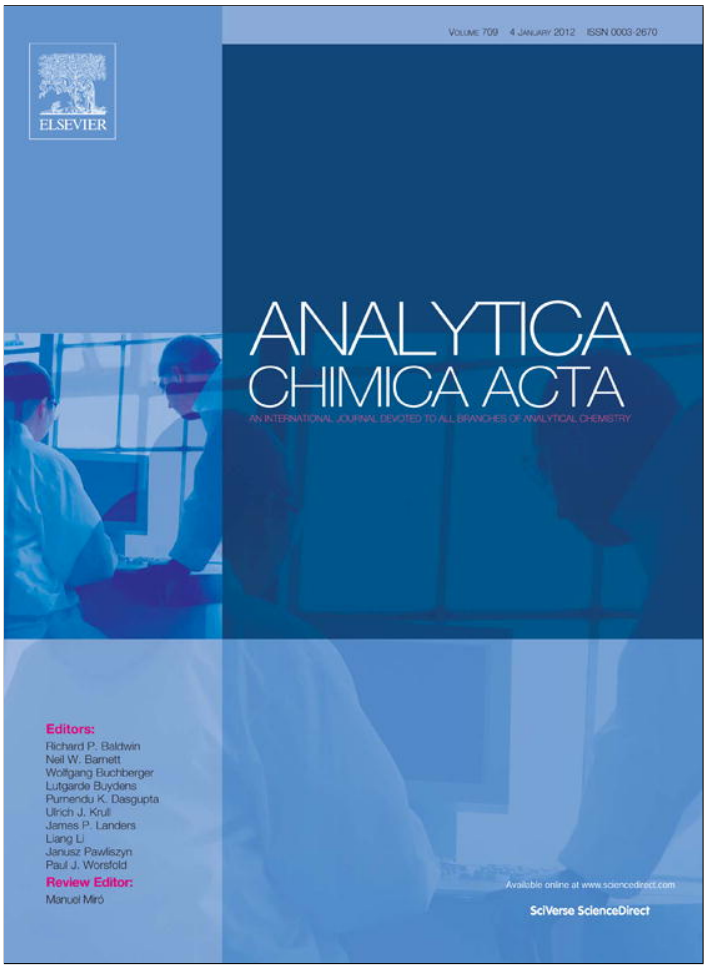

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Fabrication of tunable microreactor with enzyme modified magnetic nanoparticles for microfluidic electrochemical detection of glucose

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ABSTRACT

A microfluidic device was designed for amperometric determination of glucose by packing enzyme modified magnetic nanoparticles (MNPs) in its microchannel as an enzyme microreactor. Glucose oxidase was covalently attached to the surface of MNPs and localized in the microchannel by the help of an external magnetic field, leading to a tunable packing length. By changing the length of microreactor from 3 to 10 mm, the performance for glucose detection was optimized. The optimal linear range to glucose was from 25 μM to 15 mM with a detection limit of 11 μM at a length of 6 mm. The inter- and intra-day precisions for determination of 1.0 mM glucose were 0.8% and 1.7%, respectively, and the device-to-device reproducibility was 95.6%. The enzyme reactor remained its 81% activity after three-week storage. Due to the advantages of the device and fracture sampling technique, serum samples could be directly sampled through the fracture to achieve baseline separation from ascorbic acid, and proteins in the samples did not interfere with the detection. This work provided a promising way for pretreatment-free determination of glucose with low cost and excellent performance.

1. Introduction

Compared with typical benchtop instruments, micro-total analysis system offers a series of advantages including smaller dead volumes, shorter analysis time, higher automation, better portability, and disposability [1–3]. The micro-total analysis systems integrating with enzymatic reaction have been applied for determination of enzyme kinetic parameters [4,5], quantification of targeted substrates [6] and identification of possible inhibitors [7]. In general, the fabrication materials of enzymatic reaction channels in microfluidics were widely composed of polymers, such as polydimethylsiloxane [8] and polycarbonate [9], or glass [10] or integration of both materials [11]. Besides, silica capillary can greatly reduce the adsorption of analytes on the wall in capillary electrophoretic assays [12], which improves the separation efficiency. Thus, it remains great potential to introduce silica capillary for construction of enzyme microreaction channel of microfluidic device.

The immobilization of enzyme in microchannel is significant for good performance of enzyme microreactor [13–15]. The immobilization methods can be classified into two categories according to the immobilization location: direct immobilization on the microchannel wall [16], and immobilization on a solid support inside channel by using nanoporous scaffolds [17], microbeads [18] or magnetic nanoparticles (MNPs) [19]. Although these enzyme microreactors have exhibited the ability to quantify analytes, the immobilization capacity of the former is limited, and the preparation of the latter is time-consuming. In previous works, we proposed a microfluidic device integrated with molecularly imprinted MNPs as stationary phase for rapid enantioseparation by capillary electrochromatography [20]. MNPs not only offer large surface area, chemical and physical stability, and harmonious size [21,22], but also can be conveniently manipulated by magnets. Thus MNPs are candidates as the good enzyme support material to construct tunable enzymatic microreactor. This work used MNPs as support of enzyme molecules to prepare a tunable microreactor by packing the enzyme modified MNPs in a silica capillary with the help of external magnetic field and developed a microfluidic electrochemical method for glucose detection.

Carbohydrates are important markers for different diseases; the most common of them is glucose in diabetes [23]. Currently, analyses of glucose on enzymatic microreactor are mainly based on chaotic mixing channels with arrays of either slanted or herringbone grooves [8], on a polycarbonate microfluidic chip integrated in-channel modification of electrode [9], and a reaction chamber packed with enzyme-immobilized glass microbeads [24], and so on. Herein, we proposed a convenient and tunable enzyme microreactor for quantitative detection of glucose. By combining the advantages of microfluidic device (MD) and MNPs, fast and sensitive detection of glucose was achieved on the designed

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glucose oxidase (GO_x)–MNPs–MD. Human serum samples have successfully been determined on the GO_x–MNPs–MD without any pretreatment. Therefore, this proposed GO_x–MNPs–MD method was demonstrated to be a promising choice for glucose monitoring.

2. Experimental

2.1. Chemicals and materials

Fused-silica capillaries (360 μm o.d., 50 μm i.d.) were obtained from Yongnian Optical Fiber Factory (Hebei, China). The electrophoresis buffer for microchip electrochromatographic separation was phosphate buffer saline (PBS, pH 7.4, 25 mM). 3-Aminopropyltriethoxysilane (APTES) was purchased from Alfa Aesar (Ward Hill, MA, USA). Hydroxyl group modified superparamagnetic nanospheres (50 mg mL^{-1}) with the mean diameter of 25 nm were obtained from Kisker (Steinfurt, Germany). Glucose oxidase (GO_x, EC 1.1.3.4, type X-S, lyophilized powder, 100–250 units mg^{-1}, from *Aspergillus niger*) and *o*-dianisidine were obtained from Sigma (China). Horseradish peroxidase was obtained from Autobio Diagnostics Co., Ltd. (China). D-(+)-Glucose was bought from Sinopharm Chemical Reagent Co., Ltd. (China). Glucose stock solution was mutarotated overnight at room temperature prior to use. All solutions were kept in a freezer to prevent deterioration. All liquid samples were filtered with a 0.22 μm syringe filter to remove particulates before use. All aqueous solutions were prepared using ≥18 MΩ ultrapure water (Milli-Q, Millipore). The clinical serum samples were from Jiangsu Institute of Cancer Research, in which the concentrations of glucose have been confirmed by spectrophotometric measurements. All other chemicals were of analytical grade.

2.2. Instruments

A laboratory-built high-voltage power supply automatically controlled by computer was used to supply separation voltage and sampling voltage during experiments. Electrochemical detections were performed on a CHI 812 electrochemical station (CH Instruments Co., China). For amperometric detection, a home-made platinum (Pt) micro-disk working electrode (WE) was prepared as follows: a segment of 250 μm diameter Pt wire (Wuxi Cable Factory, Wuxi, China) was inserted into a glass capillary (400 μm o.d., 300 μm i.d.), which was then sealed with 705 glue (Nanjing Chemicals, Nanjing, China). Prior to use, the surface of WE was polished on a finer sand paper until a mirror-like appearance was observed, following with washing and sonication in a water bath. A 40 multiple light microscope (Nanjing Optics Instruments Factory, Nanjing, China) was employed to monitor the position of the WE.

A UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Kyoto, Japan) was employed for spectrophotometric determination. The packed length of GO_x–MNPs in the microchannel was observed on an inverted fluorescence microscope (Nikon Eclipse TE2000-U, Japan). Microinjection pump (Baoding Longer Precision Pump Co., Ltd., Shanghai, China) was used to operate the syringe. Ultrasonic disintegrator with a 2-mm o.d. probe from Ningbo Scientz Biotechnology (Ningbo, China) was used to prepare the sampling fracture.

2.3. Preparation and activity assay of GO_x–MNPs

The MNPs were first functionalized with APTES by adding 400 μL APTES into MNPs (5 mg) dispersed in 25 mL 95% (v/v) ethanol solution and reacted under a nitrogen environment for 12 h at room temperature. After separated from the solution and rinsed with ethanol to remove non-covalently bound APTES, the functionalized MNPs were re-suspended in a 2.5% (v/v) solution of glutaraldehyde in 3 mL PBS, and were reacted for 2 h at room temperature to activate the amine groups in APTES. Finally, the MNPs were incubated with 4 mL GO_x solution (2 mg mL^{-1} GO_x in PBS) for 1 h at room temperature with slight shaking. The prepared GO_x–MNPs were washed with PBS for 3 times and were re-suspended in 4 mL PBS, which were stored at 4 °C when not in use.

The activity of immobilized GO_x was measured by spectrophotometric determination of H_2O_2 amount gained by the catalytic oxidation of glucose. 0.5 mL glucose solution (100 mg mL^{-1}) was firstly mixed with the GO_x–MNPs in a test tube at 37 °C. 2.4 mL *o*-dianisidine solution (0.21 mM) and 0.1 mL peroxidase solution (1 mg mL^{-1} in PBS buffer) were then added to the tube. After the color change was observed, the reaction was stopped by adding 0.5 mL of 2.5 M sulfuric acid, and the UV absorbance of the solution was measured at 405 nm.

2.4. Fabrication of GO_x–MNPs–MD

The GO_x–MNPs–MD was prepared according to our previously reported procedure [20,25]. Briefly, two segments of poly(dimethylsioxane) (PDMS) matrixes with an 365 μm diameter inner channel and three reservoirs were used as the polymer retainers. The three reservoirs were buffer reservoir (BR), sampling reservoir (SR), and detection reservoir (DR), respectively. After the slurry of GO_x–MNPs was introduced into the capillary (40 mm long) using a syringe with microinjection pump, and packed in a region where the magnets were located, the packed capillary with two short magnets was then inserted into the SR and BR retainer until a small scratch made previously appeared in the area of SR. Then, DR retainer was assembled to the other side of the capillary, and the sampling fracture was obtained by sonicating the small scratch in SR [26]. Electrodes were inserted into reservoirs to achieve the sampling and separation. The sampling was performed by applying a high voltage between SR and BR with DR in floating. And during the separation process, high voltage was applied between BR and DR with SR in floating. Subsequently, the WE was placed closed to the outlet of the separation channel at an optimum distance of 15 ± 5 μm [25,27], and the position was adjusted under microscope. Besides, an Ag/AgCl reference electrode and a Pt wire as the auxiliary electrode were inserted into the DR to obtain an integrated three-electrode system for amperometric detection. The resulting GO_x–MNPs–MD is shown in Fig. 1.

2.5. Electrochemical detection

Prior to detection, the enclosed GO_x–MNPs–MD was filled with PBS and the microchannel was washed at a voltage of 1000 V. Sampling mode was performed by applying an injection voltage of 200 V between SR and BR. Then separation voltage was applied to BR with DR grounded and SR floating by automatically switching the high-voltage contacts, and the electropherogram was recorded on a CHI 812 using the "amperometric *i*–*t* curve" mode at an applied potential of +0.7 V. All experiments were performed at room temperature. In virtue of the sampling channel with a depth-to-width ratio more than 190 acting as a sieve, the entrance of macromolecules of the serum samples to the separation channel could be prohibited [26], thus serum samples were directly used for sampling without any pretreatment.

3. Results and discussion

3.1. Characterizations of GO_x–MNPs–MD

For the covalent immobilization of enzyme, MNPs were modified with APTES to provide amine groups that could serve as sites for immobilization of various enzymes. In this study, GO_x was

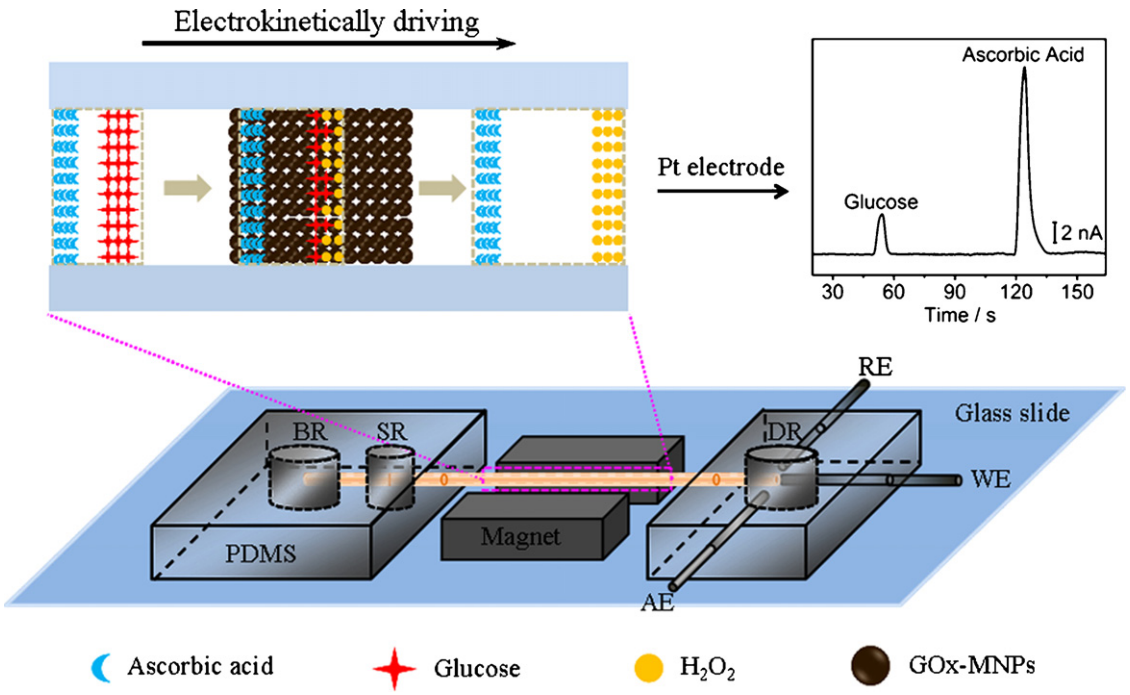

Fig. 1. Scheme representation of the construction and analytical procedure of GO_x–MNPs–MD. BR, buffer reservoir; SR, sample reservoir; DR, detection reservoir; WE, working electrode; RE, reference electrode; AE, auxiliary electrode.

covalently immobilized on the surface of the MNPs by an imine linkage formed between the aldehyde group and the primary amine on GO_x [28]. And according to the UV absorption at 405 nm by spectrophotometer in the activity assay of GO_x–MNPs, the concentration of GO_x immobilized on the MNPs was determined to be 12.2 mg enzyme per gram beads.

GO_x–MNPs were injected into the microchannel with the aid of a microinjection pump and retained by placing a magnet with tunable length adjacent to the microchannel. Compared with the microscopic image of empty capillary (Fig. 2A), a homogeneous and permeable packing of the GO_x–MNPs was obtained in the GO_x–MNPs located capillary (Fig. 2B). Besides, no adsorption of GO_x–MNPs was observed on the inner surface of the microchannel except the magnetic applied region. Moreover, the length of the GO_x–MNPs phase can be regulated by changing the magnet length, leading to different detection signal.

3.2. Detection potential and separation voltage

Amperometric detection was selected due to its excellent properties such as high sensitivity, selectivity, low energy cost, and easy to miniaturize [29]. When glucose solution was driven electrokinetically through the enzymes immobilized on the MNPs, enzymatic reaction was to occur, forming the reaction product hydrogen peroxide, which was electro-active and electrochemically detectable. Fig. 3 illustrates the effect of detection potential on current signal for 1.0 mM glucose at injection voltage of 200 V for 2 s with PBS (pH 7.4, 25 mM) as mobile phase at separation voltage of 1000 V on the GO_x–MNPs–MD. The results showed that the response current increased with the detection potential from 0.5 V to 0.7 V, then reached a pseudo-plateau for higher potentials. Since higher anodic detection potential will result in higher background current in electrophoresis-electrochemical detection, 0.7 V (vs. Ag/AgCl) was chosen for the detection in the subsequent experiments. In addition, the working electrode showed relatively good stability and reproducibility at this detection potential.

The applied separation voltage controlled the separation process and the migration time of the analytes in the microchannel by altering the electroosmotic flow. We evaluated the effect of separation voltages ranging from 800 V to 1400 V. At the separation voltages more than 1000 V, the baseline drift appeared which resulted in poor reproducibility of the analysis. While low

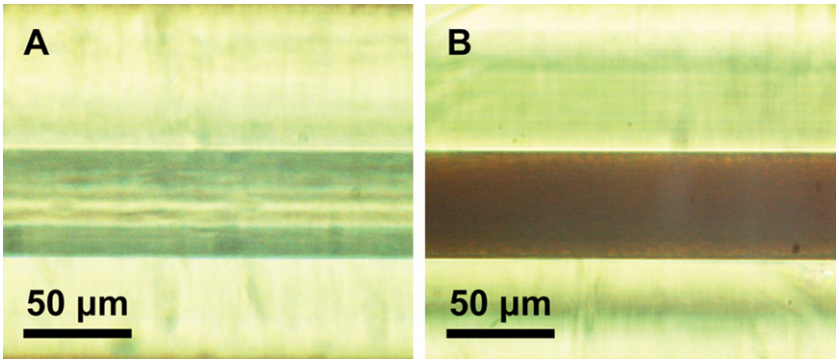

Fig. 2. Microscopic images of (A) empty capillary and (B) GO_x–MNPs located capillary.

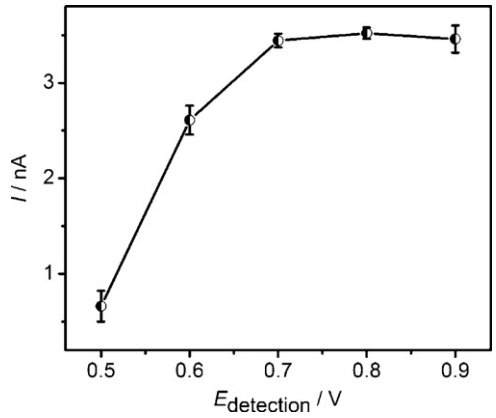

Fig. 3. Effect of detection potential on current signal for 1.0 mM glucose. Conditions: separation voltage: 1000 V; sample injection: at 200 V for 2 s; working electrode: Pt microelectrode (250 μm in diameter); electrophoresis buffer: PBS (pH 7.4, 25 mM); packing length of GO_x–MNPs: 6 mm.

separation voltage resulted in long migration time, and diffusive sample zone. Thus, an optimum separation voltage of 1000 V was selected in the experiments.

3.3. Performance of GO_x–MNPs–MD

When a sample plug of glucose electrokinetically passed through the reactor, it was enzymatically oxidized with the dissolved oxygen in the electrophoresis buffer and hydrogen peroxide was formed (Fig. 1), which can be detected at a Pt microelectrode. As shown in Fig. 4, the electropherogram of 1.0 mM glucose a sharp peak with the smooth background. The current response from 1.8 nA to 6.8 nA was much better than the data of 0.58 nA on a poly(dimethylsioxane) electrophoretic microchip with a carbon fiber electrode [30] and 0.1 nA on a poly(ethylene terephthalate)-toner microchip with a 10 μm diameter Pt electrode [16]. The high response of glucose solution on the GO_x–MNPs–MD may be due to the sufficient reaction with plenty enzyme that immobilized on the surface of the microbeads. Thus, the detection of glucose on the GO_x–MNPs–MD was highly sensitive. Besides, since this system can also be used for other oxidase system, various substrates such as cholesterol, alcohol, and glutamic acid can be detected using their counterpart oxidase enzymes.

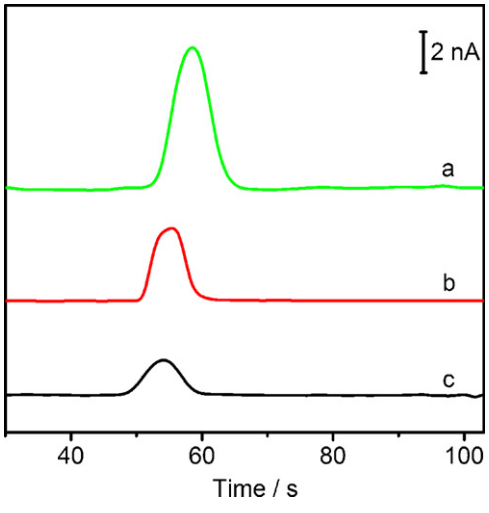

Fig. 4. Effect of packing length of GO_x–MNPs in microchannel on the detection of 1.0 mM glucose at (a) 10, (b) 6 and (c) 3 mm. Other conditions were the same.

Table 1
Properties of GO_x–MNPs–MD at three different packing lengths of GO_x–MNPs.

Packing length (mm)	Detection limit (μM)	Retention time (s)	Reaction time in microreactor (s)	Response (nA)
3	21	54	4	1.8
6	11	55	8	3.3
10	4.9	58	15	6.8

With the increasing enzyme reactor length, which was adjusted by changing the length of magnet, the electropherograms for detection of 1.0 mM glucose showed slight increase of retention time and obvious increase of current response (Fig. 4), indicating the increase of current response resulted from more enzymes contained in the enzyme reactor. Because glucose and hydrogen peroxide were neutral molecules, their migration times were relative to that of the electroosmotic flow, which only slightly changed at different lengths of microreactors. From Fig. 4, the retention times for 3 mm, 6 mm and 10 mm lengths of GO_x–MNPs packed MD were 54 s, 55 s and 58 s, respectively. This property avoided the peak broadening in long microreactors and favored the usage of MD with different packing length of GO_x–MNPs. In order to minimize the size of GO_x–MNPs–MD, the length of GO_x–MNPs packed MD was chose to be 6 mm in the next experiments.

Since the reaction time determined the amount of H_2O_2 generated during the enzyme reaction, the sensitivity of the glucose determination was not only dependent on the sensitivity of the working electrode to H_2O_2, but also on the reaction time. Considering an enzyme-modified microbeads region with the same length of sample plug as a unit part of the microreactor, the whole immobilized enzyme reactor was a sum of many such units with identical concentration of enzyme and oxygen. The reaction time (t) of glucose in the whole microreactor was dependent on the length of the whole reactor (l) and the electroosmotic velocity (ν_{eo}):

$$t = \frac{l}{n_{eo}} \quad (1)$$

where $\nu_{eo} = L/t_{eo}$. L denotes the length of the separation channel and t_{eo} denotes the migration time of the electroosmotic flow. The reaction time increased with the increasing length of the GO_x–MNPs, and the values were listed in Table 1. Thus, at a certain concentration of glucose, more H_2O_2 could be produced through a longer reactor due to the corresponding longer reaction time. As shown in Fig. 4, the signal responses for 3 mm, 6 mm and 10 mm lengths of GO_x–MNPs packed MD were 1.8 nA, 3.3 nA and 6.8 nA, respectively.

The detection limits of glucose on MD with different packing lengths of GO_x–MNPs were investigated and listed in Table 1. The detection limit of the MD decreased with the increase of the microreactor length, since the longer reactor supported more glucose to be oxidized to produce more H_2O_2, which also induced the increase of the upper detection limit.

3.4. Detection of glucose on GO_x–MNPs–MD

Fig. 5 shows the electrophoretic peak current of hydrogen peroxide as a function of the glucose concentration on a 6 mm length of GO_x–MNPs packed MD. The current response increased linearly with the glucose concentration at $C_{glucose} < 15$ mM (inset of Fig. 5). The linear response of the glucose concentration ranged from 25 μM to 15 mM ($r = 0.999$) with a detection limit of 11 μM at a signal-to-noise ratio of 3. At higher glucose concentration, the response leveled off, indicating that some glucose molecules did not react with enzyme. The upper linear range is better than those of 10 mM on a bead incorporated microfluidic device by fluorescent detection [24] and 2 mM on a polycarbonate microfluidic chip by an

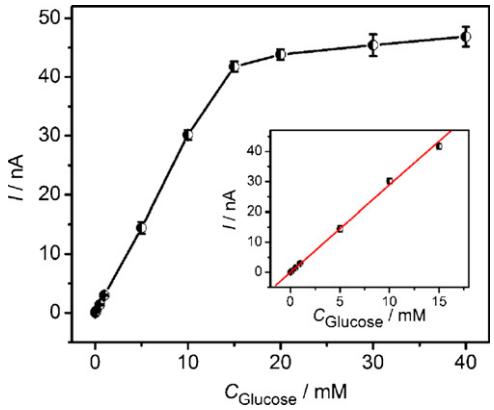

Fig. 5. The electrochemical response to glucose concentration in the enzyme microreactor. Inset: amplification signal of the linear relationship. The conditions were the same as in Fig. 4.

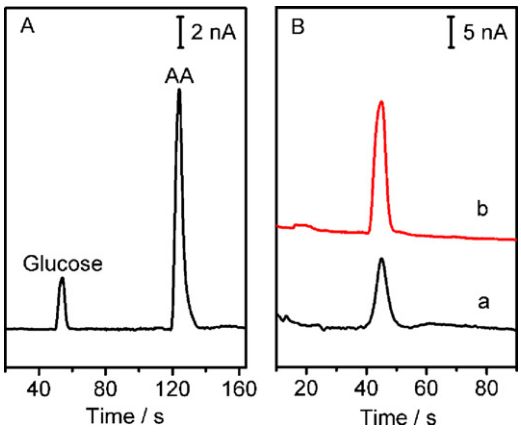

Fig. 6. Electropherograms of (A) a mixture of 1.0 mM glucose and 0.5 mM AA on the GO_x–MNPs–MD and (B) serum sample 1 before (a) and (b) after spiked with 4.0 mM glucose. The conditions were the same as in Fig. 4.

in-channel modified biosensor electrode [9]. Moreover, by changing the length of GO_x–MNPs, the sensitivity and upper detection limit of MD were reconcilable, thus could satisfy different detection requests and conditions.

3.5. Stability and reproducibility

The stability and reproducibility of the GO_x–MNPs–MD were investigated with 1.0 mM glucose, and when not in use, it was stored in PBS buffer at 4 °C. The operational stability of the device was investigated by consecutively assaying a glucose solution over 50 times and there was no obvious activity loss of the immobilized enzyme. The storage stability of the immobilized enzyme was also examined when stored in PBS at 4 °C and measured at intervals over several days. The current responses retained about 96% of its initial value after the MD was stored for a week, and 81% after stored for three weeks. This result implied that the bioactivity of the enzyme in GO_x–MNPs–MD could be perfectly retained under storage.

The relative standard deviations of the responses to 1.0 mM glucose under the same conditions were 0.8% ($n = 5$) inter-day and 1.7% ($n = 3$) intra-day. Device-to-device reproducibility was 95.6% ($n = 10$), demonstrating that consistent results could be obtained from different devices by the precise control of the number of MNPs within devices. These results indicated that the proposed method had good stability and the designed GO_x–MNPs–MD had acceptable reproducibility.

3.6. Interference experiment

Ascorbic acid (AA), as the main interferent in serum, was added to glucose solution to investigate the possible interference. The electropherogram of a mixture solution of 0.5 mM AA and 1.0 mM glucose in PBS (pH 7.4, 25 mM) was shown in Fig. 6A. It can be seen that the migration time of AA was much longer than glucose, due to its anion form in neutral or alkaline solution. Thus, AA had different electrophoretic mobilities from glucose and showed no interference from glucose detection on the GO_x–MNPs–MD. This result suggested that the proposed method had high selectivity and little interference from those endogenously coexisted electroactive substances due to the separation effect of the capillary electrophoresis.

3.7. Application in human serum

The GO_x–MNPs–MD was used to determine the glucose in human serum to investigate its feasibility for biological sample analysis. The analysis was carried out without any requirement of sample pretreatment except a standard addition step by adding glucose into the sample. As seen from Fig. 6B, the serum sample showed stable baseline without other interference peaks, indicating that the microparticles, macromolecules such as proteins and fat in the serum did not interfere with the detection. After spiking the standard solutions of glucose into the sample, electropherogram showed the enhanced current response at unchangeable retention time. From the current response, the value of recovery and the calibration curve, the concentration of glucose in the sample 1 was measured to be 4.62 mM, and the concentration of the spiked 4.0 mM glucose was measured to be 98.4% of the spiked concentration, demonstrating good accuracy for the determination of glucose in real samples. The concentrations of glucose in four serum samples were determined to be consistent with those obtained by spectrophotometric measurements (Table 2). Thus, the designed GO_x–MNPs–MD and the proposed method have good performance in the detection of glucose in human serum without any sample pretreatment.

Table 2
Glucose concentrations in human serum samples tested by the proposed method ($n = 3$) and spectrophotometric measurements.

Serum sample	Proposed method concentration (mM)	Spectrophotometric measurements concentration (mM)
Sample 1	4.62 ± 0.05	4.75
Sample 2	5.35 ± 0.03	5.16
Sample 3	6.64 ± 0.07	6.25
Sample 4	5.48 ± 0.05	5.62

4. Conclusions

An enzyme microfluidic reactor was designed using enzyme modified magnetic nanoparticles as stationary phase for glucose detection. The prepared GO_x–MNPs could be conveniently localized to the pre-nominated position in the separation channel of silica capillary by applying an external magnetic field. The silica capillary channel can greatly reduce the adsorption of analytes to achieve high accurate detection. Moreover, the length of the immobilized enzyme reactor could be controlled conveniently by changing the magnet length. The GO_x–MNPs–MD showed good anti-interference ability, stability and reproducibility, and was successfully used for quantitative determination of glucose in human serum without any sample pretreatment. Thus, this newly developed low-cost enzyme microreactor system is convenient and tunable, and offers a promising way for practical application.

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