



Amperometric glucose biosensor based on direct assembly of Prussian blue film with ionic liquid-chitosan matrix assisted enzyme immobilization

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ABSTRACT

A facile fabrication procedure was developed for the construction of glucose biosensor. Prussian blue (PB) was modified on the electrode through simple direct assembly process. The ionic liquid (IL) assisted chitosan (Chi) matrix was used for the glucose oxidase (GOx) immobilization on the PB electrode. Cyclic voltammetry (CV), chronoamperometry and electrochemical impedance spectroscopy (EIS) were used to evaluate the performance of the prepared GOx/Chi/IL/PB/Pt biosensor. The presence of IL (1-butyl-3-methylimidazolium tetrafluoroborate, [bmim]BF₄) can effectively enhance the electron transfer rate and reduce the interfacial resistance. This biosensor exhibited a fast response time within 3 s, a linear range up from 0.01 to 4.2 mM, low detection limit of 5 μM and a sensitivity of 37.8 μA mM⁻¹ cm⁻². Because of the biocompatibility of the IL-Chi composite matrix, the biosensor exhibited satisfactory storage stability over 40 days with retention of 90.4% activity. Additionally, the biosensor exhibited good selectivity which was attributed to the low operating potential employed (−0.05 V vs. Ag/AgCl).

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1. Introduction

The detection of glucose has attracted considerable interest owing to its importance in the diagnosis of diabetes [1–4]. It is reported that glucose biosensors account for approximately 85% of the entire biosensor market [5]. However, there are still many challenges which should be overcome in order to satisfy requirements such as long term stability, high sensitivity and selectivity. Enzyme electrodes have been widely investigated for a range of biomedical applications [6]. In the fabrication of glucose biosensor, glucose oxidase catalyzes the oxidation of glucose to gluconolactone and H₂O₂ in the presence of oxygen as follows:



Due to its excellent electrocatalysis toward hydrogen peroxide (H₂O₂) reduction, Prussian blue (PB) has been extensively used as an effective low-potential electron transfer mediator for the construction of oxidase-based biosensors [7–9]. The low applied potential could overcome signals due to electroactive interferences such as uric acid and ascorbic acid which commonly co-exist in blood samples. Among the various fabrication methods which may be employed, the self-assembly process has advantages for the

preparation of PB based biosensor owing to its technical simplicity and excellent control over film thickness [10–14]. Since Millward et al. [15] initially proposed the assembly of PB on Au electrode surface, this approach has been fully exploited with the assistance of pre-modification, such as anodic alumina, charged surfactants and carbon nanotubes, to tailor the nanostructure on the substrate [16–18]. Nevertheless, the majority of these substrate modification procedures was technically complicated and time consuming for PB fabrication. Therefore, the direct assembly of PB on electrodes can still be a promising approach for the construction of electron mediator based biosensors.

The immobilization strategy of enzymes is essential for the performance of oxidase-based biosensors [19–21]. Chitosan (Chi) is an abundant natural biomaterial with excellent film forming ability, high mechanical strength and biocompatibility. It is widely applied as a matrix for enzyme immobilization [22,23]. However, the poor electrical conductivity of Chi hindered the performance of the enzyme-based biosensor. Thus, compounds with good conductivity need to be combined with Chi in order to achieve a high performance biosensor [24,25]. Recently, ionic liquids (ILs), which consist entirely of ions, have attracted considerable attention in the field of electrochemistry because of their unique properties such as high conductivity, wide electrochemical window, good thermal stability and biocompatibility [26–28]. Proteins held within the microenvironment created by ILs are expected to promote the enzyme reaction process to present enhanced conductivity, stability and selectivity in this immobilization matrix.

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This paper describes a simple strategy for the construction of a glucose biosensor based on direct assembly of PB modified electrode with the IL-Chi matrix assisted GOx immobilization. Due to the excellent electrocatalytic properties of PB as well as the favorable microenvironment for enzyme immobilization, the resulting biosensor of GOx/Chi/IL/PB/Pt exhibited a wide linear range response to glucose with good selectivity, fast response time as well as long-term stability.

2. Materials and methods

2.1. Reagents and apparatus

All chemicals were of analytical purity and used as received. $K_4[Fe(CN)_6] \cdot 3H_2O$, $FeCl_3 \cdot 6H_2O$ were purchased from Sigma–Aldrich; 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF₄) were obtained from Alfa Aesar. Glucose oxidase (GOx) from *Aspergillus niger* (E.C. 1.1.3.4, 180,200 U g⁻¹) was supplied by Sigma. Glucose, chitosan, ascorbic acid and uric acid were obtained from Sinopharm Chemical Reagent Co. Ltd. (China). Glucose stock solution was stored at room temperature for 24 h before using to allow for mutarotation. 1 mg/mL chitosan solution was made with 1.0% (v/v) acetic acid in 0.05 M phosphate buffer solution (PBS, pH 6.5) including 0.1 M KCl. Pt disk electrode (0.28 cm²). Double-distilled water was used throughout.

Electrochemical measurements were performed on CHI 660C electrochemical workstation (Shanghai Chenhua, China). The electrochemical cell was consisted with a conventional three-electrode system. A saturated Ag/AgCl electrode was used as reference electrode together with a platinum wire as an auxiliary electrode for all experiments. The working electrode was a modified Pt electrode. All the characterization experiments were performed at the temperature of 25 °C.

2.2. Preparation of the modified electrodes

Prior to assembly of PB films, the Pt electrodes were first cleaned in Piranha solution (7:3 mixture of H₂SO₄/H₂O₂) for 30 min and then sonicated in water for 30 min. For the assembly of PB films, two solutions were prepared. Solution 1: 0.01 M K₄[Fe(CN)₆] + 0.1 M KCl + 0.1 M HCl. Solution 2: 0.01 M FeCl₃ + 0.1 M KCl + 0.1 M HCl. The pretreated electrode was consecutively dipped into solution 1 for 1 min, pure water for 30 s, solution 2 for 1 min, and pure water for 30 s again. The four steps lead to a single PB layer. 30 layers PB were modified on the electrode with temperature controlled at 25, 30, 35 and 40 °C.

For the immobilization of enzyme, a GOx mixture solution was prepared by adding GOx and [bmim]BF₄ in chitosan solution. The concentration of enzyme, [bmim]BF₄ and chitosan was 10 mg/mL, 4% (v/v) and 1 mg/mL, respectively. Subsequently, 5 μL of the enzyme mixture was dropped onto the surface of the PB modified electrode and allowed to dry for 2 h at room temperature. The resulting enzyme electrodes GOx/Chi/IL/PB/Pt were stored at 4 °C in PBS when not in use. The GOx/Chi/PB/Pt was obtained by placing 5 μL of the 1 mg/mL Chi solution including 10 mg/mL GOx on the PB surface.

3. Results and discussion

3.1. The influence of temperature on direct assembly of the PB modified electrode

Considering the temperature influence for the growth of nanocrystals [29] the direct formation of PB on the electrode using a range of temperatures (25–40 °C) was investigated.

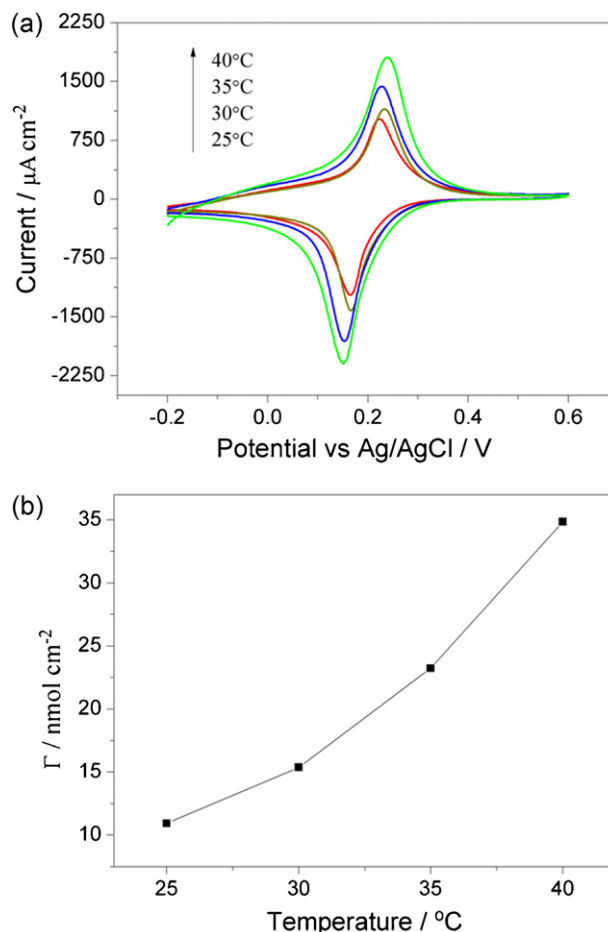


Fig. 1. (a) CV image of PB modified electrode fabricated at different temperatures (25, 30, 35 and 40 °C) and (b) the calculated concentration of PB on the electrode in 0.5 M PBS (pH 6.5) including 0.1 M KCl at the scan rate of 50 mV s⁻¹.

Characterization using cyclic voltammetry (Fig. 1a) confirmed that the variation of assembly temperatures could evidently affect the growth of PB. This peak current expressed the transformation between PB and its reduction state—Prussian white (PW) [30]. Therefore, its value was in proportion to the amount of PB on the electrode. The peak current values increased with the elevation of temperatures. On the other hand, the enhancement of peak separation ΔE_p indicated the electron transfer was restrained due to the increased thickness of PB film [31].

$$\Gamma = \frac{Q}{nAF} \quad (1)$$

The surface concentration of PB (Γ) was calculated from Eq. (1) where Q is the single peak area, A the electrode area, F the Faraday constant, and n the average electrons transfer which was calculated by $57/\Delta E$. (The potential difference between oxidation and reduction peaks.) [32] The PB concentrations shown in Fig. 1b are 10.94, 15.39, 23.22 and 34.86 nmol cm⁻² prepared using temperatures of 25, 30, 35 and 40 °C, respectively. The PB was slowly formed on the Pt surface at low temperatures. With the enhancement of temperature, the increased thermal motion of K₄[Fe(CN)₆] and FeCl₃ molecules could accelerate the reaction rate to facilitate the formation of PB on the electrode surface.

The sensitivities of the PB modified electrodes to H₂O₂ was evaluated at -0.05 V (vs. Ag/AgCl) over the range 10–400 μM H₂O₂. The sensitivities were calculated to be 477.5, 535.6, 763.4 and 662.5 μA mM⁻¹ cm⁻² using fabrication temperatures of 25, 30, 35 and 40 °C, respectively. With the increase of temperature, the

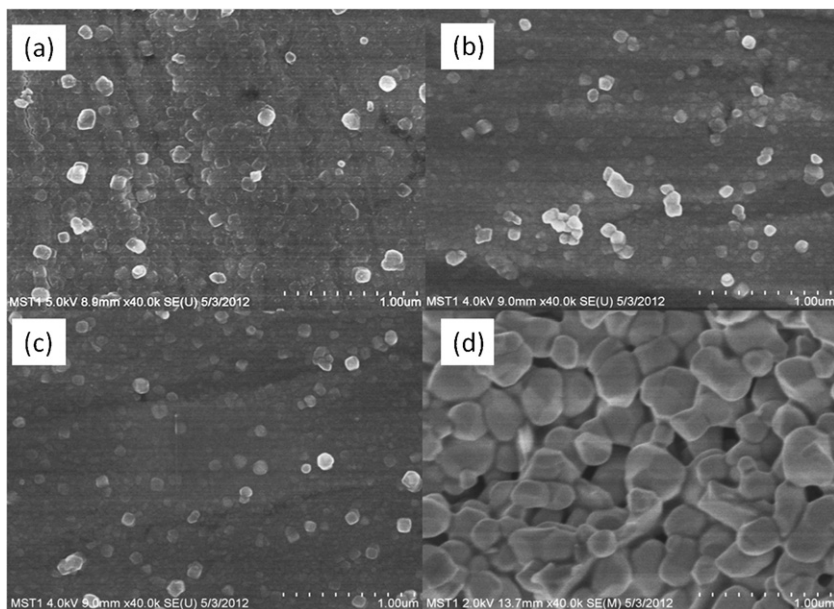


Fig. 2. The SEM images of (a) PB/Pt, (b) Chi/PB/Pt, (c) Chi-IL/PB/Pt and (d) GOx/Chi-IL/PB/Pt.

sensor demonstrated a higher sensitivity to H_2O_2 response which was attributed to the increased formation of PB. However, when the temperature further increased to 40°C , the sensitivity was slightly decreased probably due to the difficult electron transfer and blocked diffusion of H_2O_2 with the increased thickness of PB film [31]. Consequently, excessive loading of PB were adverse for the electrocatalytic reduction of H_2O_2 . Fabrication at 35°C proved to be the appropriate direct assembly condition for the PB based biosensor which was agreement with our previous research [32]. The following prepared sample PB modified electrodes were all prepared under 35°C for study.

3.2. SEM characterization of the modified electrode

Fig. 2 shows the films surface morphology of the stepwise biosensor using SEM technology. As can be seen from Fig. 2a, the PB film presents a uniform morphology with growths of cubic grains in nanosize. After Chi (Fig. 2b) and IL (Fig. 2c) modified on the PB surface, the images were similar to that of PB film. Apparently different image was observed in Fig. 2d indicating the successful immobilization of GOx on the film of Chi-IL/PB.

3.3. CV and EIS of the modified electrodes

The electrochemical behavior of the different modified electrodes was investigated in 0.1 M PBS (pH 6.5) including in 0.1 M KCl at the scan rate of 50 mV s^{-1} by CV. All the sample PB modified electrodes were prepared under 35°C for study. Fig. 3A showed the CV of the bare Pt (curve a), PB/Pt (curve b) and GOx/Chi-IL/PB/Pt electrodes (curve c). No redox peaks were observed with bare Pt electrode (curve a). After the self-assembly of PB on the electrode, a couple of well-defined redox peaks were noticed which was attributed to the transformation between PB and Prussian White (curve b) [33]. When the GOx was further immobilized on the electrode with the assistance of Chi-IL, the GOx/Chi-IL/PB/Pt electrode showed a decline in peak current value and a slight increase in ΔE compared to PB/Pt (curve c). This may be ascribed to the non-conductivity of GOx hindering the diffusion of electron towards the electrode surface.

The EIS was also used to monitor the assembly process of different electrodes. Fig. 3B showed the EIS of the bare Pt (curve a),

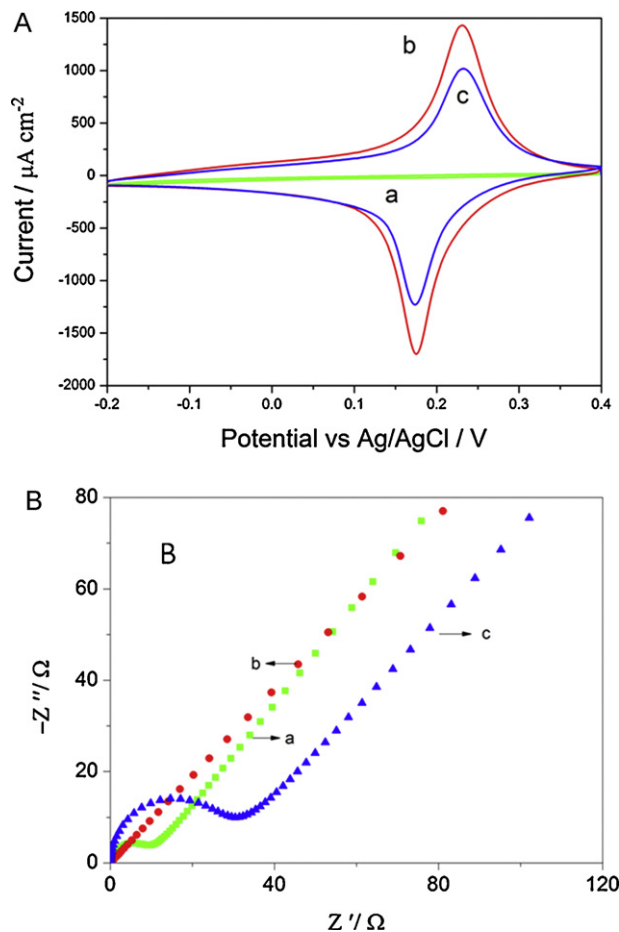


Fig. 3. (A) CV of bare Pt (a), PB/Pt (b) and GOx/Chi-IL/PB/Pt electrodes (c) in 0.1 M PBS (pH 6.5) including in 0.1 M KCl at the scan rate of 50 mV s^{-1} ; (B) EIS of the bare Pt (curve a), PB/Pt (curve b) and GOx/Chi-IL/PB/Pt electrodes (curve c) in $5\text{ mM } [\text{Fe}(\text{CN})_6]^{4-/3-}$ (1:1) containing 0.1 M KCl solution across the frequency range from 10 kHz to 100 mHz.

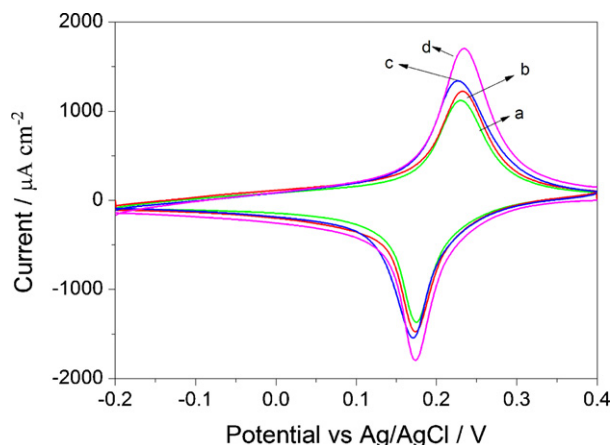


Fig. 4. CVs of GOx/Chi/PB in absence (a) and presence (c) of glucose and GOx/Chi-IL/PB modified electrodes in absence (b) and presence (d) of glucose in 0.1 M PBS (pH 6.5) including 0.1 M KCl at the scan rate of 50 mV s^{-1} .

PB/Pt (curve b) and GOx/Chi-IL/PB/Pt electrodes (curve c) in 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ (1:1) containing 0.1 M KCl solution across the frequency range from 10 kHz to 100 mHz. Bare Pt (curve a) had a small semicircle implying low R_{et} to the redox probe. For the PB/Pt electrode (curve b), the resistance decreased when compared to bare Pt which attributed to the nanosized PB had effectively promoted the electron transfer. When the GOx was immobilized on the electrode with the assistance of Chi-IL matrix (curve c), the semicircle increased because the macromolecular structure of enzyme and the poor conductivity of Chi block the electron transfer, as observed in CV [34,35].

3.4. Influence of $[\text{bmim}]\text{BF}_4$ on the modified electrode

The influence of $[\text{bmim}]\text{BF}_4$ on the enzyme immobilization layer was studied using CV. Fig. 4 showed the CVs of GOx/Chi/PB and GOx/Chi-IL/PB modified electrodes in absence and presence of glucose in 0.5 M PBS (pH 6.5) including 0.1 M KCl at the scan rate of 50 mV s^{-1} . In the absence of glucose, the reversible electrochemical behavior of PB was observed on both electrodes, characteristic of PB redox process. The peak current of GOx/Chi-IL/PB (Fig. 4, curve b) was higher in comparison with GOx/Chi/PB (Fig. 4, curve a) modified electrode. After the addition of 1 mM glucose solution, the peak current increased for both electrodes implying the catalytic properties to glucose. The detailed detection process can be expressed as follows: the GOx catalyzes the oxidation of glucose to H_2O_2 and gluconolactone in the presence of oxygen. The PB act as the effective low-potential electron transfer mediator for H_2O_2 reduction. In turn the Prussian White reoxidized at the underlying electrode with the generation of response current [36]. As can be seen, the GOx/Chi-IL/PB modified electrode (Fig. 4, curve d) exhibits more sensitive response when compared to GOx/Chi/PB electrode (Fig. 4, curve c). It can be concluded the presence of IL was effective in enhancing analytical performance of the biosensor owing to its high conductivity.

3.5. Electrochemical behavior of the enzyme electrode

Fig. 5 displays the influence of scan rates on the electrochemical response of the GOx/Chi/IL/PB/Pt. Well defined symmetrical redox peaks were observed in the CV. The redox peak current linearly increased with the increase of the scan rates in the range of 25–200 mV s, indicating a surface-controlled electrochemical process. The ΔE also gradually increased with the variation of the scan rate implying the more irreversible process. Using

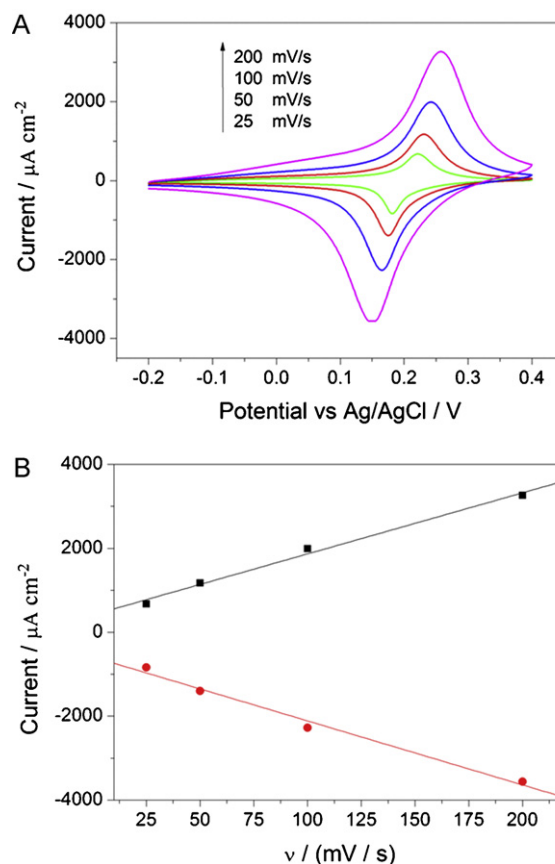


Fig. 5. (A) CV of GOx/Chi/IL/PB/Pt in 0.1 M PBS (pH 6.5) including 0.1 M KCl at the scan rates of 25 to 200 mV s from inner to outer; (B) plot of peak current versus the scan rates.

Laviron's equation [38], two straight lines were obtained with equation of $E_{\text{pc}}(\text{V}) = -0.0359 \ln \nu (\text{V s}^{-1}) + 0.230$ ($r = 0.987$) and $E_{\text{pa}}(\text{V}) = -0.0399 \ln \nu (\text{V s}^{-1}) + 0.163$ ($r = 0.996$). The diffusion coefficient of charge transfer was estimated to be 0.51 which was similar to the Nafion/PBNPs/GCE [39].

3.6. Amperometric performance of the glucose biosensor

The GOx/Chi/IL/PB/Pt biosensor showed a typical chronoamperometric response upon the successive addition of 0.2 mM glucose at -0.05 V (vs. Ag/AgCl). Fig. 6 (inset A) displays the corresponding calibration curve for glucose. The biosensor exhibited a linear range response to glucose from 0.01 to 4.2 mM with a correlation coefficient of 0.9968 and a sensitivity of $37.8 \mu\text{A mM}^{-1} \text{ cm}^{-2}$. The detection limit was $5 \times 10^{-6} \text{ M}$. According to inset B of Fig. 6, the time required to reach 95% of the steady-state current was rather fast within 3 s. Probably the existence of PB as a mediator for electron transfer and the conductivity of IL contributed to the fast response to glucose. The performance comparison of reported PB based biosensor is given in Table 1.

According to the Lineweaver–Burk equation:

$$\frac{1}{I_{\text{ss}}} = \frac{1}{I_{\text{max}}} + \frac{K_{\text{M}}}{I_{\text{max}} C} \quad (2)$$

where I_{ss} is the steady-state current for glucose response, I_{max} is the maximum current measured under saturated substrate solution and C is the buck concentration of substrate. The apparent Michael–Menten constant (K_{M}) was calculated to be $6.6 \pm 0.5 \text{ mM}$. The relatively low value of K_{M} indicated the IL-Chi composite film could provide a biocompatible microenvironment for immobilized

Table 1
Comparison of the performance of PB based glucose biosensor.

Glucose biosensor	Linear range (mM)	Detection limit (μM)	Sensitivity ($\mu\text{A mM}^{-1} \text{cm}^{-2}$)	Response time(s)	Stability	Reference
GOx/Chi/IL/PB/Pt	0.01–4.2	5	37.8	3	90.4% (40 d)	Our work
GCE/PB/MWNTs-GOx-Chi-ICPTES	0.025–1.3	7.5	15.2	10	–	[41]
GCE/PB/Chi-AuNPs/GOD	0.001–1.6	0.69	69.26	3	70% (2 weeks)	[42]
GCE/CS-PB@MWNTs/H-PtCo/GOx/nafion	0.003–3.6	0.85	21	8	83% (1 month)	[34]
AuE/PB/(Chi-GOD)n	0.006–1.6	3.1	–	10	73% (4 weeks)	[43]
GCE/PBNPs/GOx/nafion	0.18–1.1	65	1.59	–	–	[39]
GCE/PB/silica sol-gel-GOD	0–4.75	20	1.18	12	90% (45 d)	[40]
AuE/CS/CS-PB/GOD	0.002–0.4	0.397	–	10	90% (1 week)	[22]
GCE/Fe ₃ O ₄ /PB/GOD	0.0005–0.08	0.1	–	10	No loss (60 d)	[37]

GCE: glassy carbon electrode; MWNTs: multi-walled carbon nanotubes hybrids; ICPTES: 3-isocyanatopropyltriethoxysilane; Pt-NCs: platinum nanoclusters; GOD/GOx: glucose oxidase; NF: Nafion; CS: chitosan; H-PtCo: hollow PtCo; AuE: gold electrode; OSiFPB: organosilica nanosphere functionalized Prussian blue; PGNs: positively charged gold nanoparticles; PBNPs: Prussian blue nanoparticles; -: not reported.

GOx which revealed high enzymatic activity and affinity for glucose.

3.7. Effect of apparent GOD loading

The amount of enzyme immobilized on electrode surface strongly influenced the amperometric response to glucose [40]. Fig. 7 revealed the calibration curve for glucose detection performed at the electrode with different amount of GOx. The sensitivity dependence on the amount of GOx was shown in the Fig. 4b. Obviously, the response sensitivity sharply increased with increasing GOx loading. A comparatively high sensitivity of $90.4 \mu\text{A mM}^{-1} \text{cm}^{-2}$ was obtained with the amount of GOx of 36U. This indicated that the electrode response was dependent on enzyme loading. However, the linear range diminished with the amount of enzyme increased probably due to the leakage of unbound GOx and the lack of oxygen in the response [1]. A wide linear range is of benefit for clinical analysis and accordingly, the enzyme loading of 9U was more appropriate for further applications.

3.8. Anti-interferent ability, reproducibility and stability of the enzyme electrode

Oxidizable compounds such as ascorbic acid (AA) and uric acid (UA) are common electrochemical interfering species which can co-exist with glucose in real blood samples. Therefore the effect of AA and UA on the current response of 0.1 mM glucose was evaluated.

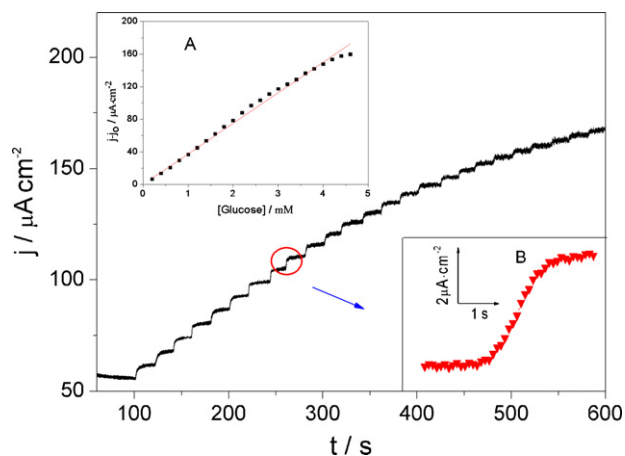


Fig. 6. The amperometric responses of glucose biosensor to successive injections of 0.2 mM glucose in 0.05 M PBS including 0.1 M KCl (pH 6.5, 25 °C) at -0.05 V (vs. Ag/AgCl). Inset A displays the corresponding calibration curve for glucose. Inset B shows the steady-state current response time of the enzyme electrode to 0.2 mM glucose.

Addition of 0.1 mM AA and 0.1 mM UA to 0.1 mM solution did not cause obvious current response which was mainly attributed to the low applied potential of -0.05 V (vs. Ag/AgCl) and the barrier effect of the enzyme layer as shown in Fig. 8. The results revealed that the GOx/Chi/IL/PB/Pt biosensor had good anti-interference ability.

The reproducibility of the enzyme biosensor was evaluated according to five electrodes prepared under the same condition with a RSD of 4.8%. The RSD of the enzyme electrode response to 1 mM glucose was 4.2% for five successive measurements. The results showed that the resulting glucose biosensor possessed satisfactory reproducibility.

The enzyme electrode was stored at 4 °C in PBS when not in use. It was found that no obvious loss was observed after 20 days and the biosensor retained 90.4% of the original response after 40 days. This

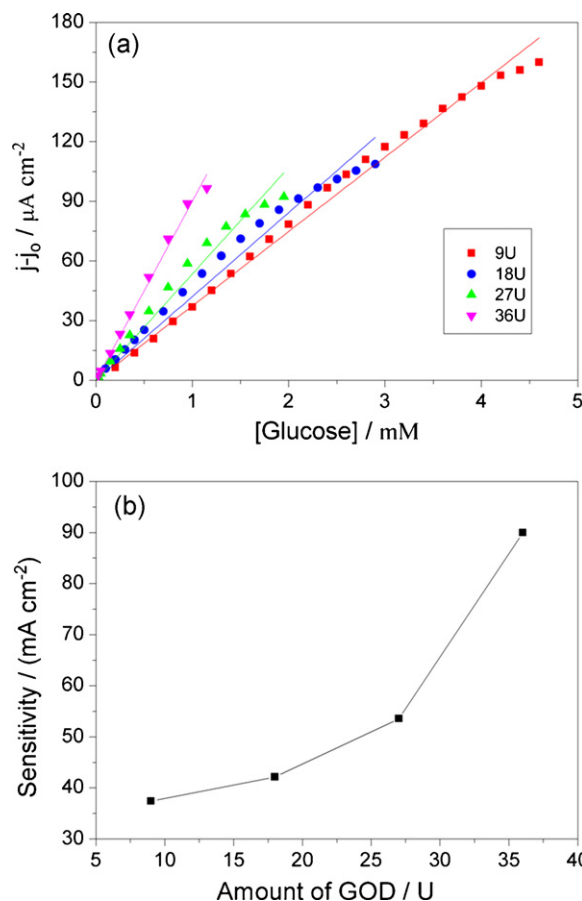


Fig. 7. (a) Calibration curves for glucose detection performed at electrode with different amount of GOx; (b) the dependence of sensitivity on the amount of GOx in 0.1 M PBS (pH 6.5) including 0.1 M KCl at the scan rate of 50 mV s^{-1} .

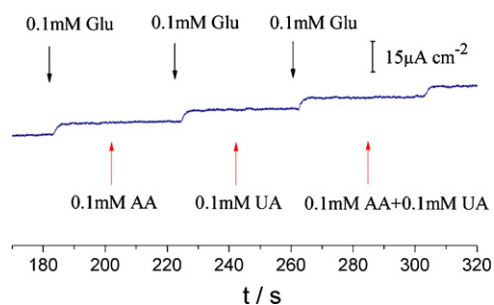


Fig. 8. Effects of AA and UA interference on the response of the GOx/Chi-IL/PB/Pt electrode in 0.1 M PBS (pH 6.5) including 0.1 M KCl at the scan rate of 50 mV s⁻¹.

Table 2
Detection of glucose in blood serum samples by GOx/Chi-IL/PB/Pt biosensor.

Sample	Found (mM)	Added (mM)	Found (mM)	Recovery (%)
1	0.40	0.12	0.53	108.3
2	0.40	0.24	0.63	95.8
3	0.40	0.36	0.77	102.7

revealed the interaction of GOx with the Chi ensured good stability of the biosensor. In addition, the good biocompatibility of the IL-Chi matrix maintained the biological activity of the immobilized enzyme.

3.9. Application of the biosensor in serum samples

To evaluate the ability of the biosensor for routine analysis, the biosensor was applied to detect glucose in animal blood serum samples utilizing the standard addition method. 2 mL serum samples were added to 40 mL 0.05 M PBS (pH 6.5) for measurement at the potential of -0.05 V (vs. Ag/AgCl). The results were listed in Table 2. The recoveries of the biosensor were in the range of 95.8%–108.3% indicating the potential for analysis in real samples.

4. Conclusions

Based on direct assembly of PB modified electrode, a high performance glucose biosensor was developed with enzyme immobilized in IL-Chi matrix. 35 °C was proved to be the appropriate temperature for the direct assembly of PB films. The interaction of GOx with Chi ensured good stability of the biosensor and the presence of IL effectively reduced the interfacial resistance of the enzyme layer. Amperometric glucose response revealed that the prepared biosensor GOx/Chi-IL/PB/Pt exhibited fast response time, wide linear range response, high sensitivity as well as good stability. The direct assembly of PB film could be a promising platform for the development of electron mediator based biosensor, and the immobilization of various enzymes could retain activity in the IL-Chi composited matrix.

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