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A novel stable amperometric glucose biosensor based on the adsorption of glucose oxidase on poly(methyl methacrylate)–bovine serum albumin core–shell nanoparticles

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ABSTRACT

A novel amperometric glucose sensor was constructed by immobilizing glucose oxidase (GOx) on poly(methyl methacrylate)–bovine serum albumin (PMMA–BSA) core–shell nanoparticles. The large surface area of these nanoparticles resulted in a high enzyme loading. The BSA protein shell provided biocompatible microenvironment to retain the native structure and bioactivity of the immobilized enzymes. Such biosensors were characterized by both cyclic voltammetry and chronoamperometry. The effects of the working potential, the amount of the immobilized enzymes and the operating pH on the response of the glucose sensor were investigated. Under an optimal preparation condition, the resultant biosensor had a short response time of less than 8 s, a sensitivity of 44.1 $\mu A\,mM^{-1}\,cm^{-2}$, and a wide linear range from 0.2 to 9.1 mM with a correlation coefficient of 0.997. More important, such biosensors were thermally stable at room temperature (~25 °C); which means there was nearly no change in their performance even after they were operated in pH 7.4 phosphate buffer solution for 23 days. The biosensor can be used directly to determine glucose in serum sample. The results show that PMMA–BSA nanoparticles provide a promising material for fabricating enzyme based amperometric biosensors.

1. Introduction

Diabetes mellitus is a worldwide public health problem, resulting from insulin deficiency and hyperglycemia, which could be reflected by blood glucose concentrations higher or lower than the normal range of 4.4–6.6 mM [1]. The diagnosis and management of diabetes mellitus thus requires a tight and precise monitoring of the blood glucose level. Amperometric enzyme electrodes with a high sensitivity and selectivity, based on glucose oxidase (GOx), are widely used for the detection of the blood glucose concentration [2–5].

Since the first glucose biosensor was reported by Clark and Lyons [6], many researchers have focused on the improvement of performance and long-term stability of enzyme electrodes [7–11]. Among numerous reports about glucose biosensors, the enzyme immobilization on electrodes is the first important step in fabrication, which also plays a vital role in the biosensor performance. Different immobilization methods have been tried, including covalent attachment [12–14], physical entrapment or encapsulation and adsorption [15–19]. Each method has its own advantages and limitations. Covalent attachment often involves some complicated synthesis under some harsh experimental conditions, leading to a significant loss of enzymatic activity. Entrapment or encapsulation inside a solid matrix usually results in a low enzymatic reaction rate because substrates have to diffuse into the matrix to interact with those entrapped enzymes. In contrast, adsorption is a much simpler way to immobilize enzymes and maintain their activity under mild experimental conditions. However, the stability of adsorption is a problem, affected by a combination of factors, such as pH, ionic strength, temperature, surface tension, charges, and matrix.

Miniaturization of enzyme electrodes, operating in a small volume, has attracted more attention in recent years [20–22]. Such kind of electrodes enhances the signal-to-noise ratio and lowers the sample volume. In order to develop better miniature biosensors, large amounts of enzyme should be immobilized on a very small electrode surface. It is a natural choice of using nanomaterials as a solid support to immobilize more enzymes on the electrode surface because of its huge interfacial area [23,24]. On the other hand, nanomaterials have unique advantages in promoting a direct electron transfer between the enzyme's active sites and the electrode [25,26]. Much research has already been reported in this direction, such as using spherical polystyrene colloids [27], carbon

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nanotubes [28–30], Pt or Au nanoparticles [31–36], ZnO nanorod [37], graphene [38], and their composite as the electrode materials [39].

Bovine serum albumin (BSA) as the most abundant globular protein in plasma is naturally biodegradable/biocompatible, nontoxic and nonimmunogenic [40]. It has been extensively used for the fabrication of biosensors [41–43]. For example, by studying the glucose biosensor prepared by co-immobilization of GOx and BSA within an alumina sol–gel matrix, Wilson et al. [44] demonstrated that BSA can improve the activity of the immobilized GOx.

Recently, we have reported a one-step method to prepare well-controlled PMMA–BSA nanoparticles [45]. Transmission electron microscopy and X-ray photoelectron spectroscopy analysis confirms that such particles have an inert PMMA core and a biocompatible BSA protein shell. Further study shows these nanoparticles could adsorb GOx by electrostatic interaction [46]. The adsorption of GOx under a mild condition can minimize the enzyme denaturation and preserve at least 80% of its activity comparing with GOx free in the dispersion.

In the current study, we have concentrated on the construction of a novel stable amperometric glucose biosensor by a simple procedure of immobilizing GOx on PMMA–BSA nanoparticles. We have investigated the effects of pH, applied potential, and enzyme loading on the sensor response to glucose. Our results shows that in such kind of biosensors, the immobilization of GOx on biocompatible PMMA–BSA particles significantly improves its thermal stability, which is one of the most important and critical requirements in the biomedical applications of biosensors.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA) and glucose oxidase (GOx from *Aspergillus niger*, 100 units/mg) purchased from Amresco were used as received. Glutaraldehyde and glucose from Shanghai Sangon Biological Engineering Co. were used without further purification. Methyl methacrylate from Shanghai Chemical Reagent was washed three times with a 5% sodium hydroxide solution to remove the inhibitor, then with deionized water until the pH in the water layer reached 7, and further purified by distillation under vacuum prior to its use. Copper chloride dehydrate, potassium ferricyanide, sodium chloride, potassium dihydrogen phosphate, and hydrochloric acid were also from Shanghai Chemical Reagent and used as received without further purification.

2.2. Preparation of PMMA–BSA particles

The preparation of spherical particles with a PMMA core and a BSA shell by using Cu^{2+}-mediated graft copolymerization of methyl methacrylate directly from bovine serum albumin was detailed before [45]. In brief, BSA was dissolved in deionized water at 25 °C in a water-jacketed flask equipped with a magnetic stirrer, a thermometer, a condenser, and a nitrogen inlet. The solution was stirred for 30 min before designated amount of copper chloride aqueous solution was introduced. The solution mixture was purged with nitrogen for 30 min prior to the addition of MMA. The polymerization was carried for 3 h with constant stirring under nitrogen. The resultant dispersion was purified by several cycles of centrifugation (11,000 rpm) and redispersion until no BSA and MMA were detectable in supernatant. Transmission electron microscopy (TEM) images (Fig. 1) reveal that such formed particles have some core–shell morphology, presumably with a water-insoluble PMMA core and a water-swollen BSA shell. Fig. 2 shows the X-ray photoelectron spectra (XPS) survey scan of the PMMA–BSA particles

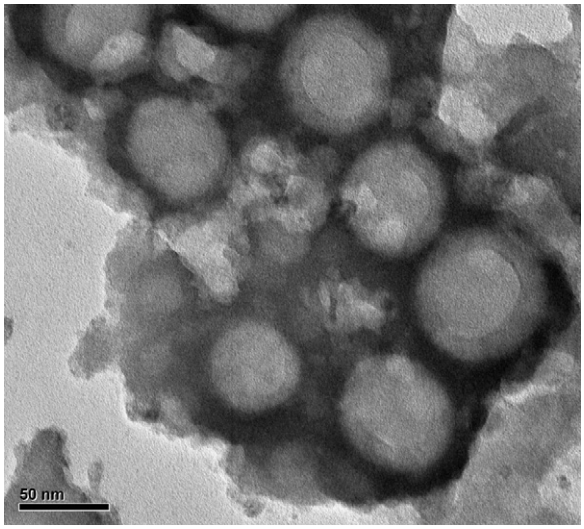

Fig. 1. TEM images of PMMA–BSA particles stained with 1% phosphotungstic acid on carbon-coated grids.

with their main peaks centered at 284.7, 399.6 and 531.6 eV, respectively, related to C 1s, N 1s and O 1s. These results clearly show that as expected the surface of the PMMA–BSA particles is BSA.

2.3. Immobilization of GOx on PMMA–BSA nanoparticles

Our results showed that GOx could strongly adsorb on spherical PMMA–BSA core–shell nanoparticles at pH 4.5, between the *p*I values of GOx and PMMA–BSA particles, at which they are oppositely charged [46]. Here, different amounts of GOx and PMMA–BSA dispersions were mixed in 0.05 M phosphate buffer solution (PBS, pH 4.5). The dispersions were incubated at room temperature for 3 h. The mixture was stored at 4 °C for electrode preparation.

2.4. Preparation of the enzyme electrode

Platinum disk electrodes (2.0 mm in diameter) were polished with 1.0, 0.3 and 0.05 μm α-alumina powder, respectively, rinsed thoroughly with deionized water between each polishing step, sonicated in 1:1 nitric acid, acetone, and deionized water successively, and then dried in air before use. The enzyme electrode was prepared by dropping 5 μL of the PMMA–BSA and GOx mixture described before onto the surface of the platinum disk electrode to form a thin film. After dried in air for 1 h, 3 μL glutaraldehyde solution (0.5%) was added on the film. Finally, the enzyme electrode

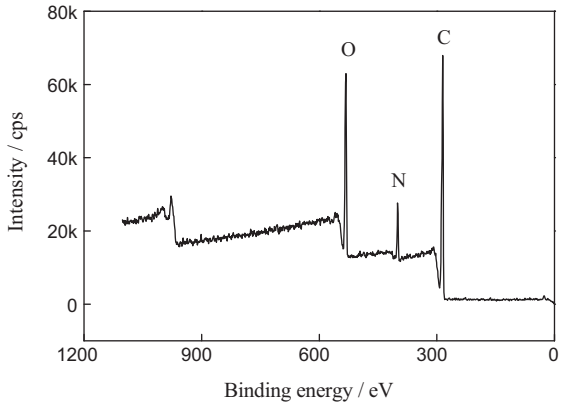

Fig. 2. XPS survey scan of PMMA–BSA particles.

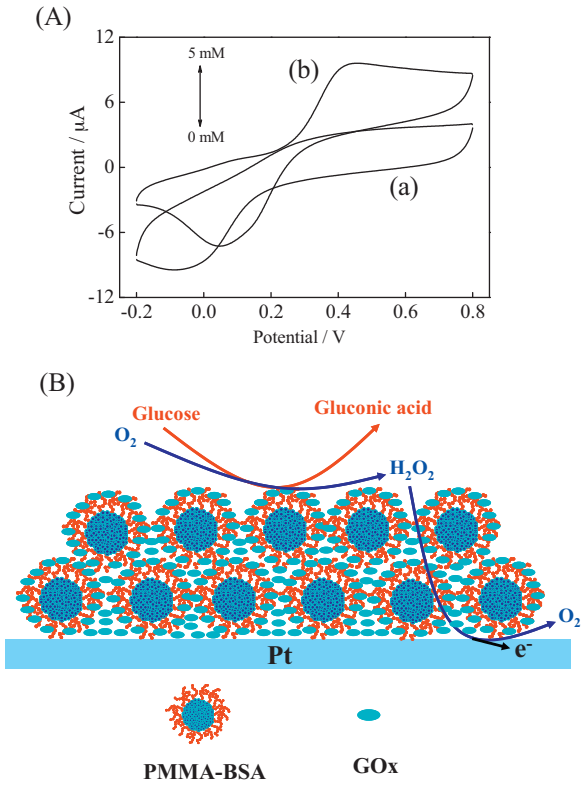

Fig. 3. (A) Cyclic voltammograms of GOx electrode measured (a) without and (b) with 5 mM glucose in pH 7.4, 0.05 M PBS at 50 mV s^{-1}. (B) Schematic of immobilization of enzyme GOx on spherical PMMA–BSA (core–shell) particles on a Pt electrode and its corresponding charge transfer reaction.

was rinsed and immersed in pH 7.4, 0.05 M PBS in order to wash out those free particles and enzymes from the electrode film.

2.5. Characterization and electrochemical measurements

The particle morphology was viewed with a JEOL JEM-2010 TEM. The TEM sample was prepared by adding a drop of dilute aqueous dispersion of PMMA–BSA particles on a carbon-coated copper grid. After 10 min, excess liquid was blotted away using a strip of filter paper, and further, a drop of 1% (w/w) phosphotungstic acid was added to the grid. After incubating at the room temperature for 2 min, excess liquid was removed and the grid was dried at the room temperature before the TEM analysis. XPS was performed by using a VGESCALAB MKII spectrometer with monochromatic Al K_α radiation as the excitation source. The binding energies obtained in the XPS analysis were standardized for specimen charging using C 1s as the reference at 284.6 eV. Amperometric and cyclic voltammetric experiments were performed with a CHI 660A instrument. A three-electrode system comprising the enzyme electrode as working electrode, a platinum wire as auxiliary electrode and Ag/AgCl as reference was employed for all electrochemical experiments. All the potential given in this study were relative to the Ag/AgCl (saturated KCl) reference electrode. The glucose stock solutions prepared in the phosphate buffer were allowed to mutarotate at room temperature overnight before use.

3. Results and discussion

3.1. Cyclic voltammetric characterization

Fig. 3A shows cyclic voltammograms of the enzyme electrode measured without and with the addition of 5 mM glucose in pH 7.4, 0.05 M PBS. In comparison with the background (without glucose), the cyclic voltammogram dramatically changes after the addition of 5 mM glucose. An obvious increase of the oxidation current starts from ~0.25 V, indicating the response of the enzyme electrode to glucose. The observed current increase is from the electrochemical oxidation of hydrogen peroxide generated by the reaction of glucose and oxygen catalyzed by GOx [1,2], as schematically shown in Fig. 3B.

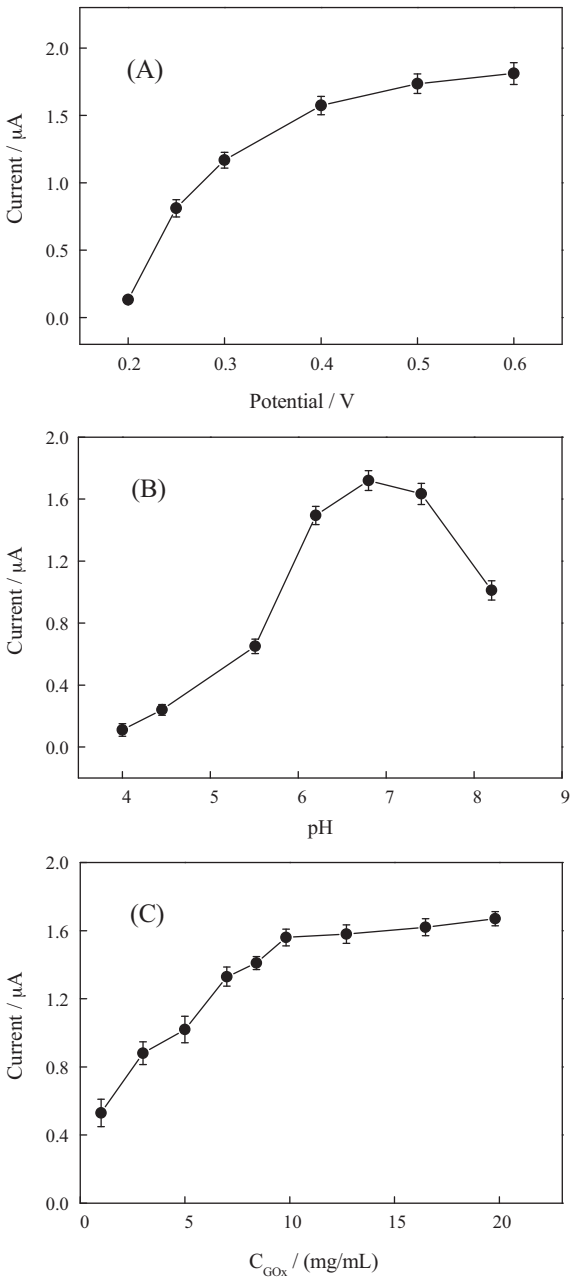

Fig. 4. (A) Effect of applied potential on the steady-state response current of GOx electrode in pH 7.4, 0.05 M PBS with 1 mM glucose. (B) Effect of solution pH on the steady-state response current of GOx electrode under an applied potential of 0.4 V in 0.05 M PBS with 1 mM glucose. (C) Effect of GOx concentration on the steady-state response current of enzyme electrode under an applied potential of 0.4 V in pH 7.4, 0.05 M PBS with 1 mM glucose.

3.2. Effect of applied potential and operating pH on the biosensor

Effect of the applied potential and the operating pH was investigated to optimize the enzyme electrode's performance. Fig. 4A

shows that the current of the enzyme electrode in pH 7.4, 0.05 M PBS containing 1 mM glucose rapidly increases with the potential in the range 0.0–0.4 V, indicating that the response of the enzyme electrode is controlled by the electrochemical oxidation of hydrogen peroxide. When the potential is higher than 0.5 V, the current reaches a plateau, presumably due to the rate-limiting process of the enzyme kinetics and substrate diffusion [47,48]. Considering at the high applied potential, the base current is large and various of electroactive substances (such as ascorbic acid and uric acid in blood) can interfere with the glucose detection, so a low potential of 0.4 V is selected hereafter as the working potential for the test of the enzyme electrodes prepared in this study.

The response of the glucose sensor also depends on the activity of immobilized GOx, which is related to the pH value of the solution used. Fig. 4B shows that the response of our testing sensor increases with pH in the range 4.0–6.8 and reaches its maximum at pH ~6.8. It has been known that GOx works over a wide pH range 2.7–8.5 and the optimum pH is usually between 4.8 and 6.0 for free enzyme molecules when oxygen is used as the electron acceptor [41]. It was also reported that the immobilization of GOx on electrode shifts the optimum pH [49]. Therefore, the optimum response at pH 6.8 may be attributed to the adsorption of GOx on the PMMA–BSA particles. We also found that the response was still reasonably good even at the physiological pH (~7.4). Hereafter, the testing results of our electrodes were obtained in a buffer solution of pH 7.4.

3.3. Effect of enzyme loading

Fig. 4C shows the effect of the GOx concentration in the GOx and PMMA–BSA mixture on the steady-state response current of the final enzyme electrode at 0.4 V in pH 7.4, 0.05 M PBS containing 1 mM glucose. It is clear that for a given amount of PMMA–BSA, the response current increases with the GOx concentration and reaches a plateau at the enzyme concentration of ~9.6 mg/mL, indicating that the enzyme loading capacity of the PMMA–BSA particles is saturated. The excess of GOx molecules is free in the mixture and removed by the rinsing procedure. This saturated concentration is higher than 8.4 mg/mL calculated on the basis of our previous study [46]. It can be attributed to (1) the physical entrapment of some enzyme molecules in the gaps among of the particles; and (2) some GOx molecules are cross-linked by glutaraldehyde on the BSA shell. When the enzyme concentration is higher than ~9.6 mg/mL, the excess of GOx molecules are free in the mixture and must be removed by the following rinsing procedure.

3.4. Chronoamperometric response

Fig. 5A shows a typical current–time plot of our newly developed biosensors on a successive addition of glucose under optimized experimental conditions. When glucose was added into the stirring buffer solution, the response current raised quickly and steeply to a stable value. The biosensor can achieve 95% of the steady state current within eight seconds, much faster than previously reported results on the electrodeposition of GOx with pure BSA followed by a subsequent cross-linking with glutaraldehyde [50]. Such a fast response indicates a fast mass transfer of the substrate across the film as well as a quick electron exchange between GOx and its substrate, attributing to the exposition of GOx molecules on the surface of PMMA–BSA (core–shell) nanostructure and the film is thin.

Fig. 5B shows the corresponding calibration curve of the enzyme electrode as a function of the glucose concentration. The response current increases linearly with an increase of glucose concentration and gradually reaches a saturation value at a high glucose concentration, which suggests that the active sites of enzyme units are saturated at those glucose levels. The calibration for the glucose response in Fig. 5B has a linear range between 0.2 and 9.1 mM

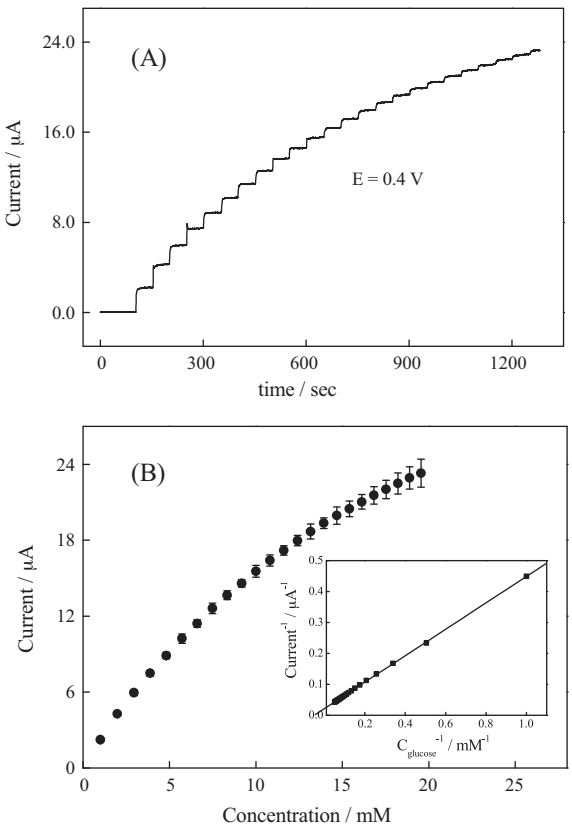

Fig. 5. (A) Current response of GOx electrode after a successive addition of glucose in pH 7.4, 0.05 M PBS under an applied potential of 0.4 V. (B) Calibration curve of GOx electrode as a function of glucose concentration, where the insert is a Lineweaver–Burk plot.

with a correlation coefficient of 0.997. The sensitivity of the biosensor obtained from the slope of this linear part of the calibration is approximately 44.1 μA mM^{-1} cm^{-2}, much higher than those reported values of 15.3 μA mM^{-1} cm^{-2} [37] and 7.2 μA mM^{-1} cm^{-2} [49]. The high sensitivity can be attributed to the biocompatible microenvironment provided by the BSA protein shell that leads to a higher catalytic activity of the adsorbed GOx molecules.

The apparent Michaelis–Menten constant (K_m^{app}), an indication of the enzyme–substrate kinetics, is generally used to evaluate the biological activity of immobilized enzymes. It can be obtained by an amperometric method suggested by Wilson et al. [51,52]. From the Lineweaver–Burk plot based on the inset in Fig. 5B, the calculated K_m^{app} is 19.2 mM, much lower than that of free GOx molecules in solution (27 mM) [53]. These results indicate that the GOx immobilized on the PMMA–BSA nanoparticles possess a good biological affinity to glucose.

3.5. Reproducibility, interference and stability of the glucose biosensor

The good reproducibility of a glucose biosensor is important for real applications. For the biosensors developed in this study, successive measurements of 1 mM glucose for 10 times showed a relative standard deviation of only 3.6%. Even after 50 successive scans, the response still remains 93.7% of the initial value, an acceptable durability. Further, we tested the reproducibility of the biosensor preparation by using six electrodes prepared in the same way to detect an identical glucose concentration (1 mM). The relative standard deviation is 4.9%, showing a good reproducibility of the biosensor preparation. Moreover, we examined the influence of the electroactive interferent commonly present in physiological samples

Table 1
Influence of electroactive interferents on the response of the biosensor.

Interferent	Physiological content (mM)	$(i_{G+I} - i_G)/i_G$ (%)
Ascorbic acid	0.1	2.8 ± 0.2
Uric acid	0.5	2.3 ± 0.1
p-Acetaminophenol	0.1	4.6 ± 0.2

i_G, response current to 5 mM glucose; i_{G+I}, response current to 5 mM glucose in the present of interferent at the physiological normal content.

of glucose under an applied potential of 0.4 V. The result is listed in Table 1. The normal physiological level of glucose is between 4.4 and 6.6 mM, much higher than the concentrations of these interferents so that their effects on the detection of glucose are insignificant. Note that the interference can be further reduced by using a lower working potential. The response of these interferences was nearly eliminated at the potential of 0.25 V by coating a preselective Nafion film on the outside of the enzyme electrode [54–57]. However, the response current of the biosensor with Nafion reduced about 17% comparing with that without the Nafion covered one. The decrease of the response could be attributed to the denaturation of the GOx enzyme caused by the organic solvent of Nafion solution.

Finally, we come to the mostly important problem of the long-term stability of a glucose sensor. Many researchers have reported the performance of glucose sensor stored in PBS at 4 °C [58–61]. However, such a low storing temperature is not always practical in some real application, especially when these sensors are carried and used on patients who are on trips. In the current study, we stored the enzyme electrode in pH 7.4, 0.05 M PBS at 25 °C instead of 4 °C and measured its stability over one month by repeat measurements of its response to 1 mM glucose. The result is shown in Fig. 6, where i_0 is the difference between the steady-state current and the background current of the enzyme electrode freshly fabricated and i is the difference after a given storage time. After 23 days, the response current of the biosensor only decreased ~4%. Even after 30 days, the biosensor still retained ~82% of its original response. We think that such an excellent stability is attributed to four following aspects. First, our biosensors are prepared under mild conditions to avoid the denaturation of GOx; second, the enzyme is immobilized on a biocompatible BSA shell of each nanoparticle, which maintains its bioactivity; third, the BSA shell stabilizes the enzyme nature structure to improve its thermal stability; and fourth, the relative strong physical interactions between the PMMA–BSA nanoparticles and GOx and the entrapment of GOx among the particles prevents the leaking of enzyme from the electrode during the repeated applications.

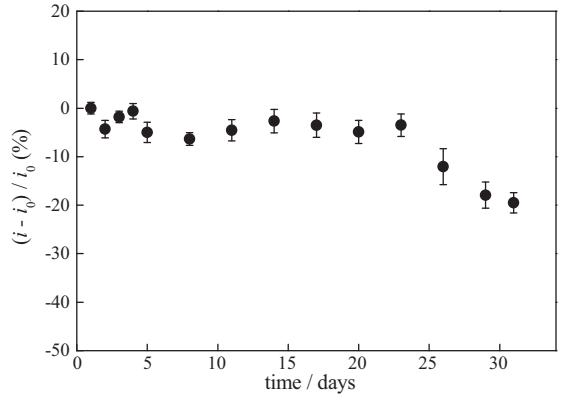

Fig. 6. Stability of GOx electrode stored at 25 °C in pH 7.4, 0.05 M PBS, where each data point represents an enzyme electrode response to 1.0 mM glucose under an applied potential of 0.4 V.

Table 2
Determination of glucose in human serum samples with the biosensor.

Sample No.	Determined by biosensor (mM)	Given by hospital (mM)	Relative error (%)
1	4.3	4.2	2.4
2	5.9	6.0	1.7
3	6.0	6.2	3.2

3.6. Application of the glucose biosensor in real samples

The accuracy of glucose sample detection was evaluated by comparing the analytical results of three human serum samples determined by the glucose biosensor and measured by the spectrophotometric method in the hospital. Before measured by the glucose biosensor, the serum samples were firstly diluted 5 times with phosphate buffer to ensure the glucose concentrations located in the linear range. As shown in Table 2, the concentration of glucose in human serum samples determined by the glucose biosensor was close to the value measured by the hospital, and the relative error was less than 3.22%. In addition, the recovery was investigated by standard additions of glucose to the serum samples and the value was between 97.35% and 103.14%. In future works, we will focus on improving the performance of the glucose biosensor by involving some metal nanoparticles with good conductivity.

4. Conclusions

We have developed a novel stable amperometric glucose biosensor by immobilizing glucose oxidase (GOx) on the PMMA–BSA (core–shell) nanoparticles to form a thin film on a platinum electrode. The biocompatible BSA protein shell does not only immobilize GOx via the relative strong physical interaction, but also retain its bioactivity and native structure and provides a long thermal stability at room temperature. The testing shows that the enzyme electrode has a quick response time, a low transport barrier and a high affinity to glucose with a wide linear detection range. The biosensor made of this kind of electrodes is highly sensitive to glucose. The simple preparation procedure leads to an excellent reproducibility among different biosensors prepared under the same conditions. More remarkably, our testing results demonstrate that when such prepared biosensors are stored at room temperature for more than three weeks, they can still maintain 96% of their original response, which also show a good thermal stability. The general idea and preparation method presented in this study may offer a facile way to fabricate other type of enzyme based amperometric biosensors.

Acknowledgments

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