A stable and controllable Prussian blue layer electrodeposited on self-assembled monolayers for constructing highly sensitive glucose biosensor

Xueping Ji,*[a] Jujie Ren,*[b] Ruixing Ni[a] and Xiaohong Liu[a]

Received 20th January 2010, Accepted 12th May 2010
DOI: 10.1039/c0an00019a

A stable and controllable Prussian blue (PB) layer electrodeposited on self-assembled monolayers (SAMs) of thioctic acid amide (T-NH_2) on gold electrode was prepared. The PB, combining with biocompatible matrix chitosan, was used for the highly efficient immobilization of glucose oxidase (GOD) for sensitive biosensing of glucose. The PB on SAMs exhibits a good stability and a much higher catalytic ability toward the reduction of hydrogen peroxide in neutral medium, compared with that of PB directly electrodeposited on bare Au electrode. The biosensor at an applied potential of 0.0 V exhibits good *anti*-interferent ability, with a low detection limit of 2 μM and a much higher sensitivity (59.9 mA M^{-1} cm^{-2}). The apparent Michaelis–Menten constant (K_m^{app}) and apparent activation energy of enzymatic reaction were found to be 5.4 mM and 24.1 kJ mol^{-1}. The present method provides a promising platform for the development of new biosensors and bioelectronic devices.

Introduction

The glucose biosensors based on immobilized glucose oxidase (GOD) on electrode surface have attracted special interest for researchers due to their great application in the clinical, environmental, and chemical analysis. The enzymatically generated H_2O_2 can be detected electrochemically by the electrooxidation of H_2O_2 at high applied potential.[1] However, many reduced species, such as ascorbic acid (AA), uric acid (UA) and so on, are easily co-oxidized at this potential and may produced interfering current.[2,3]

For now, increasing attention has been devoted to avoiding or decreasing such interferences to enhance sensitivity. The focus is mainly on two aspects. One is to adopt a redox mediator as a catalyst for reduction of H_2O_2, instead of its oxidation, to decrease the H_2O_2-detecting potential. Another is to use perm-selective layer to diminish or inhibit the electroactivity of interferents, while maintaining a high permeability of H_2O_2 across the layer. Peroxidase,[4,5] proteins containing heme groups[6,7] and hexacyanoferrate[8] are usually used as transducers for the reduction of H_2O_2 at low applied potential. Among them, Prussian blue (PB), a typical hexacyanoferrate, is an efficient redox mediator for selective reduction of H_2O_2. Its high chemical stability, facile preparation and rich electrochemical, optical and magnetic properties make it more extensive in the application of biosensor.[8–11] However, the PB was not stable on the surface of bare electrode at neutral solution, which limited its use in biosensor fabrication. Thus, it is necessary to find a suitable method for the immobilization of PB to improve its operational stability and to enhance its sensitivity for the reduction of H_2O_2 in neutral solution.

Self-assembled monolayers (SAMs) is a very general and powerful technique for modifying Au electrodes. SAMs, with amino groups, can offer an excellent microenvironment for the immobilization of PB and make PB stable due to electrostatic attraction between positively charged amino groups on the SAMs and negatively charged $[Fe(CN)_6]^{3-/4-}$ during the formation of PB layer. A disulfide-containing base, thioctic acid amide (T-NH_2), has distinct advantages for this purpose.[12–14] The disulfide-containing base yields two gold-sulfur bonds, and gives additional stability, compared with the single gold-sulfur bond formed by alkanethiols with a gold surface. Up to now, there are no reports about the PB electrodeposited directly on the SAMs of T-NH_2.

On the other hand, chitosan (Chit), a polyelectrolyte with amino groups, displays an excellent film forming ability, biocompatibility, high mechanical strength and a susceptibility to chemical modification, it has been extensively used for immobilization of enzymes to construct the amperometric biosensors in recent years.[3,15,16] Chitosan can not only provide a suitable microenvironment for enzyme, but also make PB more stable in neutral solution due to the protection of amino groups on PB.

Taking account of the advantages of SAMs, PB, and the biocompatibility of chitosan, constructing amperometric biosensor with them will be a good promising prospect of application. It is expected that the combination of PB and Chit will provide a suitable microenvironment for the immobilization of GOD and make the sensor stable and sensitive for sensing glucose. On this basis, we have developed a novel amperometric glucose biosensor based on Chit/GOD/PB/SAMs modified Au electrode. The proposed biosensor exhibits a highly sensitive and selective response toward glucose sensing.

Experimental

Reagents and chemicals

Chitosan (Chit, MW 1.5×10^5, 75% ~85% deacetylation) was obtained from Yuhuan Chemical Factory (Zhejiang, China). Glucose oxidase (GOD, from *Aspergillus niger*, 10000 unit mg^{-1}) obtained from Sigma, was used without further purification. Glucose (Glu), ascorbic acid (AA) and uric acid (UA) were

[a]*Department of Medical Chemistry, Hebei Medical University, Shijiazhuang, 050017, China. E-mail: xpji03@yahoo.com.cn; Tel: +86-311-86265593*
[b]*Department of Chemistry, School of Sciences, Hebei University of Science and Technology, Shijiazhuang, 050018, China. E-mail: renjujie@hebust.edu.cn.*

purchased from Tianjin Kaitong Chemical Reagent Co. Ltd. (China). Thioctic acid amide (T-NH_2), obtained from Aldrich Chemical Co. Inc., was dissolved in anhydrous ethanol. Glutaraldehyde (GA) solution (50%) was obtained from Tianjin Kemiou Chemicals Factory (China). Potassium ferricyanide was the product of Shanghai Chemical Reagent Factory (Shanghai, China). Hydrogen peroxide (H_2O_2, 30%, w/v solution) was purchased from Tianjin Yongda Chemical Reagent Development Center (China), and used as received. The exact concentration of H_2O_2 was obtained by titration with a standard potassium permanganate solution. All other chemicals were of analytical reagent grade, and used as received.

Phosphate buffer solutions (PBS, 0.10 M, pH 6.5) were prepared from disodium hydrogen phosphate and potassium dihydrogen phosphate (Tianjin Chemical Reagent Factory, Tianjin, China), with the pH adjusted with potassium hydroxide or phosphoric acid.

A 1.0 wt.% chitosan solution was prepared by dissolving 1.0 g of chitosan flakes into 100 ml of 1.0% acetic acid and stirred for 10 h at room temperature until complete dissolution. The chitosan solution was filtered by a 0.45 μm syringe filter, and then stored in a refrigerator when not in use.

A 1.0 M glucose stock solution was prepared in 0.10 M PBS (pH 6.5), and allowed to mutarotate for 24 h at room temperature before use.

Electrochemical measurement

All electrochemical experiments were carried out using a computer-controlled CHI 750C electrochemical workstation (CH Instruments, Chenhua Co., Shanghai, China) at ambient temperature (23 ± 2 °C). A conventional three-electrode system was used. The working electrode was a modified Au electrode, and the reference electrode was an Ag/AgCl (3 M KCl). The counter electrode was a platinum wire.

In amperometric experiments, the current-time data were recorded by applying a potential of 0.0 V on a stirred cell after a constant residual current had been established and a glucose solution was successively added into the buffer solution. The response current was marked with the change value of the steady state current and the background current.

Electrochemical impedance spectroscopy (EIS) measurements were carried out in the presence of 5.0 mM $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1 : 1) and 0.10 M PBS (pH 7.0) by applying an AC voltage with 5 mV amplitude in a frequency range from 5.0×10^{-2} to 1.0×10^5 Hz at the potential of 0.14 V.

Preparation of modified electrode

The gold disk electrodes (2 mm Φ) were carefully polished first with 1200 grit Carbimet disk, and then followed by 1.0, 0.3 and 0.05 μm alumina slurry on microcloth pads. After removing the trace alumina from the surface by rinsing with water at each step, the electrodes were ultrasonicated for 10 min in a fresh Piranha solution ($H_2SO_4 : H_2O_2 = 3 : 1$(v/v)). *Warning*: *Piranha solution reacts violently with organic solvents.* The electrodes were then ultrasonicated in water, acetone, and water again for 5 min, respectively. After being rinsed with water and dried, the electrodes were incubated in a 10 mM anhydrous ethanol solution of T-NH_2 at 4 °C for *ca.* 15 h to form monolayer and introduce amino groups on the surface of gold electrodes. The modified electrodes were rinsed in turn with ethanol and water, to remove any physically adsorbed T-NH_2. Then electrochemical deposition of PB on the surface of modified electrodes was carried out by potential cycling in an unstirred fresh 0.050 M $K_3[Fe(CN)_6]$ + 0.050 M $Fe_2(SO_4)_3$ + 0.10 M K_2SO_4 + 0.050 M H_2SO_4 solution in the potential range of $-0.05 \sim +0.5$ V at a scan rate of 20 mV s^{-1}. The amount of the PB electrodeposited on SAMs-modified electrode was controlled by scanning time, which usually took *ca.* 10 cycles to obtain the optimal amount of the PB. The PB/SAMs/Au electrode was then rinsed carefully with water and dried. After that, an aliquot of 5 μL of 5.0 mg mL^{-1} GOD solution was dropped onto the surface of the PB/SAMs/Au electrode and dried in a refrigerator at 4 °C. Then an aliquot 5 μL of 1% chitosan solution was dropped onto the surface of the GOD/PB/SAMs/Au electrode and dried. Finally, the enzyme electrode without rinse was dip-coated by 5 μL of 0.25% glutaraldehyde (GA). After 30 min, the resulting Chit/GOD/PB/SAMs/Au electrode was thoroughly washed with water, and then stored in 0.10 M PBS (pH 6.5) at 4 °C for future use.

Results and discussion

Electrochemical impedance characterization of PB-modified electrodes

Electrochemical impedance spectroscopy (EIS) is an effective method to probe the features of surface-modified electrodes.[17–19] In order to obtain more information about the electrical properties of interfaces between electrodes and solution, the working electrode was modelled as an equivalent circuit (inset of Fig. 1), as already used for other modified electrodes.[20] In the equivalent circuit, it was assumed that the resistance to electron transfer (*R*et) and the diffusion impedance (*W*) were both in parallel to the interfacial capacity (*C*dl), which are in series with an electrolyte solution resistance *R*s. This parallel combination of *R*et and *C*dl gave rise to a semicircle in the Nyquist plot of Z'' *vs.* Z'. When various substances are assembled on the surface of the electrode, the resistance value varies accordingly.[20] In this paper, the assembly process of Chit/GOD/PB/SAMs layers on the Au electrode was monitored by EIS experiment, and the results are shown in Fig. 1. At a bare gold electrode, only a very small semicircle could be observed, showing a low transfer resistance (Fig. 1(a)). For T-NH_2 modified electrode, the diameter of the semicircle increased greatly (Fig. 1(b)), implying that the SAMs of T-NH_2 obstruct electron-transfer of the redox probe. The results indicate that the SAMs inhibit penetration of the $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ redox species toward the electrode. After the electrodeposition of PB, the interfacial resistance decreased greatly (Fig. 1(c)), indicating that the PB layer forms high electron conduction pathways between the electrode and the redox couple, and promoted the electron transfer of the redox couple ($[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$). This may be ascribed to the excellent conductivity of the PB and facilitated electron-transfer rate. When GOD was assembled on the PB layer, the interfacial electron-transfer resistance increased (Fig. 1(d)). This result is attributed to the non-conductive properties of GOD, which

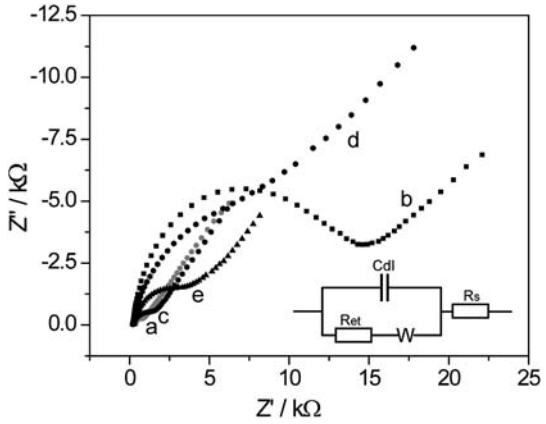

Fig. 1 The Nyquist plot of various modified electrodes in the presence of 5.0 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ and 0.10 M PBS (pH 7.0). The frequency range was 5.0×10^{-2} to 1.0×10^{5} Hz. (a) ● Bare Au electrode, (b) ■ SAMs/Au electrode, (c) ● PB/SAMs/Au electrode, (d) ⬣ GOD/PB/SAMs/Au electrode, and (e) ▲ Chit/GOD/PB/SAMs/Au electrode. Inset is the Randles circuit.

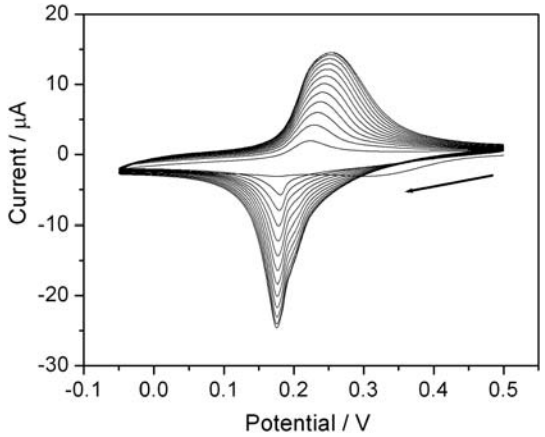

Fig. 2 Cyclic voltammograms of SAMs-modified Au electrode in a solution containing 0.050 M $K_3[Fe(CN)_6]$ + 0.050 M $Fe_2(SO_4)_3$ + 0.10 M K_2SO_4 + 0.050 M H_2SO_4. Scan rate, 20 mV s^{-1}.

hinders the access of the redox probe to the electrode, and obstruct electron-transfer of the redox couple. It is similar to the previous report.[19] With the assembly of Chit on GOD layer (Fig. 1(e)) the interfacial resistance decreased again, but it is larger than that of bare Au and PB layer, suggesting that Chit layer promoted the electron transfer compared with the GOD layer. This may be ascribed to the weak conductivity of chitosan film. These results above proved that SAM, PB GOD and Chit have been successfully assembled onto the Au electrode.

Cyclic voltammetry characterization of PB electrodeposited on the SAMs

Cyclic voltammetry was used to characterize the electrochemical behaviour of PB electrodeposited on the surface of modified electrode. Fig. 2 shows the cyclic voltammograms of the electrochemical deposition of PB on SAMs-modified Au electrode in 0.050 M $K_3[Fe(CN)_6]$ + 0.050 M $Fe_2(SO_4)_3$ + 0.10 M K_2SO_4 + 0.050 M H_2SO_4. It can be seen that a pair of well-defined redox peaks grow with potential cycling during the first 10 cycles in the potential range from −0.05 to +0.5 V, and then decreased gradually. The continuously increasing current indicated that PB was gradually accumulating around amino groups in the surface of SAMs-modified electrode, until the electrodeposition reached saturation state. The further electrochemical deposition of PB made peak currents decrease, which indicated that a thicker PB layer was unstable and easy to drop down from the surface of SAMs-modified electrode. The forming processes of PB layer can be inferred that $[Fe(CN)_6]^{3-}$ ions are attracted by positively charged amino groups, and gather together around them. The $[Fe(CN)_6]^{3-}$ is electroreduced to $[Fe(CN)_6]^{4-}$ while capturing an electron. Subsequently, the PB molecules are deposited around amino groups, while $[Fe(CN)_6]^{4-}$ combines with Fe^{3+} in solution.

Electrocatalytic reduction of H_2O_2 on PB-modified electrode

The insoluble form of PB, $Fe_4^{III}[Fe^{II}(CN)_6]_3$, can be reduced to soluble Prussian White (PW), $K_4\{Fe_4^{II}[Fe^{II}(CN)_6]_3\}$, at approximately −0.1 V *vs.* SCE.[21] The reduced form of PB (Prussian White) has a catalytic activity for the reduction of H_2O_2 and can chemically reduce H_2O_2 in an acidic solution,[22,23] thus acting as an electron-transfer mediator between the electrode and H_2O_2 generated by the enzymatic oxidation of glucose.[24] The mechanism of catalytic reduction of H_2O_2 on PB/SAMs/Au electrode can be expressed as the follows.[10,22]

$$Fe_4^{III}[Fe^{II}(CN)_6]_3 + 4\,K^+ + 4e^- \rightleftarrows K_4\{Fe_4^{II}[Fe^{II}(CN)_6]_3\} \quad (1)$$

$$K_4\{Fe_4^{II}[Fe^{II}(CN)_6]_3\} + 2H_2O_2 + 4H^+ \rightarrow Fe_4^{III}[Fe^{II}(CN)_6]_3 + 4\,K^+ + 4H_2O \quad (2)$$

Fig. 3 shows cyclic voltammograms of the PB/SAMs/Au electrode in the presence or absence of H_2O_2 at 20 mV s^{-1} in a pH 6.5 PBS. In the absence of H_2O_2, the PB/SAMs/Au electrode only gave the electrochemical response of PB itself (Fig. 3(a)). Upon addition of 4.0 mM H_2O_2, the cyclic voltammogram changed with an increase in the reduction current and a decrease in the oxidation current (Fig. 3(b)), displaying an electrocatalytic behavior of PB in response to the reduction of H_2O_2 at a low applied potential, which is possible to eliminate the interfering effect of oxidizable species, such as AA, UA and so on.

Thermal stability of GOD immobilized on modified electrode

Temperature is an important factor effecting activity of immobilized enzyme. The effect of temperature on the biosensor response to 0.50 mM glucose was evaluated from 10 to 60 °C, as shown in Fig. 4. It can be found that the response current increased with the increase of temperature, and reached a maximum value at *ca.* 50 °C, which may result from the increase of the activity of the immobilized GOD with the increase of temperature. Then, the enzyme activity decreased over 50 °C due to the deactivation of GOD at higher temperatures.

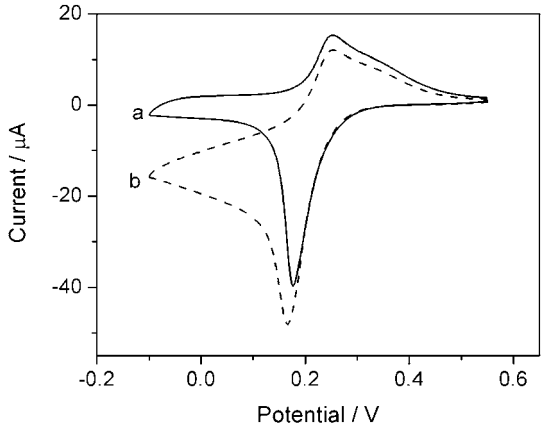

Fig. 3 Cyclic voltammograms of the PB/SAMs/Au electrode recorded in 0.10 M PBS + 0.10 M K_2SO_4 solution (pH 6.5) containing 0.0 (a) and 4.0 mM H_2O_2 (b). Scan rate, 20 mV s^{-1}.

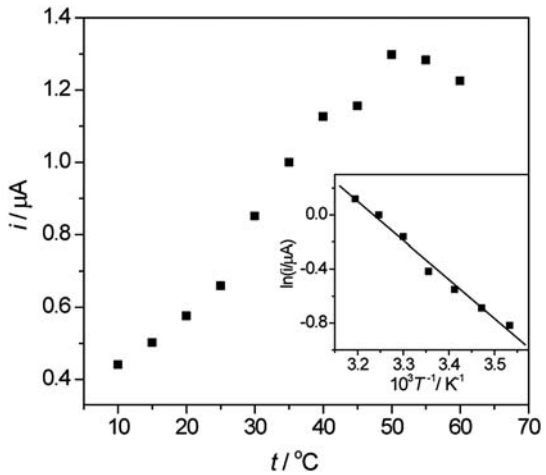

Fig. 4 Effect of temperature on the current response of the enzyme electrode in 0.10 M PBS + 0.10 M K_2SO_4 (pH 6.5) containing 0.50 mM glucose. Inset, the plot of ln i against $T^{-1} \times 1000$ between 10 and 40 °C. Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

According to the Arrhenius formula,

$$\ln k = \ln A - (E_a/RT) \quad (3)$$

where k is the rate constant, E_a is the activation energy, A is the frequency factor, R is the universal gas constant and T is the absolute temperature. Since the steady state current (i) is proportional to the rate constant k, the Arrhenius formula can be expressed as follows.[25]

$$\ln i = \ln i_o - (E_a/RT) \quad (4)$$

The apparent activation energy was calculated from the slope of Fig. 4 and found to be 24.1 kJ mol^{-1}, which is similar to 23.9 and 25.3 kJ mol^{-1} obtained by previous work.[26,27]

Effects of the applied potential and the solution pH

The effect of the applied potential on the enzyme electrode response was investigated in the range from −0.3 to +0.2 V in a PBS containing 1.0 mM glucose. The results showed that along with the decrease of the applied potentials the response current gradually increased and simultaneously operational stability decreased. No maximum current response was obtained. When the potential was below 0.0 V, the response currents of the electrode became obviously unstable. It is similar to the previous report.[28] Taking into account the response sensitivity, operational stability and effective avoiding interference, a potential of 0.0 V was selected as the applied potential for glucose sensing in further experiments.

The effect of the solution pH on the sensitivity of the biosensor is of great importance due to the bioactivity of GOD and the stability of PB depending on pH. The response behavior of the biosensor to 1.0 mM glucose in PBS with different pH values ranging from 5.0 to 8.0 was investigated, as shown in Fig. 5. The results showed that the response current increased from pH 5.0 to 6.5, and then decreased sharply from pH 7.0 to 8.0. The maximum response current was observed in pH range from 6.5 to 7.0. The decrease of the current response when pH more than 7.0 is attributed to the decrease of both PB stability[29,30] and GOD activity.[31] The increase of current response from pH 5.0 to 6.5 is due to more negative charges of GOD (pI = 4.2),[32] which makes the electrostatic interaction between GOD and Chit increase, and enzyme activity enhance. Considering the stability of PB and the practical biosensing applications, the PBS of pH 6.5 was selected as the optimal supporting electrolyte.

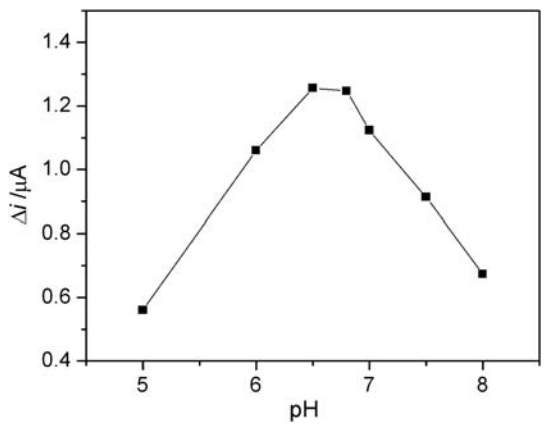

Fig. 5 Effect of the solution pH on the stable current response of the enzyme electrode in 0.10 M PBS + 0.10 M K_2SO_4 containing 1.0 mM glucose. Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

Effect of electroactive interference

Fig. 6 shows the current responses to the biosensor upon the injection of glucose and various electroactive interferences at the low applied potential. The results showed that upon addition of 0.10 mM AA or 1.0 mM UA the observed current responses were very small, contrast to the large response to the addition of 0.50 mM glucose, indicating that the effects of AA and UA on the response current are really not obvious at the low applied potential. It is consistent with the previous reports.[10,33] Additionally, the chitosan-crossed film, with small pore size and permselectivity, can restrain electroactive interferents from permeating into it. The response currents for chitosan-crossed film coated electrode and chitosan-crossed film free electrode to

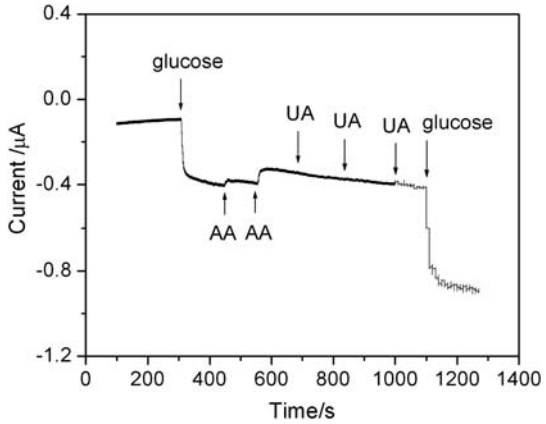

Fig. 6 Amperometric response obtained at the Chit/GOD/PB/SAMs/Au electrode in 0.10 M PBS + 0.10 M K_2SO_4 solution (pH 6.5) on successive injection of 0.50 mM glucose, 0.10, 0.20 mM AA, 0.10, 0.40, 1.0 mM UA, and then 1.0 mM glucose. Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

Table 1 Response current to H_2O_2 and various interferents at Au/SAMs/PB and Au/SAMs/PB/Chit electrodes

	Response current to substrates/nA[a]					
Electrode	AA	UA	AP	L-tyr	L-cys	H_2O_2
Au/SAMs/PB	110.8	20.0	3.3	53.4	3.3	333.1
Au/SAMs/PB/Chit	47.3	0.9	0.8	12.9	0.8	327.1

[a] Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

H_2O_2 and various interferents are investigated at an applied potential of 0.0 V, and the results are listed in Table 1. As seen from Table 1, the response currents decreased remarkably to ascorbic acid, uric acid, acetaminophen (AP), L-tyrosine (L-tyr) and L-cysteine (L-cys) after coating the electrode with chitosan-crossed film. However, the response current to H_2O_2 was no significant reduction. The results indicate that chitosan-crossed film is a good permselective film to diminish or inhibit interferents, while a high permeability for H_2O_2. The Chit/GOD/PB/SAMs/Au electrode is thus of good *anti*-interferent ability due to the low applied potential used and the selective permeability of analyte across the chitosan film.

Amperometric response of the biosensor toward glucose

In the presence of dissolved oxygen, H_2O_2 is generated during the oxidation of glucose catalyzed by GOD. The enzyme-dependent catalytic process can be expressed as follows.[34,35]

$$\text{GOD(ox)} + \text{glucose} \rightarrow \text{GOD(red)} + \text{gluconolactone} \quad (5)$$

$$\text{GOD(red)} + O_2 \rightarrow \text{GOD(ox)} + H_2O_2 \quad (6)$$

The H_2O_2 generated by the enzymatic oxidation of glucose increased correspondingly along with the increase of glucose concentration, and then H_2O_2 is reduced at PB-modified electrode and the intensity of reduction current is proportional to the concentration of glucose in solution. Therefore, the current response of the electrocatalytic reduction of H_2O_2 can be as the analytical signal for determination of glucose.

In this study, SAMs play an important role for the stability of PB. The effect of SAMs on the response of enzyme electrode was investigated. A SAMs-free enzyme electrode, as a controlling electrode, was prepared by direct electrochemical deposition of PB on the surface of bare Au electrode. Fig. 7 shows the amperometric response of Chit/GOD/PB/SAMs/Au electrode and Chit/GOD/PB/Au electrode at an applied potential of 0.0 V upon successive addition of glucose. It can be found that the amperometric response of SAMs-modified electrode was much higher, with a good stability in the presence of 0.10 mM glucose (Fig. 7(a)). In contrast, for the SAMs-free electrode the amperometric response decreased significantly, with very low signal-to-noise ratio (S/N) and low operating stability in the presence of 0.20 mM glucose (Fig. 7(b)), which confirmed that the SAMs in the biosensing interface could enhance the stability of PB and efficiently improve the electron transfer between analyte and electrode surface. The Chit/GOD/PB/SAMs/Au electrode exhibited a rapid and sensitive response to the change of glucose concentration.

The results above proved that the PB electrodeposited on the SAMs could make GOD have high activity and provide an efficient conduction pathway to accelerate electron transfer, compared whit that of PB on bare Au electrode.

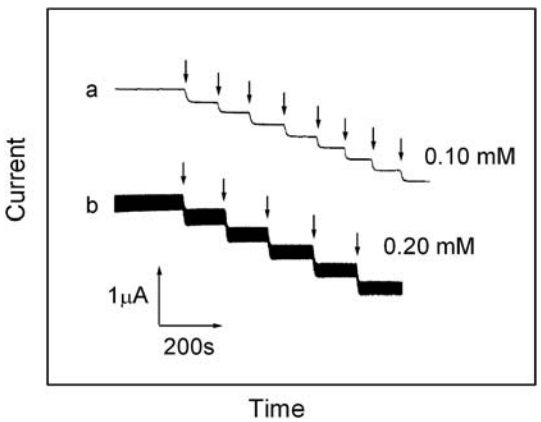

Fig. 7 Amperometric response of Chit/GOD/PB/SAMs/Au electrode (a) and Chit/GOD/PB/Au electrode (b) upon successive addition of 0.10 or 0.20 mM glucose to 0.10 M PBS + 0.10 M K_2SO_4 solution (pH 6.5) as indicated. Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

Fig. 8 shows the response of the biosensor to glucose concentration in PBS at an applied potential of 0.0 V. Upon the addition of an aliquot of glucose to the PBS, the reduction current increased and then reached a stable value. The modified electrode achieved 95% of the maximum steady-state current in less than 8 s, which demonstrated clearly that the electrocatalytic response was very fast.

As shown in Fig. 8 (inset), the biosensor signal was proportional to the glucose concentrations from 0.01 to 1.0 mM with a correlation coefficient of 0.9996. From the slope (1.88 μA mM^{-1}) of the calibration curve, a sensitivity of 59.9 mA M^{-1} cm^{-2} was obtained. Compared with the sensitivities reported for a PB based on chitosan and carbon nanotube by layer-by-layer self-assembly technique (8.017 mA M^{-1} cm^{-2})[28] and a poly(o-aminophenol)

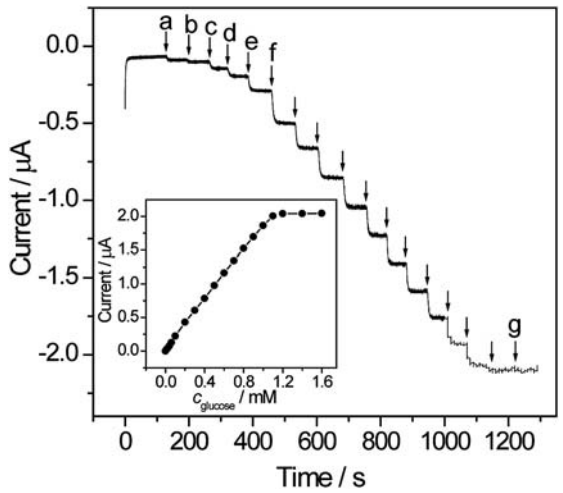

Fig. 8 Current-time curves obtained with the Chit/GOD/PB/SAMs/Au electrode upon successive addition of glucose (0.010 mM (a,b), 0.020 mM (c,d), 0.040 mM (e), 0.10 mM (f∼g)) in 0.10 M PBS + 0.10 M K_2SO_4 solution (pH 6.5). Inset, calibration curve of the biosensor as a function of glucose concentrations. Applied potential, 0.0 V *vs.* Ag/AgCl (3 M KCl).

and PB film modified Pt electrode (24 mA M^{-1} cm^{-2}),[36] the results we obtained in this study are better by factors of 7.5 and 2.5, respectively. With a further increase in glucose concentration, a plateau was observed, displaying the characteristics of Michaelis–Menten kinetics. The apparent Michaelis–Menten constant, K_m^{app}, a reflection of enzymatic affinity, can be calculated according to the Lineweaver–Burk equation.[37–39]

$$i^{-1} = i^{-1}_{max} K_m^{app}[S]^{-1} + i^{-1}_{max} \quad (7)$$

where [S] is the concentration of the substrate.

The apparent constant, K_m^{app}, for immobilized GOD was found to be 5.4 mM, which is smaller than an earlier reported value for free enzyme,[40] and even smaller than 10.5 mM for a PB based nonconducting poly(o-aminophenol) film electrode.[36] K_m^{app}approximates the affinity of enzyme for the substrate. A small K_m^{app}implies that the GOD in the modified electrode retains its catalytic activity and exhibits a higher affinity for glucose.

Stability of the Chit/GOD/PB/SAMs/Au electrode

The stability of enzyme electrode under storage conditions (PBS of pH 6.5 at 4 °C) was investigated in 0.10 M PBS + 0.10 M K_2SO_4 solution (pH 6.5) containing 0.50 mM glucose. The results showed that only 10% loss of the enzyme activity was observed in the first 2 weeks. Two weeks later, there was a slight decrease of the response current. However, 87% response current was still retained after 30 days. The good storage stability of the enzyme electrode can be attributed mainly to the biocompatible chitosan film. It can provide a suitable microenvironment to maintain the biological activity of the enzyme immobilized on the electrode. On the other hand, chitosan cross-linking by glutaraldehyde on the outer layer of the electrode forms a more compact network structure, which effectively prevents the leakage of the enzyme.

Conclusions

In this work, a highly sensitive glucose biosensor has been developed based on the efficient immobilization of GOD on a stable and controllable Prussian blue layer electrodeposited on the SAMs of T-NH_2, combining with biocompatible matrix chitosan. The PB electrodeposited on the SAMs could not only offer a friendly microenvironment for the immobilization of GOD but also efficiently improve the electron transfer between analyte and electrode surface, and the chitosan coated on the surface of the enzyme electrode was helpful for maintaining the activity of GOD and effectively preventing the leakage of the enzyme, enhancing the stability and sensitivity of the sensor. The resulting biosensor exhibited high *anti*-interferent ability, fast current response and extremely high sensitivity for biosensing glucose.

Acknowledgements

This work was financially supported by the Scientific Research Foundation of State Education Ministry of China (Jiaowaisiliu [2008] 890), the Merit-Based Science and Technology Project Foundation of Personnel Department of Hebei Province of China (No. 200702), and the Research Foundation of Education Department of Hebei Province of China (No. 2007132).

References

1 R. Garjonyte and A. Malinauskas, *Sens. Actuators, B*, 2000, **63**, 122–128.
2 M. Yang, Y. Yang, B. Liu, G. Shen and R. Yu, *Sens. Actuators, B*, 2004, **101**, 269–276.
3 B. Wu, S. Hou, F. Yin, J. Li, Z. Zhao, J. Huang and Q. Chen, *Biosens. Bioelectron.*, 2007, **22**, 838–844.
4 B. S. B. Salomi and C. K. Mitra, *Biosens. Bioelectron.*, 2007, **22**, 1825–1829.
5 Y. Song, L. Wang, C. Ren, G. Zhu and Z. Li, *Sens. Actuators, B*, 2006, **114**, 1001–1006.
6 J. Feng, G. Zhao, J. Xu and H. Chen, *Anal. Biochem.*, 2005, **342**, 280–286.
7 L. Chen and G. Lu, *J. Electroanal. Chem.*, 2006, **597**, 51–59.
8 L. Wang, S. Guo, X. Hu and S. Dong, *Colloids Surf., A*, 2008, **317**, 394–399.
9 Y. Xian, Y. Hu, F. Liu, Y. Xian, L. Feng and L. Jin, *Biosens. Bioelectron.*, 2007, **22**, 2827–2833.
10 C. Deng, M. Li, Q. Xie, M. Liu, Y. Tan, X. Xu and S. Yao, *Anal. Chim. Acta*, 2006, **557**, 85–94.
11 X. Wang, H. Gu, F. Yin and Y. Tu, *Biosens. Bioelectron.*, 2009, **24**, 1527–1530.
12 T. M. Willey, A. L. Vance, C. Bostedt, T. Van Buuren, R. W. Meulenberg, L. J. Terminello and C. S. Fadley, *Langmuir*, 2004, **20**, 4939–4944.
13 X. Ji, B. Jin, J. Jin and T. Nakamura, *J. Electroanal. Chem.*, 2006, **590**, 173–180.
14 X. Ji, J. Ren, J. Jin and T. Nakamura, *Biosens. Bioelectron.*, 2007, **23**, 241–247.
15 L. Gerbino, J. Riva, M. Strumia, R. A. Iglesias and A. M. Baruzzi, *Sens. Actuators, B*, 2008, **131**, 455–461.
16 Y. Du, X. Luo, J. Xu and H. Chen, *Bioelectrochemistry*, 2007, **70**, 342–347.
17 A. J. Bard and L. R. Faulkner, in *Electrochemical Methods: Fundamentals and Applications*, ed. John Wiley & Sons, Inc., New York, 2nd edn, 2001, ch.10, pp.368–388.
18 L. Wang and E. Wang, *Electrochem. Commun.*, 2004, **6**, 49–54.
19 W. Yang, J. Wang, S. Zhao, Y. Sun and C. Sun, *Electrochem. Commun.*, 2006, **8**, 665–672.
20 Y. Liu, F. Yin, Y. Long, Z. Zhang and S. Yao, *J. Colloid Interface Sci.*, 2003, **258**, 75–81.

21 A. Boyer, K. Kalcher and R. Pietsch, *Electroanalysis*, 1990, **2**, 155–161.
22 R. Garjonyte and A. Malinauskas, *Sens. Actuators, B*, 1998, **46**, 236–241.
23 I. L. Mattos, L. Gorton, T. Ruzgas and A. A. Karyakin, *Anal. Sci.*, 2000, **16**, 795–798.
24 W. Zhao, J. Xu, C. Shi and H. Chen, *Langmuir*, 2005, **21**, 9630–9634.
25 D. Shan, M. Zhu, H. Xue and S. Cosnier, *Biosens. Bioelectron.*, 2007, **22**, 1612–1617.
26 L. Li, Q. Sheng, J. Zheng and H. Zhang, *Bioelectrochemistry*, 2008, **74**, 170–175.
27 S. Mu, H. Xue and B. Qian, *J. Electroanal. Chem.*, 1991, **304**, 7–16.
28 Y. Zou, C. Xiang, L. Sun and F. Xu, *Electrochim. Acta*, 2008, **53**, 4089–4095.
29 Q. Chi and S. Dong, *Anal. Chim. Acta*, 1995, **310**, 429–436.
30 F. Ricci, A. Amine, G. Palleschi and D. Moscone, *Biosens. Bioelectron.*, 2003, **18**, 165–174.
31 S. Rauf, A. Ihsan, K. Akhtar, M. A. Ghauri, M. Rahman, M. A. Anwar and A. M. Khalid, *J. Biotechnol.*, 2006, **121**, 351–360.
32 J. H. Pazur and K. Kleppe, *Biochemistry*, 1964, **3**, 578–583.
33 L. V. Lukachova, E. A. Kotel'nikova, D. D'Ottavi, E. A. Shkerin, E. E. Karyakina, D. Moscone, G. Palleschi, A. Curulli and A. A. Karyakin, *Bioelectrochemistry*, 2002, **55**, 145–148.
34 M. V. Deshpande and D. P. Amalnerkar, *Prog. Polym. Sci.*, 1993, **18**, 623–649.
35 S. Berchmans, R. Sathyajith and V. Yegnaraman, *Mater. Chem. Phys.*, 2003, **77**, 390–396.
36 D. Pan, J. Chen, L. Nie, W. Tao and S. Yao, *Anal. Biochem.*, 2004, **324**, 115–122.
37 L. D. Mell and J. T. Maloy, *Anal. Chem.*, 1975, **47**, 299–307.
38 F. R. Shu and G. S. Wilson, *Anal. Chem.*, 1976, **48**, 1679–1686.
39 R. A. Kamin and G. S. Wilson, *Anal. Chem.*, 1980, **52**, 1198–1205.
40 B. E. P. Swoboda and V. Massory, *J. Biol. Chem.*, 1965, **240**, 2209–2215.

