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Determination of serum glucose using flow injection analysis and highly selective glucose sensor based on composite films

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ABSTRACT

A novel flow injection analysis (FIA) system suitable for measurements of glucose in blood serum is developed. In the proposed FIA system, a new kind of glucose sensor based on composite polymer films and well-immobilized enzyme was fabricated. An electrochemical technique of scanning electrochemical microscopy (SECM), and electrochemical impedance spectroscopy (EIS) were used for the characterization of the newly fabricated biosensor. A wide linear range of 0.1–50 mM for glucose detection was reported in virtue of the new configuration of the sensor and the developed FIA system. The reproducibility of signals was quite good with relative standard deviation (RSD) values for $n = 4$ injections (typically 5.7%). Animal blood serum was directly injected and assayed in this simulative physiological system. Good analytical recovery of glucose spiked into serum samples, with recoveries in the range of 96.7–105.0%, was exhibited. Under optimized conditions, detection of serum glucose for normal people and diabetics using our proposed method is possible.

1. Introduction

Diabetes mellitus is a group of dysmetabolic syndrome resulted from genetic cause, metabolic diseases, autoimmune disorders, microorganism infection, toxins, etc. Diabetic emergency such as hyperglycemia and hypoglycemia needs to be avoided, and it is also quite necessary to confirm the effectiveness of clinical treatment. Therefore, testing of physiological glucose levels for normal people and diabetics is critical. Glucose sensors, as one of the most popular electrochemical biosensors, have been extensively developed and applied in clinical testing of diabetes, biological and chemical analysis and food industry [1–3].

FIA technique was first introduced in 1975, presenting quantitative information of samples reproducibly in flowing streams, and has been widely applied in many fields including biochemistry, environmental science and medical science. A certain volume of samples was injected into a reagent stream continuously flowing through a tube, forming a sample area, and further mixed with the reagent(s) in streams, and finally reached the detector that typically quantitates the product of the chemical reaction. Meyerhoff et al. have reported the measurement of *S*-nitrosothiols (RSNOs) in animal blood plasma using FIA method, in which a differential experiment step and an amperometric nitric oxide (NO) sensor were applied [4]. Kolev et al. successfully developed a novel FIA system that used a polymer inclusion membrane (PIM) for the on-line extractive separation and determination of Zn(II) in the presence of a range of other metal ions [5]. The FIA method has been used by some groups for glucose measurements [6,7] as well, however, problems such as poor ability of anti-interference and narrow linear range are difficult to resolve, producing complicated process. In the present work, a kind of glucose sensor with new configuration was fabricated and further applied as the detector in the FIA system, resulting in good ability of anti-interference and wide linear range for sample detection, and the process for glucose measurements was very simple. Animal serum samples containing glucose were directly injected into the system and further carried by the flowing streams to the position of detector where samples reacted with oxygen and enzyme. Consequently, hydrogen peroxide generated by the enzymatic reaction would be detected and measured by the biosensor in proportion to the amount of glucose in samples [8]. A typical recording of output has the form of a current peak, and the height of the peak is related to the concentration of the analyte according to a standard calibration curve obtained with glucose standards prepared [9].

Polymer films have been widely employed in biosensors to increase permselectivity, to prevent the electrode surface from fouling, to entrap or immobilize a mediator, to extend the linear range of biosensors, and to improve the biocompatibility of biosensors [10–12]. Polymer films, categorized as conducting, nonconducting and composite, are mostly fabricated using solvent

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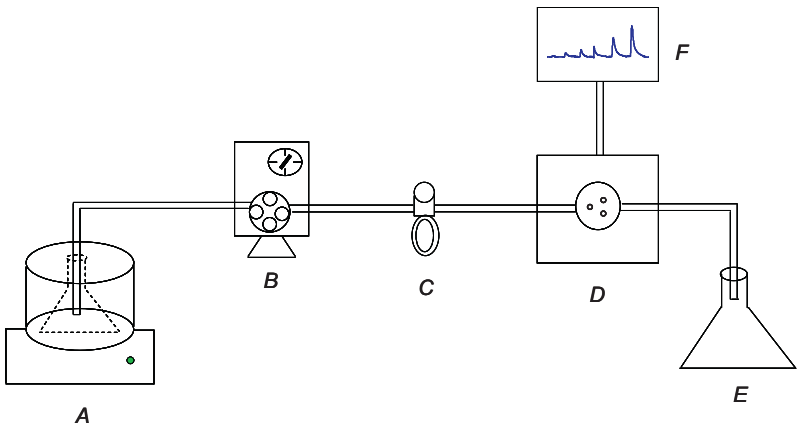

Fig. 1. Schematic diagram of flow injection analysis system. (A) Temperature controlled carrier stream (PBS buffer); (B) peristaltic pump; (C) six-port rotary injection valve; (D) temperature controlled flow-through cell equipped with detector (amperometric glucose sensor); (E) waste; (F) recorder.

casting, electropolymerization, and adsorption. Belanger et al. electropolymerized a conducting glucose oxidase/polypyrrole film onto the Pt disk of a rotating ring-disk electrode to fabricate a glucose sensor [13]. Yacynych et al. evaluated the effectiveness of various electropolymerized films and reported that poly(1,3-phenylenediamine/resorcinol) film covered electrode was not fouled by serum solution. In that work, glucose oxidase (GOD) was immobilized onto the electrode surface by crosslinking with glutaraldehyde followed by an electropolymerized film, and the resulted sensor had a linear response (2.5–10 mM) using FIA method [14]. In the present work, we simultaneously immobilized GOD into the interspace of poly(1,3-phenylenediamine/resorcinol) molecules during electropolymerization followed by modification of Nafion perfluorinated ion exchange resin (5 wt.% solution in lower aliphatic alcohols/H_2O mix containing 45% water) film. A wider linear range of 0.1–50 mM for serum glucose detection was achieved in FIA system, and such configuration of glucose sensor and so wide linear range for detection have rarely been reported by now.

2. Experimental

2.1. Materials and reagents

Glass carbon working electrode (1.5 mm in radius), platinum wire counter electrode (0.5 mm in radius) and Ag/AgCl reference electrode were purchased from CH Instrument (Chenhua Instrument Co., Ltd., Shanghai, China). All potentials were reported against the Ag/AgCl reference electrode.

All chemicals were of analytical grade or better and used as received without further purification. D-(+)-Glucose and glucose oxidase (GOD) were obtained from Sigma–Aldrich. Potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA), 1,3-phenylenediamine (m-PD) and resorcinol were purchased from a chemical supplier (J&K Scientific Co., Ltd., Beijing, China). Alumina polishing powder (1.0 μm, 0.3 μm, 0.05 μm) was obtained from CH Instrument (Chenhua Instrument Co., Ltd., Shanghai, China). Potassium chloroplatinate was obtained from a commercial chemical supplier (Boyuan Chemicals Co., Ltd., Jinan, China). Nafion perfluorinated ion exchange resin was purchased from Dupont Company (DE, USA). 0.01 M phosphate buffer solution (PBS) was prepared freshly from appropriate reagents. All aqueous solutions were prepared with 18.2 MΩ cm ultrapure water using a Milli-Q filter (Research UV, Hetai Instrument Co., Ltd., Shanghai, China).

2.2. Apparatus and method

Electrochemical experiments were carried out using a developed FIA system, the diagram of which is shown in Fig. 1. A peristaltic pump (BT01-Y21515, Tianjin Xieda electron. Co., Ltd., Tianjin, China) was applied to pump the carrier stream (PBS), and the thermostat (85-2, Siyue Instrument, Co., Shanghai, China) was used to keep the required temperature. The sample loop and polyetheretherketone (PEEK) tubing used for carrier stream were purchased from a commercial supplier (RUSH Science & Technology Co., Ltd., Hangzhou, China). All current measurements from the glucose sensor were performed using a potentiostat (CHI 920C, Chenhua Instrument Co., Ltd., Shanghai, China). Samples were injected into the carrier stream through the six-port rotary valve (Rhenodyne 7725i) and loaded into the fitted 200 μL sample loop. Immediately, samples would be carried to the thermostatic flow-through cell equipped with detector in the oxygen atmosphere, and glucose contained in samples would be reacted to generate hydrogen peroxide as a product. Glucose sensor, as the detector, would respond to this hydrogen peroxide, and a current peak related to glucose concentration was displayed by the recorder. Calibration curves were obtained by injecting freshly prepared standard glucose solutions into the FIA system. All samples and all components of the FIA system including the carrier stream, tubing and electrochemical cell were prevented from light with aluminum foil. Furthermore, EDTA was added to blood samples as an anticoagulant. Construction procedure of polymer films was analysed by means of the technique of scanning electrochemical microscopy (SECM, Chenhua Instrument Co., Ltd., Shanghai, China) and electrochemical impedance spectroscopy (EIS, CHI 920C, Chenhua Instrument Co., Ltd., Shanghai, China).

2.3. Fabrication of glucose sensors

The glass carbon working electrode was mechanically polished with successively finer grades of deagglomerated alumina slurries down to 0.05 μm in particle size. An ultrasonic cleaner was used to remove residual alumina loosely bound to the electrode surface. The polishing procedure was repeated until the working electrode was electrochemically clean for use. The ensuing working electrode was platinized in a saturated potassium hexachloroplatinate solution by cycling the potential from +0.70 to −0.35 V (vs. Ag/AgCl) at a scan rate of 20 mV/s for 8 min using the potentiostat. Poly(m-PD/resorcinol) films were electrochemically grown from a fresh solution containing 1.5 mM m-PD and 1.5 mM resorcinol

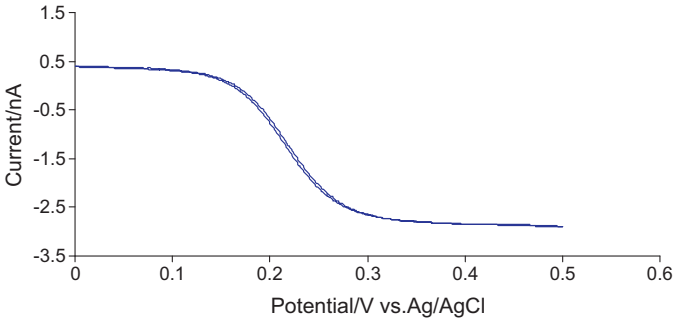

Fig. 2. Cyclic voltammogram of ferrocenemethanol in 0.1 M KCl solution.

monomers dissolved in deoxygenated buffer. Enzyme immobilization was performed by adding 700 U/mL of GOD to the monomers solution prior to electropolymerization. Films were grown gradually by cycling the potential from 0.00 to +0.90 V (vs. Ag/AgCl) at a scan rate of 2 mV/s for 18–21 h. During electropolymerization, the solution was protected from light and bubbled with nitrogen all the time. After electropolymerization, the working electrode was further modified with a thin layer of Nafion via dip-coating and then dried in the nitrogen atmosphere for 10 min. The fabricated biosensor was stored in the PBS at 4 °C.

3. Results and discussion

3.1. Analysis of construction procedure of polymer

Scanning electrochemical microscopy (SECM) is a kind of novel technique for in situ electrochemical study. It was developed by

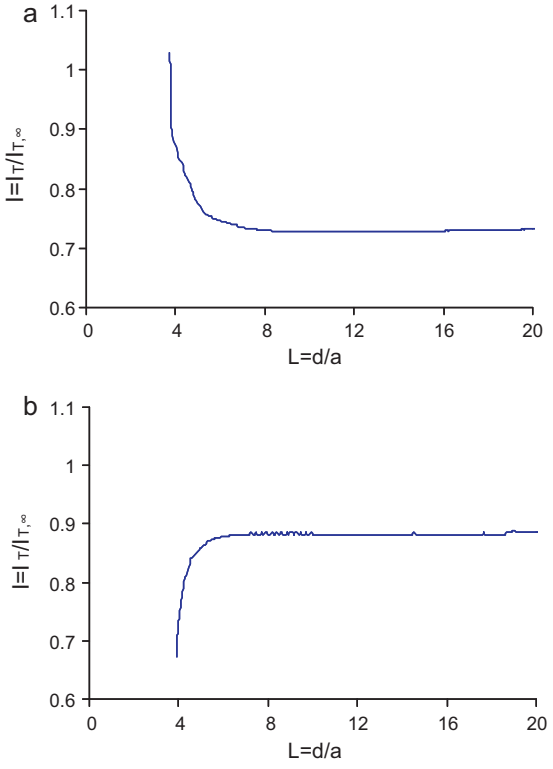

Fig. 3. Experimental approach curves on platinized glass carbon electrode as substrate (a) and on substrate of platinized glass carbon electrode modified with polymers (b). *I*, normalized tip current; I_T, current at the tip electrode; $I_{T,\infty}$, diffusion-limiting steady-state current at the tip electrode; *L*, normalized distance between tip and substrate; *d*, distance between tip and substrate; *a*, radius of the tip electrode.

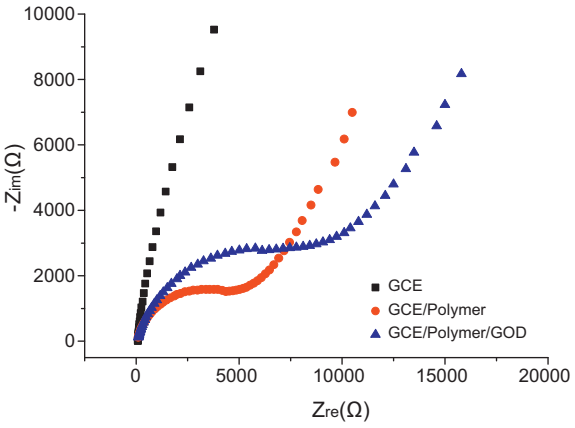

Fig. 4. EIS for the growth of poly(m-PD/resorcinol) polymers with and without GOD on glass carbon electrode (GCE) in PBS with 10 mM $Fe(CN)_6^{4-/3-}$. Signal amplitude 5 mV; frequency range 60 kHz–1 Hz.

Allen J. Bard group based on scanning tunneling microscopy (STM) and the ultramicroelectrode (UME), and was suggested to give chemical information of systems investigated [15,16]. SECM can operate in many working modes, and the widely used one is positive/negative feedback mode [17]. Chemical information such as conductive or nonconductive of substrate can be obtained by means of the tip current. As shown in Fig. 2, the cyclic voltammogram of ferrocenemethanol presents typical steady-state curves, indicating that the tip working electrode is ideal for use. Fig. 3 exhibits the experimental results when platinized glass carbon electrode and platinized glass carbon electrode modified with polymers were applied as the substrate respectively. As shown in Fig. 3a, the current on tip electrode increased when the tip approached to platinized glass carbon electrode substrate, demonstrating that the substrate is conductive. This is in agreement with the positive feedback working mode of SECM. However, as illustrated in Fig. 3b, the current decreased during the same approaching process when the platinized glass carbon electrode modified with polymers was applied as the substrate. This is in agreement with the negative feedback working mode of SECM, and it demonstrates that the nonconductive composite polymer films have been modified onto the surface of conductive platinized glass carbon electrode.

Electrochemical impedance spectroscopy (EIS) is frequently used in the characterization of electrode surface during the procedure of construction [18,19]. The semicircle diameter in the impedance spectrum equates to the charge-transfer resistance, R_{ct}, at the electrode surface [20]. As shown in Fig. 4, the impedance spectrum of bare glass carbon electrode presents an approximate straight line, demonstrating that electron transfer to the surface of glass carbon electrode is a diffusion-control step. After electropolymerization, the charge-transfer resistance increased to approximate 7000 Ω. It is suggested that the nonconductive poly(m-PD/resorcinol) films have been modified onto the surface of the electrode, and that this kind of composite polymer films do not facilitate electron transfer. This result is consistent with the analysis performed by SECM. This nonconductive polymer was applied onto biosensors to prevent interferences, to prevent fouling of the electrode surface by proteins and other substances and to entrap biocomponent [13]. The charge-transfer resistance further increased to about 12,000 Ω when GOD was in the presence of monomers solution during electropolymerization, which manifests that GOD had been well immobilized into the composite poly (m-PD/resorcinol) films.

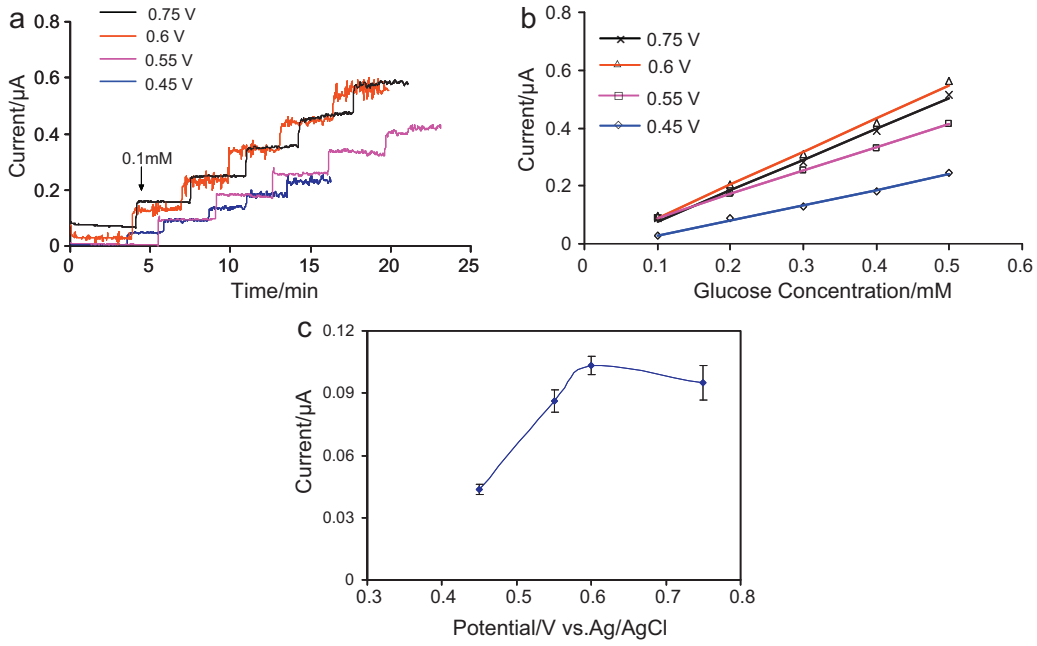

Fig. 5. The amperometric response to consecutively added 0.1 mM glucose (a), the corresponding calibration curves (b), and amperometric current (with RSD for $n = 5$ injections) of the biosensor varies with the applied potential (c).

3.2. Optimum applied potential for glucose measurements

In order to obtain better performance in terms of sensitivity to samples, optimum applied potential has to be determined prior to FIA experiments. Amperometric response to successively added standard glucose at different applied potential and the corresponding calibration curves are shown in Fig. 5. Response to standard glucose became higher when applied potential increased from 0.45 V to 0.6 V (vs. Ag/AgCl). However, response to samples decreased when the applied potential was further increased to 0.75 V, indicating that the optimal potential for glucose detection is 0.6 V. Herein, glucose measurements using FIA system were performed at the potential of 0.6 V.

3.3. Optimum pH for glucose measurements

Affinity of GOD to substrate electrode and stability of enzyme in immobilized state can be affected by the pH value of the reacting solution [21]. Herein, the effect of pH value on biosensor performance was investigated by measuring the current response to

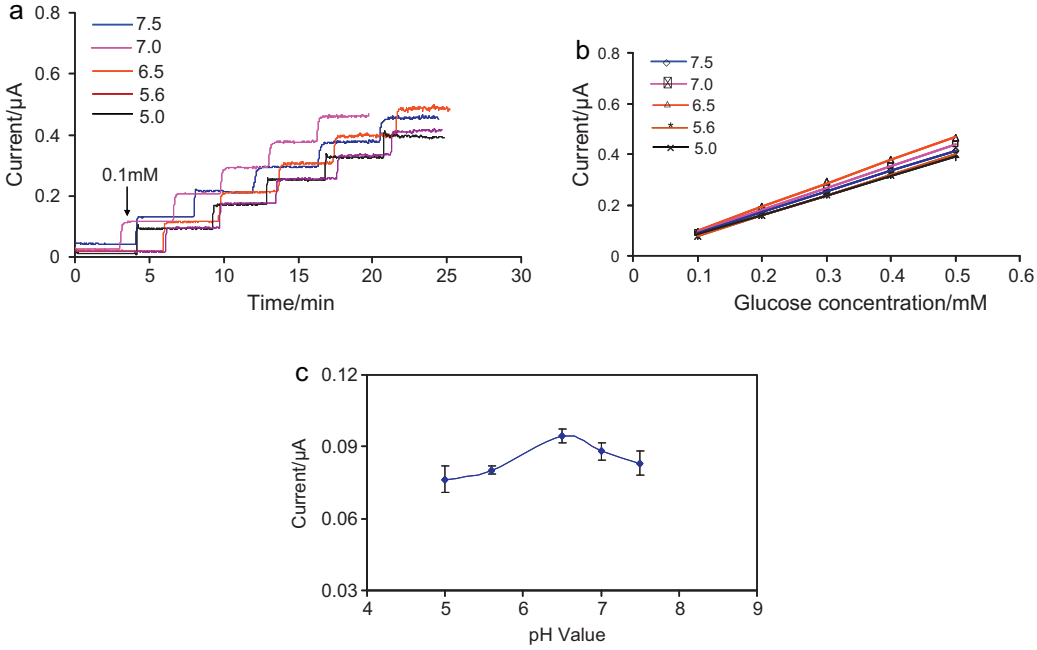

Fig. 6. The amperometric response to consecutively added 0.1 mM glucose (a), the corresponding calibration curves (b), and amperometric current (with RSD for $n = 5$ injections) of the biosensor varies with the pH value (c).

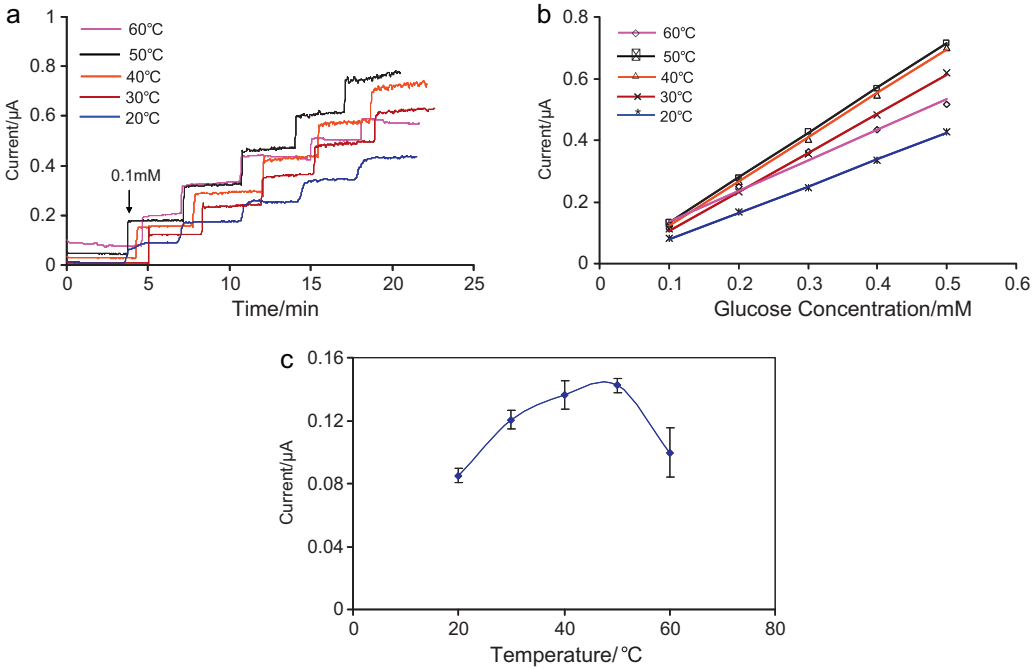

Fig. 7. The amperometric response to consecutively added 0.1 mM glucose (a), the corresponding calibration curves (b), and amperometric current (with RSD for $n = 5$ injections) of the biosensor varies with the temperature(c).

consecutively added 0.1 mM glucose at optimum potential. As can be seen in Fig. 6, the biosensor presents the optimal response to samples at pH 6.5, indicating that pH 6.5 is the optimum acidic condition for enzyme activity. However, in this work, the buffer solution was adjusted to pH 7.3 in consideration of the physiological conditions.

3.4. Optimum temperature for glucose measurements

Enzyme stability and activity are susceptible to thermal conditions. In case of thermal denaturation, the effect of varying temperature on the biosensor was examined by measuring the response to successively added 0.1 mM glucose between 20 °C and 60 °C. As illustrated in Fig. 7, the biosensor response to glucose gradually increased with increasing temperature and reached its maximum value at 50 °C. This is because enzyme activity increases at higher temperature. However, response to glucose decreased when temperature further increased to 60 °C. It is likely that natural thermal degradation of enzyme occurred at much higher temperature. As a result, the optimum temperature for GOD is 50 °C corresponding to a series of temperature values. During measurements of samples, obtaining good response to glucose and minimizing possible experimental errors such as solution evaporation have to be considered. In addition, it is better to keep physiological conditions when performing experiments. Therefore, 37 °C was chosen as the working temperature as a compromise in this study.

3.5. Response to glucose using flow injection analysis system

As shown in Fig. 8a, the amperometric detector yielded a fast response to glucose and the response current returned to the baseline within 5 min. A linear range of 0.1–50 mM using FIA system is presented in Fig. 8b. As shown in Fig. 9, the reproducibility of the signals is quite good, with RSD values for $n = 4$ injections typically 5.7%. It is not difficult to find out that this kind of glucose sensor applied in FIA system possesses quite good accuracy, reproducibility and wider linear range for glucose detection.

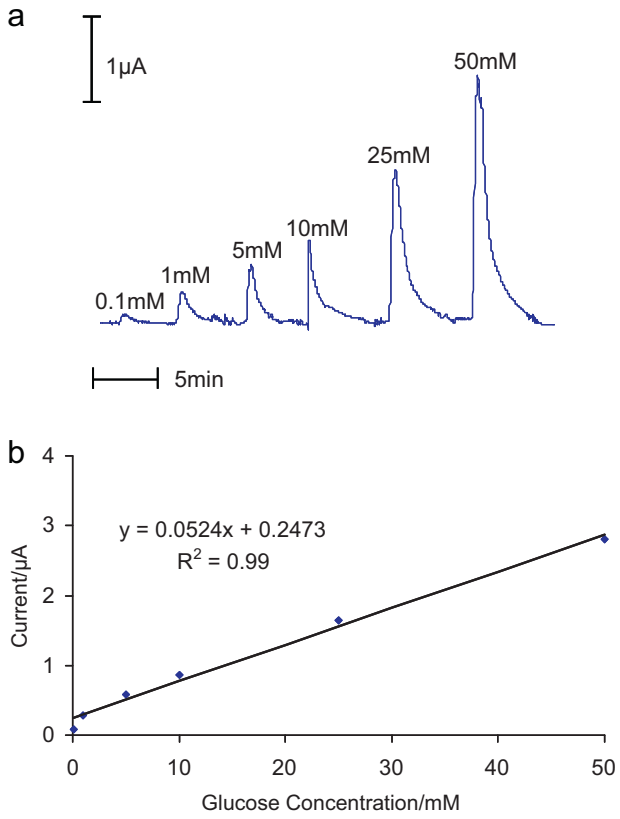

Fig. 8. Response to standard glucose and the corresponding calibration curve. Flow rate, 2 mL/min.

3.6. The ability of anti-interference using FIA system

It is well known that some electroactive species in serum, such as ascorbic acid (AA), L-cysteine (L-Cys) and urea may influence the performance of a biosensor [8]. Therefore, the anti-interference

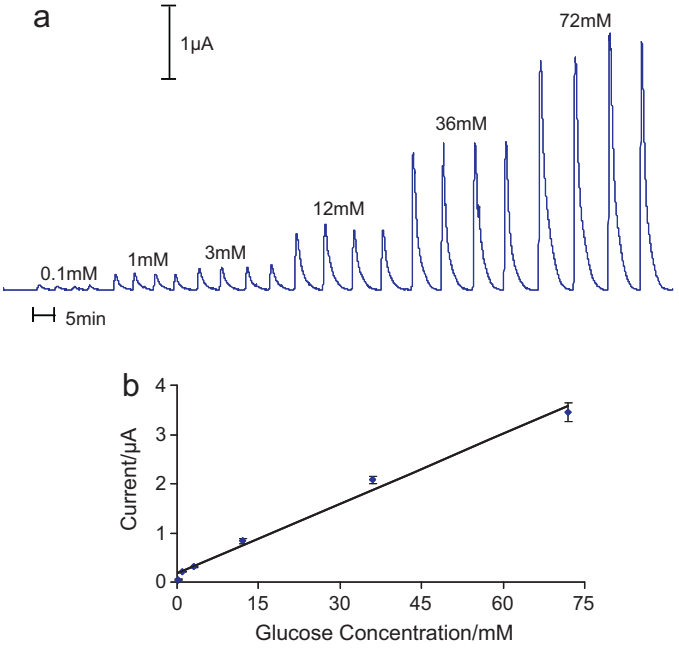

Fig. 9. Response to different glucose standard solutions (a) and calibration curve using FIA system (b). Flow rate, 2 mL/min. Data are represented as means ± SD (n = 4).

ability of the fabricated biosensor applied in FIA system was investigated. In this work, 5 mM urea (more than physiological levels), 0.1 mM L-Cys (more than physiological levels) and 0.1 mM AA (around physiological levels) were injected into the FIA system consecutively, followed by injection of various concentrations of glucose as a comparison. As can be seen from Fig. 10, these interferences did not have any obvious effect on the biosensor compared with successively injected standard glucose samples using the FIA method. Such good ability of anti-interference can be attributed to two factors. On the one hand, interfering species are likely blocked by the composite polymer films and Nafion film modified on the biosensor surface. On the other hand, different from electrochemical reaction of hydrogen peroxide, interferences can probably not be oxidized at the low applied potential, and this is the catalytic function of the platinized platinum. These results indicate that the developed glucose biosensor and the proposed FIA method exhibit good quality in anti-interference, and hence the biosensor applied in FIA system can be used for blood serum measurements.

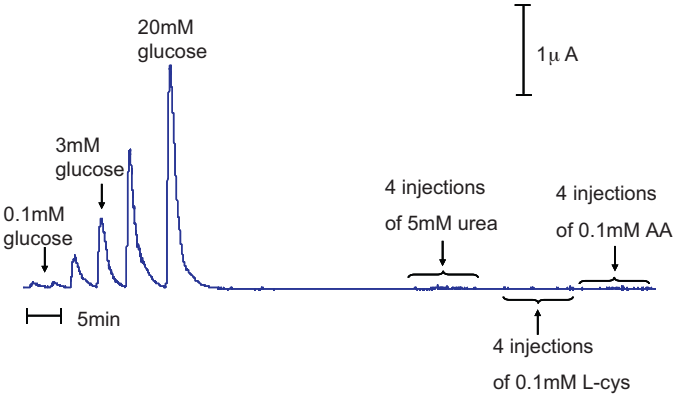

Fig. 10. Response to standard glucose solutions and interferences using FIA system. Flow rate, 2 mL/min.

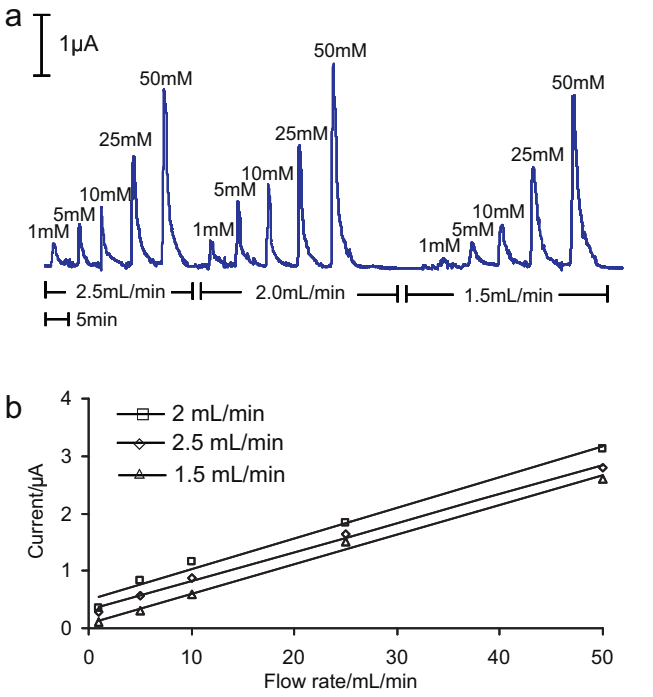

Fig. 11. Effect of the flow rate of carrying buffer on the response to different concentrations of glucose.

3.7. Optimum flow rate for glucose measurements using FIA system

Biosensor performance would be influenced by the flow rate when glucose sensor was applied in FIA system. The key factors considered during method development were the rate of enzymatic reaction and the sensor's response time toward hydrogen peroxide. The optimum flow rate should not only be slow enough to allow complete oxidation of the glucose by the enzyme but also fast enough to detect hydrogen peroxide within the response time of sensor. Three different flow rates were chosen and examined considering of not causing pressure problems and not producing noisy amperometric signals. As clearly shown in Fig. 11, increasing the flow rate from 1.5 mL/min to 2 mL/min, the sensitivity of the system increased significantly. This effect is likely due to the reduced dispersion and hence dilution of the glucose sample plug within the FIA system when the flow rate is greater [4]. As a result, lower amounts of glucose can be detected at the higher flow rates. However, when further increasing the flow rate to 2.5 mL/min, the sensitivity of the system decreased. It is likely that carrier stream containing samples passed by the detector at such high flow rate and there is not adequate time for enzyme to completely oxidize glucose. As a result, 2.0 mL/min was determined to be the optimum flow rate and was used for sample measurements.

3.8. Detection of glucose in blood serum

Fresh blood serum samples (from rabbits) were obtained from School of Life Science and Technology at Beijing Institute of Technology and centrifuged (2000 rpm) for 15 min. The supernatant (serum) was carefully aspirated at room temperature and pooled into a tube for assay using the FIA system. The levels of glucose in the serum samples as determined from calibration of the FIA system were 4.8 ± 0.09 mM (Table 1) which are within the normal range of serum glucose of rabbits. The accuracy of this FIA method was evaluated by performing recovery experiments with serum

Table 1
Summary of glucose concentration in animal serum using the FIA system.

Injections	Glucose concentration (mM)
1	4.7
2	4.8
3	4.9
Average	4.8 ± 0.09

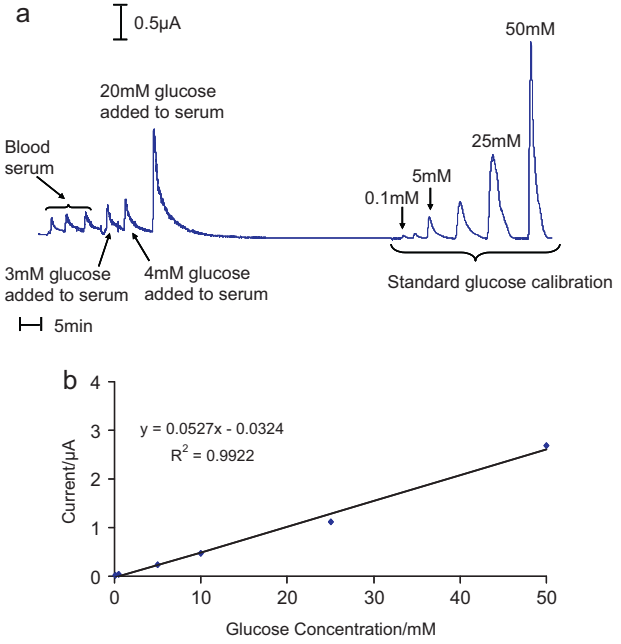

Fig. 12. Typical response to serum, standard glucose and serum that was spiked with varying amounts of standard glucose (a), and the corresponding standard glucose calibration curve (b). Flow rate, 2.0 mL/min; temperature, 37 °C; pH 7.3; applied potential, 0.6 V (vs. Ag/AgCl).

Table 2
Summary of glucose recovery experiments in animal serum.

Amount of glucose		% Recovery
Added (mM)	Found (mM)	
3	2.9	96.7%
4	3.9	97.5%
20	21	105.0%

samples that were spiked with varying amounts of standard glucose, the result of which is shown in Fig. 12 and Table 2. Note that in Fig. 12, when the spiked samples were injected into the FIA system, the peak height increased in proportion to the amount of glucose added to the sample. Based on these recovery data, it is evident that the method can accurately quantitate the levels of glucose added to the serum samples.

4. Conclusion

In summary, we have described a relatively simple FIA system to detect serum glucose in a wide linear range of 0.1–50 mM using a fabricated glucose sensor that was modified with composite films to prevent interferences and to entrap enzyme. Animal blood serum was directly injected and assayed in the FIA system. Good reproducibility and analytical recovery were achieved using the proposed method. The main advantages of this new FIA system are the wide linear range for detection and the potential to have a high throughput assay system, which can be referred as the simulation of the flowing physiological system when compared to the measurement of glucose in blood serum or whole blood using the previously reported method [22,23]. In addition, electrochemical arrangement used here for FIA measurements of glucose could also be employed as a simplified HPLC method [24], and could be applied for detection of serum glucose for normal people and diabetics under optimized conditions.

Acknowledgements

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