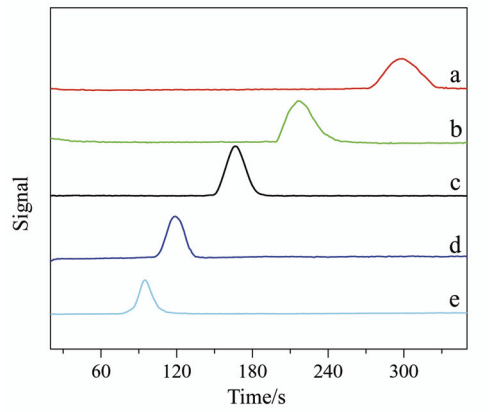


Figure 4 Effect of separation voltage on the detection of 2.5 mmol•L^{-1} glucose at (a) 600, (b) 900, (c) 1200, (d) 1500 and (e) 1800 V using 20 mmol•L^{-1} pH 7.4 PBS as the running buffer at a detection potential of 0.7 V.

Analytical performance of microreactor

Under the optimum separation voltage and detection potential, glucose solution with different concentration could be amperometrically detected on the chip. Figure 5 shows electrophoretic peak current of hydrogen peroxide as the function of glucose concentration on the

microreactor with a 6-mm length of GOx immobilized channel. The current response increased linearly with the increasing glucose concentration in the range from 0.05 to 7.5 mmol•L^{-1} with a detection limit of 23 μmol•L^{-1} at a signal-to-noise ratio of 3. The upper detection limit was better than those of 2 mmol•L^{-1} on a polycarbonate microfluidic chip by an in-channel modified biosensor,[24] 2.5 mmol•L^{-1} on integrated microfluidic chip by UV detection,[25] 0.9 mmol•L^{-1} on glass chip as homogeneous microreactor with amperometric detection,[26] 0.5 mmol•L^{-1} on enzyme-immobilized magnetic microreactor in flow injection analysis,[27] and 0.5 mmol•L^{-1} on enzymatic microreactor packed with GOx modified porous glass beads.[28]

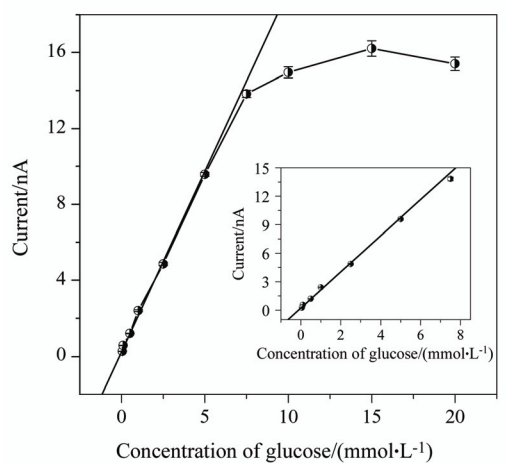


Figure 5 Linear relationship between amperometric response and glucose concentration. Conditions: 20 mmol•L^{-1} PBS as the running buffer; injection voltage: 200 V; injection time: 2 s; separation voltage: 1200 V; detection potential: 0.7 V (n=3).

The stability and reproducibility of the chip-based microreactor were investigated at 2.5 mmol•L^{-1} glucose, and when not in use, the chip-based microreactor was stored in PBS buffer at 4 ℃. The operational stability of the device was investigated by consecutively assaying a glucose solution over 60 times, and no obvious activity loss of the immobilized enzyme was observed. The inter- and intra-chip reproducibilities for determination of 2.5 mmol•L^{-1} glucose were 98.5% (n=5) and 96.0% (n=5), respectively. The acceptable performance of the proposed microreactor ensured the reliability in sample analysis.

Under the selected condition, the mixture of 3.5 mmol•L^{-1} glucose and 2 mmol•L^{-1} citric acid or 3.5 mmol•L^{-1} glucose and 2 mmol•L^{-1} uric acid was studied on the chip-based microreactor. As shown in Figure 6A, the citric acid did not show electrochemical signal on WE and a single peak was obtained for glucose. When the mixture of glucose and uric acid was injected in the channel, a peak of uric acid was observed before the glucose with completely base-line separation. This result suggested that the proposed method had good selectivity from those coexisted substances due to the good separation efficiency of the capillary electrophoresis.

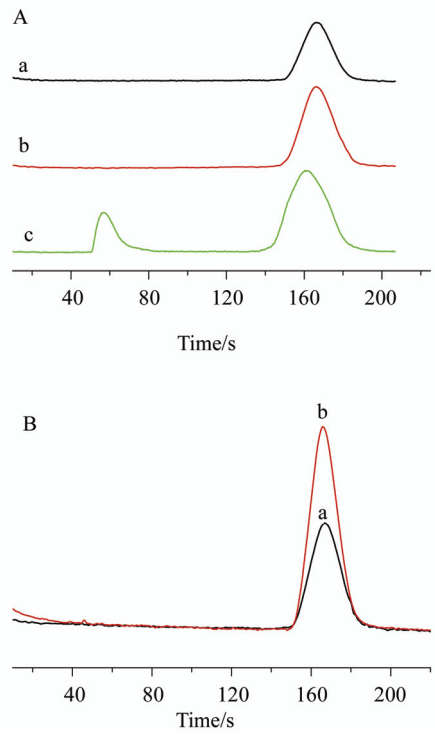


Figure 6 Electrophoregrams of (A) 2.5 mmol•L^{-1} glucose (a), a mixture of 3.5 mmol•L^{-1} glucose and 2 mmol•L^{-1} citric acid (b), and a mixture of 3.5 mmol•L^{-1} glucose and 2 mmol•L^{-1} uric acid (c), and (B) a 100-fold dilution of 5% pharmaceutical injection before (a) and after (b) spiked with 2.5 mmol•L^{-1} glucose. The conditions were the same as in Figure 5.

Application

Although ascorbic acid interferes the detection of glucose, considering the fact that glucose and ascorbic acid are not coexisting species in pharmaceutical injection, the chip-based microreactor was used to determine glucose in pharmaceutical injection for investigating its feasibility. As shown in Figure 6B, the 100-fold dilution of 5% glucose injection was found to contain 2.5 mmol•L^{-1} of glucose. After spiking with 2.5 mmol•L^{-1} of glucose, the response current increased with a recovery of over 96.4% (Table 2). These acceptable results

Table 2 Determination of glucose in 5% commercial glucose injections containing glucose and sodium chloride

Sample	Dilution	Added/(mmol•L^{-1})	Founded/(mmol•L^{-1})	Recovery/%
1	100 fold	0	2.48±0.05	96.4
		2.5	4.89±0.07	
2	100 fold	0	2.50±0.04	97.6
		2.5	4.94±0.06	

illustrated the microreactor was successfully applied in the analysis of real sample.

Conclusions

A flexible assembled hybrid chip as open tubular microreactor was designed by using enzyme modified microchannel for glucose detection. The resultant open tubular silica capillary as microchannel was used for immobilization of enzyme without extra special designed structures. Moreover, the *in situ* polymerization on the inner wall of capillary afforded the rough surface for large loading of enzyme. The proposed chip-based microreactor showed good sensitivity, rapid separation, acceptable stability and reproducibility and the need for small amount of sample, and was successfully used for quantitative determination of glucose in real sample without any sample pretreatment. Therefore, the proposed method for *in situ* modification of enzyme on the channel wall provided a promising way for constructing enzyme microreactor in practical application.

Acknowledgement

This work was funded by the National Natural Science Foundation of China (Nos. 21121091, 21135002, 21075060), and the Natural Science Foundation of Jiangsu Province (No. BK2010302).

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