Electrochemical quartz crystal impedance study on immobilization of glucose oxidase in a polymer grown from dopamine oxidation at an Au electrode for glucose sensing

Mingrui Li, Chunyan Deng, Qingji Xie, Yang Yang, Shouzhuo Yao

Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education of China), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, PR China

Received 19 November 2005; received in revised form 19 February 2006; accepted 20 February 2006

Abstract

Glucose oxidase (GOD) was codeposited into a polymer grown from oxidation of dopamine (DA) at an Au electrode in a neutral phosphate aqueous solution for the first time. The electrochemical quartz crystal impedance analysis (EQCIA) method was used to monitor the GOD-immobilization process. Effects of concentrations of phosphate buffer, DA and GOD were investigated, and the optimal concentrations were found to be 20.0 mM phosphate buffer (pH 7.0), 30.0 mM DA and 5.00 mg ml\(^{-1}\) GOD. A glucose biosensor was thus constructed, and effects of various experimental parameters on the sensor performance, including applied potential, solution pH and electroactive interferents, were examined. At an optimal potential of 0.6 V versus the KCl-saturated calomel electrode (SCE), the current response of the biosensor in the selected phosphate buffer (pH 7.0) was linear with the concentration of glucose from 0.05 to 9 mM, with a lower detection limit of 3.9262 M (S/N = 3), short response time (within 15 s) and good anti-interferent ability. The Michaelis constant (\(K_{m}\)) was estimated to be 9.6 mM. The biosensor exhibited good storage stability, i.e. 96% of its initial response was retained after 7-day storage in the selected phosphate buffer at 4\(^{\circ}\)C, and even after another 3 weeks the biosensor retained 86% of its initial response. In addition, the enzymatic specific activity and enzymatic relative activity of the GOD immobilized in the polymer from dopamine oxidation (PFDO) were estimated from the EQCIA method to be 1.43 kU g\(^{-1}\) and 3.7%, respectively, which were larger than the relevant values obtained experimentally using poly(o-aminophenol) and poly(N-methylpyrrole) matrices, suggesting that the PFDO is a better matrix to immobilize GOD.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Immobilization of glucose oxidase; Quantification of enzymatic activity; Polymer from dopamine oxidation; Electrochemical quartz crystal impedance analysis; Amperometric glucose biosensor

1. Introduction

The amperometric glucose biosensor based on immobilized glucose oxidase (GOD) has received great attention for its great application interests. In the presence of dissolved oxygen, H\(_2\)O\(_2\) is generated during the oxidation of glucose catalyzed by GOD [1–3]. The generated H\(_2\)O\(_2\) can be monitored amperometrically, and the current response is related to the concentration of glucose. The direct electrochemical detection of hydrogen peroxide is usually accomplished at anodic potentials at 0.6 V (versus Ag/AgCl) or above [4,5]. However, many substances normally present in biological fluids, such as ascorbic acid (AA), uric acid (UA), are easily co-oxidized at similar potentials and may cause interfering responses. Consequently, the way to avoid interfering responses has been attracting widespread attention. The permselective film, such as poly(o-phenylenediamine), poly(toluidine blue), Nafton, or poly(o-aminophenol) (PoAP), has been utilized as an electrode-modification material to diminish or inhibit the electroactivity of the interferents [2,6–8], whereas the high electroactivity of H\(_2\)O\(_2\) at the relevant electrode is still retained for the film’s high permeation for H\(_2\)O\(_2\).

The immobilization of enzyme is the key step in constructing a biosensor. A variety of techniques have been utilized for enzyme immobilization on solid surfaces, involving electropolymerization [9], self-assembly [10] and Langmuir-Blodgett (LB) film methods [11]. Particularly, the electropolymerization
protocol receives much attention because of the convenience in controlling the film thickness for high experimental reproducibility. The immobilization of enzymes by electrochemical codeposition with conducting polymers, such as polypyrrole [12–14], poly(N-methylpyrrole) (PNMP) [15] and polyaniline [16,17], has been extensively studied, being due to the easiness in growing multilayer film for entrapping more enzyme ions. Amperometric biosensors based on the immobilization of enzyme in non-conducting polymers, which generally possess excellent anti-interferent permselectivity, are also worthy of attention [18]. Some non-conducting polymers, such as poly(o-phenylenediamine) [19], polyphenol [20], poly(o-aminophenol) (PoAP) [6], etc., have commonly been utilized to immobilize various enzymes, and the melanin-like polymers have also been proposed as enzyme-immobilization matrices [21,22].

The electrochemical oxidation of dopamine (DA) has been intensively investigated [23,24], which proceeds via the well-known ECET mechanism, namely, DA is oxidized to dopaminequinone (DAQ), the intramolecular cyclization of DAQ via 1,4-Michael addition leads to the more readily oxidizable leucodopaminechrome (LDAC), and then LDAC is oxidized to dopaminechrome (DAC). The final oxidation product can further undergo polymerization reactions on the electrode surface, yielding a deposited melanin-like polymer responsible for the gradual loss of electrode activity. The polymer from dopamine oxidation (PDDO) has been successfully utilized as a matrix to immobilize anti-human immunoglobulin G (IgG) in a neutral phosphate buffer [25]. Also, the PDDO was found to be of cationic permselectivity [24], and thus the interfering currents from the oxidation of anionic AA and UA may be notably decreased at a PDFO-modified electrode. Consequently, this polymer is anticipated to be a good matrix to immobilize GOD in a neutral aqueous solution.

The electrochemical quartz crystal microbalance (EQCM) is a powerful tool to quantify the immobilized enzyme and monitor in situ various modification procedures for the fabrication of an enzyme electrode [26]. The EQCM, with a piezoelectric quartz crystal (PQC) sensor as its central sensing element, can detect a change in mass loading on the electrode surface down to the monolayer or submonolayer level, the viscoelasticity of a foreign film modified on the electrode, and the solution viscodensity near the electrode during an electrochemical perturbation [27–30]. In vivid contrast to the common research motivation, we here attempt the utilization of PDFO as an enzyme-immobilization matrix for the first time, and the developed enzyme (here GOD) electrode exhibits good performance toward glucose sensing. The EQCIA method is used here to track various modification processes for better understanding of them.

2. Experimental

2.1. Instrumentation and reagents

All electrochemical experiments were conducted on a CHB660 electrochemical workstation (CH Instrument Co., USA). A conventional three-electrode cell was used. A computer-interfaced HP4395A impedance analyzer was used in the EQCIA experiments [30]. AT-cut 9 MHz quartz crystals of 12.5 mm crystal diameter were used in experiments. The gold electrode with 6.0 mm diameter on one side of the PQC contacted the solution and served as the working electrode, while the other side of the PQC was located in air. The reference electrode was a KCl-saturated calomel electrode (SCE), and all the potentials in this paper are reported versus this reference electrode. A carbon rod served as the counter electrode.

Glucose oxidase (GOD, EC 1.1.3.4; type II from Aspergillus niger, activity ≈ 100,000 U g⁻¹) was purchased from Amresco. Dopamine hydrochloride was purchased from Fluka. Lysozyme was purchased from Shanghai Chemicals Co. Glucose was purchased from Shanghai Chemicals Reagent Station. Other chemicals were of analytical grade, and all the solutions were prepared using double-distilled water.

2.2. Procedures

For cleaning the gold electrode surface, one drop of H₂SO₄ + H₂O₂ (3:1, v/v) was added on the electrode surface for 15 s, and then the electrode was rinsed thoroughly with double-distilled water and dried with a stream of pure nitrogen. The treatment was generally repeated thrice. Prior to electrochemical experiments, the gold electrode was subjected to continuous potential cycling (0–1.5 V, 20 mV s⁻¹) in 0.2 M aqueous phosphate (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use. Amperometric measurements of the enzyme electrode were carried out in a neutral phosphate buffer (pH 7.0) + 30.0 mM DA + 5.00 mg ml⁻¹ GOD aqueous solution for 20, 30, 40 or 60 cycles (not plotted). The resulting enzyme electrodes were thoroughly washed with double-distilled water and stored in 0.10 M aqueous K₂HPO₄–KH₂PO₄ and 0.10 M K₂SO₄ (pH 7.0) at 4 °C for future use.
phosphate buffer solution (pH 7.0) containing 0.10 M aqueous K$_2$HPO$_4$–KH$_2$PO$_4$ and 0.10 M K$_2$SO$_4$ under solution-stirred conditions, and the response current was marked with change value between the steady-state current and the background current. All experiments were performed at room temperature.

For comparison, matrices of PoAP and PNMP were examined in this work. The details of the construction of PoAP–GOD/Au electrodes will be given in Section 3.1. The PNMP/GOD/Au electrode was constructed as follows: (1) the “dry” frequency of a bare EQCM Au electrode was measured in air (here $f_{Na} = 9,020.853$ Hz); (2) 10.0 μl of 5.00 mg ml$^{-1}$ GOD aqueous solution (pH 7.0) containing 0.1 M N-methylpyrrole, and the PNMP deposition at the electrode was immediately conducted by applying a constant bias to the working electrode (0.85 V). The polymer growth was stopped when the frequency change was close to $-1.6$ kHz (exactly $\Delta f_{PNMP} = -1649$ Hz here); (4) the obtained PNMP/GOD/Au electrode was thoroughly washed with double-distilled water to remove any loosely bound enzyme and then dried with a stream of pure nitrogen, and its “dry” frequency ($f_{Na}/f_{PNMP}$ = 9,018,808 Hz) was recorded again. Thus, the immobilized enzyme can be quantified from the frequency decrease, i.e. $\Delta f_{GOD} = f_{PNMP}/f_{GOD} - f_{Na} - \Delta f_{PNMP} = -396$ Hz.

3. Results and discussion

3.1. Immobilization of GOD in PFDO and the comparisons with PoAP and PNMP matrices

Fig. 1 shows the simultaneous records of current (i), $\Delta f_0$, $\Delta R_1$ and $\Delta f_{GOD}$ during 40-cycle potential cycling in 20.0 mM aqueous K$_2$HPO$_4$–KH$_2$PO$_4$ (pH 7.0) containing 1.50 mM (a) and 30.0 mM (b) DA, respectively. It is seen from Fig. 1a that the current peak for the oxidation of DA to dopaminequinone in the first positive scan occurred at 0.17 V versus SCE ($P_{1/2}$), while in the first negative scan two current peaks at 0.10 ($P_{1}$) and $-0.28$ ($P_{2}$) V were observed, which can be attributed to the re-oxidation of reduced dopaminechrome. The cycle-by-cycle decrease in $f_0$ was obvious during the DA oxidation, however, the simultaneous increases in $R_1$ ($\Delta \delta/\Delta R_1 \approx -133$ Hz $\Omega^{-1}$) and $\Delta f_{GOD}$ ($\Delta \delta/\Delta f_{GOD} \approx -7$) were small, suggesting that a rigid film was growing at the Au electrode during the DA oxidation, and thus the accurate mass estimation is possible according to the Sauerbrey equation [27-30]. Simultaneously, the heights of all current peaks were decreased cycle by cycle, and the peak separations of $P_{1}$/$P_{1/2}$ and $P_{2}$/$P_{1/2}$ were also increased, indicating a gradual decrease in the electrode activity toward DA oxidation due to the deposition of a polymer layer as an electron-transfer barrier. In comparison with the cyclic voltammogram recorded in 1.50 mM DA solution, the current peaks for the reduction of dopaminequinone in 30.0 mM DA solution became more indistinct (Fig. 1b), being due probably to the faster polymer growth and thus a more significant decrease in the electrode activity.

Shown by Panel a in Fig. 2 is the in situ responses of $i$, $\Delta f_0$, $\Delta R_1$ and $\Delta f_{GOD}$ to 40-cycle potential sweep between $-0.6$ and 0.6 V versus SCE in the phosphate buffer (pH 7.0) containing 30.0 mM DA + 5.00 mg ml$^{-1}$ GOD. In contrast to the GOD-free case with a final frequency decrease of $-1.39$ kHz (Fig. 1b), the final decrease in the resonant frequency in the presence of GOD was as large as $-3.79$ kHz, indicating that the enzyme had been incorporated into the film, as further validated by the current response during subsequent glucose detection. Although larger increases of $\Delta R_1$ and $\Delta f_{GOD}$ were observed here, the absolute values of $\Delta \delta/\Delta R_1$ ($\approx -52$ Hz $\Omega^{-1}$) and $\Delta \delta/\Delta f_{GOD}$ ($\approx -5$) are still larger than their characteristic
values for a net viscous effect [27–30], and thus it is believed that the mass estimation via the Sauerbrey equation is still valid here.

To make clear the immobilization mechanism, we conducted a control experiment by replacing GOD with lysozyme, and the results are shown in Fig. 2b. Since it is well known that the isoelectric point is 4.3 for GOD [34] and 11 for lysozyme [35], GOD and lysozyme should carry negative and positive charges in the phosphate buffer (pH 7.0), respectively. As is seen from Fig. 2b, in the phosphate buffer (pH 7.0) containing 30.0 mM DA + 5.00 mg ml$^{-1}$ glucose oxidase (x) or lysozyme (b), $\Delta f = 20$ kHz $^{-1}$.

![Fig. 2. Simultaneous records of current ($\Delta i$), $\Delta f$, and $\Delta \delta$ during potential cycling in 20.0 mM K$_2$HPO$_4$–KH$_2$PO$_4$ (pH 7.0) + 30.0 mM DA aqueous solution containing 5.00 mg ml$^{-1}$ glucose oxidase (x) or lysozyme (b). $\Delta f_{AP} = 20$ kHz $^{-1}$.](Image)

The results are shown in Fig. 2b. Since it is well known that the isoelectric point is 4.3 for GOD [34] and 11 for lysozyme [35], GOD and lysozyme should carry negative and positive charges in the phosphate buffer (pH 7.0), respectively. As is seen from Fig. 2b, in the phosphate buffer (pH 7.0) containing 30.0 mM DA + 5.00 mg ml$^{-1}$ lysozyme, the change of frequency after 40 cycles was almost identical to that in the enzyme-free solution (Fig. 1b). This finding suggests that lysozyme, a cationic species in the neutral solution, was almost not incorporated into the film. Nevertheless, GOD, an anionic protein in the neutral buffer, should have been incorporated into the film, as indicated by the larger frequency decrease observed in Fig. 1b than in Fig. 1a. Therefore, it is believed that the incorporation of GOD into the polymer during the oxidation of DA proceeded mainly through the electrostatic affinity, namely, the possible anionic “doping” effect during the growth of polymer chains that might be positively charged then.

It is very interesting to estimate the mass of the entrapped GOD. Because of the electric insulation of the PFDO film, its growth was self-limiting. According to the Sauerbrey equation, the mass values of rigid PFDO ($\Delta m_{PFDO} = 2.20$ µg) equivalent to the observed frequency decrease of 1.39 kHz shown in Fig. 1b) and PFDO + GOD ($\Delta m_{PFDO+GOD} = 6.00$ µg, equivalent to the observed frequency decrease of 3.79 kHz shown in Fig. 2a) can be obtained. So the mass of the immobilized GOD was roughly estimated to be 3.80 µg via a comparison between the two mass values.

Moreover, in this work the PFDO was replaced with PoAP, a common enzyme-immobilization matrix that has been widely used in constructing enzymatic amperometric sensors [36,37], for comparatively discussing their performances in immobilizing GOD. We constructed a PoAP–GOD/Au electrode in 20.0 mM aqueous K$_2$HPO$_4$–KH$_2$PO$_4$ (pH 7.0) containing 10.0 mM o-aminophenol and 5.00 mg ml$^{-1}$ GOD by cyclic voltammetric method (0–0.9 V, 20 mV s$^{-1}$, 40 cycles), and a PoAP/Au electrode was similarly prepared in the absence of GOD too. The mass of immobilized GOD was similarly estimated to be 0.980 µg. The linear calibration range of the PoAP–GOD/Au biosensor was from 0.050 to 6.0 mM, with a slope of 0.56 µA mM$^{-1}$ cm$^{-2}$ and a correlation coefficient of 0.9933. If the concentration of o-aminophenol was increased to 30 mM for similar potential cycling, the amount of electrode-captured PoAP ($\sim$2730 Hz) became equivalent to that of the above PFDO deposition, and the mass of immobilized GOD here was similarly estimated to be 1.8 µg. However, the current response to glucose became inconspicuous.

These findings can be explained by the fact that the PoAP has a ladder structure and thus the insulating PoAP film may be easily developed to a compact network structure, making the permeation of glucose and enzymatically generated H$_2$O$_2$ more difficult, especially at a higher o-aminophenol concentration that is favorable for depositing a relatively thick film [38]. In contrast to the PoAP film, the PFDO is melanin-like amorphous deposits, as proven by other reports and our scanning electron microscope examinations [23–25], and thus it exhibits good penetrability even for a relatively thick film with more immobilized GOD and higher anti-interferent permselectivity, namely, a higher glucose-detecting sensitivity is expected for a relatively thick PFDO film. In addition, to experimentally evaluate the H$_2$O$_2$ penetrability across the PoAP and PFDO films, we recorded the currents for oxidation of 2.5 mM H$_2$O$_2$ at 0.6 V at electrodes modified with 732-Hz PoAP and 886-Hz PFDO, respectively. The current at the former electrode was found to be 1.55 µA, while that at the latter electrode was 9.65 µA, proving the above expectation that the thicker PoAP film is notably unfavorable for the permeation of H$_2$O$_2$. Therefore, the advantage of the PFDO is noticeable for its capability of immobilizing more...
enzymes or other biological molecules at a smaller sacrifice of the electrode activity for detecting H$_2$O$_2$.

Also, we constructed a PNMP/GOD/Au electrode following the procedures given in Section 2 for comparison. The sensitivity of the PNMP/GOD/Au enzyme electrode was found to be as small as 9.33 nA mM$^{-1}$ cm$^{-2}$. If the immobilization of GOD was conducted by direct potential-cycling codeposition (0.0–0.9 V versus SCE, 20 mV s$^{-1}$) with PNMP in 20.0 mM phosphate buffer (pH 7.0) + 30.0 mM N-methylpyrrole + 5.00 mg ml$^{-1}$ GOD, the sensitivity of so prepared enzyme electrode was found to be as small as 1.24 nA mM$^{-1}$ cm$^{-2}$ under our experimental conditions. We note that small responses of saturated currents (nA-scale) to glucose were also reported previously for polypyrrole-GOD amperometric biosensors [39,40]. These observations should suggest that the PFDO is a very good matrix to immobilize GOD, being due probably to its high biocompatibility with GOD and favorable porosity for the permeation of substrate and H$_2$O$_2$.

3.2. Effects of concentrations of phosphate buffer, DA and GOD in the polymerization bath on immobilization of GOD for glucose sensing

In order to optimize the polymerization bath for immobilization of GOD and glucose sensing, effects of concentrations of phosphate buffer, DA and GOD were investigated. We conducted 40-cycle potential sweeps between −0.6 and 0.6 V at 20 mV s$^{-1}$ in a specified polymerization solution (see below), then the steady-state current ($i_{\text{ss}}$) response for each enzyme electrode constructed was measured at 0.6 V (versus SCE) in 0.10 M K$_2$HPO$_4$–KH$_2$PO$_4$ + 0.10 M K$_2$SO$_4$ aqueous solution (pH 7.0) containing 10.0 mM glucose. Total f$0$ and resistance ($\Delta R_1$) for the cyclic voltammetric polymerization (40 cycles) are also shown here, see text for operation details.

Effect of concentration of the phosphate buffer (pH 7.0) is depicted in Fig. 3a, where 20.0 mM DA and 5.00 mg ml$^{-1}$ GOD were always involved in the polymerization bath. The $i_{\text{ss}}$ increased with concentration of the phosphate buffer from 1.00 to 5.00 mM, and then decreased with its further increase. Meanwhile, both frequency ($\Delta f_0$) and resistance ($\Delta R_1$) exhibited response tendencies similar to the glucose-detecting current. The findings may be explained as follows. With the increase of concentration of the phosphate buffer, the mass of PFDO was increased as a result of the favorable polymerization at an elevated concentration of supporting electrolyte, and the mass of immobilized GOD was increased accordingly. However, with the further increase of concentration of the phosphate buffer, the competitive codeposition of phosphate anions might decrease the mass of immobilized GOD and the glucose-detecting current to some extent, since the phosphate as anions might also be incorporated in the PFDO film. So 20.0 mM phosphate buffer is chosen as the optimal concentration for constructing the enzyme electrode in this work.

Effect of concentration of DA is depicted in Fig. 3b, where 20.0 mM phosphate buffer and 5.00 mg ml$^{-1}$ GOD were always involved in the polymerization bath. As is seen, $\Delta f_0$, $\Delta R_0$, and $\Delta R_1$ increased with DA concentration from 5.00 to 30.0 mM, and then decreased with its further increase. The observations may be explained by the fact that with the increase of DA concentration from 5.00 to 30.0 mM, the mass of immobilized GOD increased due to the increase of current density. However, with the further increase of DA concentration, the immobilization of GOD turned to be more difficult, being due probably to a too rapid deposition rate of PFDO, as is seen from Fig. 1. Similar phenomenon was also reported for a polypyrrole/GOD biosensor [40]. So 30.0 mM DA concentration of the maximum current response is chosen in this work.

Effect of concentration of GOD is depicted in Fig. 3c, where 20.0 mM phosphate buffer and 30.0 mM DA were always involved in the polymerization bath. As is seen, $\Delta f_0$, $\Delta R_0$, and $\Delta R_1$ increased with the increase in GOD concentration from 1.00 to 5.00 mg ml$^{-1}$, and then decreased with its further increase. These responses may be explained as follows. The quantity of PFDO-entrapped GOD should be increased with the increase in GOD concentration, which improved the response...
Fig. 4. Effect of applied potential on the stable response current of the enzyme electrode in 0.10 M K2HPO4–KH2PO4 + 0.10 M K2SO4 aqueous solution (pH 7.0) containing 2.0 mM glucose (a) and effect of solution pH on the stable response current of the enzyme electrode at 0.6 V vs. SCE in 0.10 M K2HPO4–KH2PO4 + 0.10 M K2SO4 aqueous solution containing 2.0 mM glucose (b). The solution pH was changed by adjusting the molar ratio of K2HPO4–KH2PO4.

3.3. Effects of the applied potential and solution pH

The effect of the applied potential from 0.2 to 0.8 V on the enzyme electrode response was studied in 0.10 M phosphate buffer solution (pH 7.0), and the corresponding current response of the PFDO–GOD/Au electrode in 2.0 mM glucose is shown in Fig. 4a. The response current increased with the increase of the applied potential from 0.2 to 0.6 V, and the response current was saturated since 0.6 V. By taking into account the response sensitivity, selectivity and operational stability, 0.6 V is selected in subsequent experiments. Also, the effect of the pH value of the detection solution on the response behavior of the PFDO–GOD/Au electrode was investigated, and the result is shown in Fig. 4b. By considering the maximum response sensitivity and the possible clinical application of the sensing protocol at the physiological pH, pH 7.0 is selected for following experiments.

3.4. Effect of electroactive interference

Fig. 5 shows the response currents of AA at various electrodes at the operational potential of 0.6 V. As is seen, the response current of AA was obvious at the bare Au electrode, whereas the observed response to AA at the PFDO or PFDO–GOD modified electrode was very small. The similar situation was also observed for uric acid. The effects of electroactive interferents on the response current were really not obvious in the presence of the PFDO film, being due probably to the significant cationic permselectivity of PFDO to inhibit the electroactivity of anions on it [24]. Therefore, the PFDO–GOD/Au electrode has an intrinsic anti-interferent nature against the electrooxidation of electroactive anions, without the requirement of outer-layer modification of any other permselective films, e.g. Nafion.

3.5. Electrode response characteristic and enzymatic activity of immobilized GOD

Fig. 6 shows typical steady-state current responses of the enzyme electrode to successive additions of glucose under the optimized experimental conditions. The current response of the...
enzyme electrode increased along with glucose concentration. The linear range of the biosensor was from 0.05 to 9 mM, with a slope of 3.81 μA mM\(^{-1}\) cm\(^{-2}\) and a linearity correlation coefficient of 0.9945. The lower detection limit of 3 mC was obtained at a signal to noise ratio of 3. Moreover, the enzyme electrode reached 96% of the steady-state current within 15 s, which allows the convenient quantification of glucose. The improvement of response time is due probably to the fact that the non-conducting PFDO film is highly permeable to H\(_2\)O\(_2\) and allows the convenient quantification of glucose. The improved efficacy of the immobilized GOD here may at least be ascribed to the following factors: (1) possible deactivation of GOD during its immobilization; (2) obvious oxygen depletion via the enzymatic reaction, but its limited and slow supply from outside solution due to the polymer barrier; (3) partial capture of the enzymatically generated H\(_2\)O\(_2\) by the electrode (the others might move into the stirred solution).

According to our recent work on glucose sensing based on a new poly(o-phenylenediamine)/GOD–glutaraldehyde/Prussian blue/Au electrode [26], though here we detected the oxidation current of enzymatically generated H\(_2\)O\(_2\), while previously we detected the reduction current of Prussian blue re-generated through the catalyzed oxidation of Prussian white by enzymatically generated H\(_2\)O\(_2\). The low effective activity of the immobilized GOD here is estimated to be 3.7%, according to Eq. (3) with ESA\(_n\) = 1.43 kU g\(^{-1}\) and ESA\(_o\) = 38.5 kU g\(^{-1}\), where ESA\(_o\) is the enzymatic specific activity of native GOD under our experimental condition [26].

\[
\text{ERA} = \frac{\text{ESA}_o}{\text{ESA}_n}
\]  

(3)

The ERA value obtained here is comparable with the reported one of 6.2% for a poly(o-phenylenediamine)/GOD–glutaraldehyde/Prussian blue/Au electrode [26], though here we detected the oxidation current of enzymatically generated H\(_2\)O\(_2\), while previously we measured the reduction current of Prussian blue re-generated through the catalyzed oxidation of Prussian white by enzymatically generated H\(_2\)O\(_2\). The effective activity of the immobilized GOD here may at least be ascribed to the following factors: (1) possible deactivation of GOD during its immobilization; (2) obvious oxygen depletion via the enzymatic reaction, but its limited and slow supply from outside solution due to the polymer barrier; (3) partial capture of the enzymatically generated H\(_2\)O\(_2\) by the electrode (the others might move into the stirred solution).

In order to estimate the effective ESA of the immobilized GOD, the saturated state of enzyme reaction was chosen. The hydrogen peroxide produced in the 60-s enzymatic reaction under our experimental conditions, and the H\(_2\)O\(_2\) was amperometrically detected. The effective ESA can thus be written as:

\[
\text{ESA} = \frac{n_i H_2O_2}{\Delta m}
\]  

(1)

where \(n_i H_2O_2\) in μmol is the quantity of hydrogen peroxide produced in 60 s enzymatic reaction under our experimental conditions, and \(\Delta m\) in gram is the mass of enzyme (here GOD).

The Lineweaver Burk form of the Michaelis–Menten equation is

\[
\frac{1}{i_{ss}} = \frac{1}{i_{max}} + \frac{1}{A_{MMGOD} K_{MMGOD}} c
\]

where \(i_{ss}\) is the steady-state current after the addition of substrate, \(i_{max}\) the maximum current under substrate-saturated conditions, \(c\) the concentration in mM of substrate (glucose), and \(A_{MMGOD}\) the Michaelis–Menten constant. By drawing a plot of \(i_{ss}\) versus \(K_{MMGOD}\) and then linearly regressing the data according to this equation, we obtained a slope value of 0.559 mM μA\(^{-1}\) and an intercept value of 0.0501 μA\(^{-1}\), with a linearity correlation coefficient of 0.9996. Thus, the value of \(K_{MMGOD}\) is calculated to be 9.6 mM. This value is smaller than the
Fig. 7. Stability of the constructed enzyme electrode under storage conditions (in 0.10 M K2HPO4–KH2PO4 + 0.10 M K2SO4 aqueous solution of pH 7.0 at 4°C). The measurements were conducted in the phosphate buffer (pH 7.0) solution containing 5.0 mM glucose at 0.6 V vs. SCE.

reported one (24 mM) [42], indicating that the GOD immobilized in the PFDO well retained its bioactivity and possessed a high biological affinity to glucose.

3.7. Stability of the PFDO–GOD/Au electrode

The stability of the constructed enzyme electrode under storage conditions (in 0.10 M K2HPO4–KH2PO4 + 0.10 M K2SO4 aqueous solution of pH 7.0 at 4°C) was studied in the phosphate buffer containing 2.0 mM glucose, as shown in Fig. 7. After 1 week the biosensor retained 96% of its initial sensitivity. With the experiment prolonged the decrease of the response current was observed. However, even after another 3 weeks the biosensor retained 86% of its original response, which is equally satisfactory or a little better compared with other reports [20,26].

4. Conclusions

In this work, we have proposed a non-conducting film grown from DA oxidation as a new and effective matrix for immobilization of glucose oxidase. The electrochemical quartz impedance analysis method has been utilized to track various procedures involved in the immobilization process. An amperometric glucose biosensor of high response sensitivity, fast response time, and good selectivity and stability has been constructed. Also, the presented PFDO matrix has following interesting characteristics: (1) it can be electrodeposited to form a relatively thick film to immobilize more enzyme; (2) it is highly permeselective to H2O2 and well inhibits the electrooxidation of ascorbic acid and uric acid; (3) we also experimentally found that it can be conveniently removed by rinse with H2SO4 + H2O2 (3:1, v/v) for decades of seconds, enabling the recycling of the EQCM Au electrode. Consequently, the new immobilization matrix is strongly recommended for immobilization of many other enzymes. Although the PFDO has been suggested as a GOD-immobilization matrix better than commonly used PsoAP and PNMP, we speculate that it is very interesting to further increase the effective activity of immobilized GOD and the glucose-detection sensitivity using platinization [43], or other protocols. Such researches are in progress in this laboratory based on the new quantitative gauges of ESA and ERA.

Acknowledgements

This work was supported by the National Science Foundation of China (20275010, 20335020), the Basic Research Special Program of the Ministry of Science and Technology of China (2003CCCG0700), and the Foundation of the Ministry of Education of China (jiaorensi [2000] 26, jiaojisi [2000] 65).

References