Self-gelatinizable copolymer immobilized glucose biosensor based on Prussian Blue modified graphite electrode†

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A novel poly(vinyl alcohol) grafting 4-vinylpyridine self-gelatinizable copolymer was adapted to immobilize glucose oxidase. The reduction of hydrogen peroxide (H_2O_2) was detected at a Prussian Blue (PB) modified graphite electrode. A stable and sensitive glucose amperometric biosensor is described. The copolymer is a good biocompatible polymer in which the glucose oxidase retains high activity. Moreover, the copolymer can adhere firmly to the inorganic PB membrane. The sensor showed an apparent Michaelis–Menten constant of 18 ± 0.2 mM and a maximum current density of 1.14 μA cm^{-2} mM^{-1}. The linear range is from 5 μM to 4.5 mM glucose and the detection limit is 0.5 μM glucose. The catalytic efficiency of PB for the reduction of H_2O_2 is higher than that for the oxidation of H_2O_2. Glucose concentrations in serum samples from healthy persons and diabetic patients were determined using the sensor. The results compared well with those provided by the hospital using a spectroscopy method.

Introduction

Since the work of Clark and Lyons,[1] hundreds of papers have been published regarding the design of practical amperometric enzyme electrodes for the analysis of clinically important metabolites and industrial monitoring. Among these reports, two kinds of problems are always the main subject. One is the immobilization of enzymes. Different immobilization methods and materials have been developed including absorption, cross-linking, self-assembly methods and incorporation within carbon paste, conducting or non-conducting polymers, as well as different types of gels.[2] The aim of all these efforts was to obtain an easy handling method with cheap and stable materials which can retain high biological activity of the enzyme. In our laboratory, we synthesized a new self-gelatinizable copolymer, PVA-g-PVP, which is 4-vinylpyridine grafted on to the skeleton of poly(vinyl alcohol).[3] This copolymer can firmly adhere to the electrode surface. Using this copolymer, the immobilizing procedure for the construction of a biosensor is simple and gentle, just dropping an appropriate amount of the enzyme–copolymer complex onto the electrode surface and allowing water to evaporate at 4 °C. The retention of the biological activity of the enzyme was high because no cross-linking reagent or other harsh conditions were adopted.

However, a good biosensor depends not only on a good immobilization method but also on a sensitive transducer. This is the other focus in the development of amperometric enzyme electrodes. Until now, three generations of biosensors have been fabricated.

It is relatively easy and more convenient to fabricate a first generation biosensor than to construct other types of biosensors. Moreover, oxygen is the most efficient mediator. However, the detection of oxidation of H_2O_2 suffers from interference and is expensive because of the use of noble metal electrode materials. Recently, the detection of H_2O_2 on chemically modified electrodes gained considerable attention. Wang *et al.*[4,5] used ruthenium or iridium-dispersed carbon-paste electrodes to detect the reduction of H_2O_2. The interfering reactions are negligible because of the low operating potential. Prussian Blue (PB) is a well known catalyst for both the oxidation and reduction of H_2O_2.[6] The catalytic activity of a PB modified electrode towards H_2O_2 reduction was nearly as high as the activity of a platinum electrode, while it was insensitive to oxygen reduction when the deposition was optimized.[7] Therefore, several biosensors were constructed based on PB film modified electrodes.[8–11] All these electrodes used Nafion as an enzyme immobilization material. However, the organic solvents used for dissolving the polymer can be harmful to the enzyme activity. Moreover, the weak adhesion between the Nafion and the inorganic precipitate on smooth surfaces results in decreased stability of the sensor.[9]

In this study, we chose a graphite electrode to deposit a PB film, using a self-gelatinizable copolymer to immobilize the enzyme and to develop a stable glucose sensor. The characteristics of the sensor and the interference effects on its performance have been examined.

Experimental

Materials

Glucose oxidase (GOD, EC 1.1.3.4) from *Aspergillus niger* (VII, 250 U mg^{-1}) and 4-vinylpyridine were purchased from Sigma (St. Louis, MO, USA). Poly(vinyl alcohol) was PVA 124 (A. R., Guangzhou, China). All other chemicals were of analytical reagent grade or better. Doubly distilled water was used in all aqueous solutions. The phosphate buffered potassium salt (0.05 M KH_2PO_4 + 0.05 M K_2HPO_4 + 0.1 M KNO_3) was used as supporting electrolyte. The exact concentration of H_2O_2 was determined by titration against a standard potassium permanganate solution. Glucose stock solutions were left at room temperature for 24 h to mutarotate.

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Electrochemical measurements

The Bioanalytical Systems, Inc. (West Lafayette, IN, USA) BAS 100 B/W electrochemical analyzer and software package were employed for all voltammetric and chronoamperometric experiments. The galvanostatic deposition of PB film was carried out with an Eg & G Princeton Applied Research (Princeton, NJ, USA) PAR Model 173 potentiostat and galvanostat and Model 179 digital coulometer. A conventional three-electrode system was used. A Pt wire served as the auxiliary electrode. An Ag/AgCl electrode (saturated KCl) was used as the reference electrode against which all potentials were referred. All chronoamperometric determinations were performed under conditions of continuous stirring using a magnetic stirring bar at 25 ± 1 °C.

The blood samples were supplied by the Hospital of Jilin Province. The results supplied by the hospital were obtained with a Hitachi Ltd (Tokyo, Japan) 7150 automatic analyzer using a standard spectroscopy method.

Preparation of the Prussian Blue layer

The spectroscopic graphite rods (Φ = 5 mm) were impregnated with paraffin wax and then inserted into a Teflon tube. Before use, the graphite electrodes were polished with fine wet emery paper and 1.0 μm alumina–water slurry oxide power successively, ultrasonicated in distilled water and then dried at room temperature.

The PB films on the graphite electrodes were prepared in an equal volume mixture of 2 mM $FeCl_3$ and 2 mM $K_3Fe(CN)_6$ in 0.01 M HCl by means of a galvanostatic condition with a current density of 20 μA cm^{-2} for 30 s. After deposition the PB films were activated in 0.1 M KCl (pH 4.0) by cyclic potential scans between −0.1 and +0.5 V. The total coverage of PB at the electrode surface was calculated according to the literature.[6]

Preparation of enzyme membranes

The solution of 5% copolymer was prepared as previously described.[3] An amount of 3 mg GOD was dissolved in 20 μl of pH 6 potassium phosphate buffer, then 80 μl of 5% copolymer solution was added and mixed thoroughly. A volume of 10 μl of mixture was dropped onto the surface of the PB film modified electrode. Water inside this mixture was evaporated at 4 °C overnight. The resultant enzyme electrode was stored dry in a refrigerator at 4 °C.

Results and discussion

Optimisation of biosensor design

It is well known that PB film can be easily formed on different electrode materials such as platinum, In_2O_3 glass, glassy carbon and graphite electrodes. In the case of biosensors, especially using the detection of the reduction of H_2O_2, the response current will be interfered with by the reduction of oxygen at a platinum electrode even at 0 mV, while at glassy carbon and graphite electrodes the interference from O_2 can be ignored when the applied potential is higher than −200 mV. The electrochemical behavior of a PB film deposited on a glassy carbon electrode shows a better reversibility compared to the deposition on a graphite electrode. However, the PB film and the immobilized enzyme layer are less stable using glassy carbon electrodes. The surface of a graphite electrode contains more carboxylic acid groups, quinone groups and carboxyl groups compared to other carbon electrodes. These groups are of benefit in the formation of PB films.[12] Moreover, these groups are essential for the formation of hydrogen bonds with pyridine groups in the PVA-g-PVP immobilization material. The electrochemical active surface of a graphite electrode is much larger than a geometric one. The copolymer can partly adhere directly to the graphite electrode. This enhances the stability of the enzyme layer. Considering the stability and the catalytic effect of the PB layer deposited in different ways, we deposited the PB film galvanostatically in an equal volume mixture of 2 mM $FeCl_3$ and 2 mM $K_3Fe(CN)_6$ in 0.01 M HCl with a current density of 20 μA cm^{-2} for 30 s.

As shown in the literature,[9] the PB membrane was a better catalyst for hydrogen peroxide electroreduction than pretreated platinum and its catalytic efficiency was comparable to that of a biological catalyst. Our PB modified graphite electrode also shows this catalytic effect for the reduction of H_2O_2 as illustrated in Fig. 1. Oxygen shows no interference during this potential range in pH 4–8 phosphate buffer. However, the smooth inorganic surface of the PB membrane makes it difficult to attach the immobilized enzyme membrane. All publications recommend Nafion as the immobilization material when using a PB membrane. However, the organic solvents of the Nafion solution can be harmful to the enzyme activity. The self-gelatinizable grafting copolymer synthesized in our laboratory provided a new biocompatible material on which to immobilize the enzyme. The preparation method is as simple as using Nafion, but it is more gentle to the enzyme. From the IR spectroscopy results we can see the interaction of GOD and the copolymer. This enhances the stability of the enzyme layer and the activity of the immobilized enzyme is higher than the activity of immobilized enzyme crosslinked with glutaraldehyde and BSA.[13]

Characteristics of the electrode response

Effect of pH. As known from the literature, the presence of K^+ leads to an increased reversible voltammetric response of the PB membrane, especially for the peak appearing at 0.2 V. Therefore, we chose potassium phosphate electrolyte as the buffer solution. The amperometric measurements of glucose were carried out at a constant potential of −50 mV *vs.* Ag/AgCl using the PB copolymer–GOD graphite electrode. The sensitivities of a typical biosensor to glucose as a function of pH are shown in Fig. 2. No stable signal was observed in solutions of pH greater than 7.5 because the PB film is very stable in acidic and neutral solution, but unstable in alkaline media. The sensitivity of the sensor increased with increasing pH of the buffer from pH 5 to 7.5. As shown in our previous results,[13] the highest activity of the copolymer immobilized GOD was

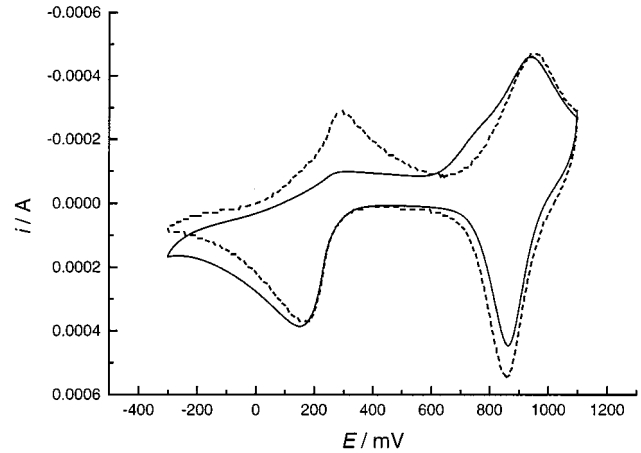

Fig. 1 Catalytic reduction and oxidation of 1 mM H_2O_2 on a PB-modified graphite electrode in pH 6 potassium phosphate buffer. Scan rate: 100 mV s^{-1}. (- - - -) buffer; (———) 1 mM H_2O_2.

obtained between pH 7–8. That is to say, the pH effect of the sensor was controlled by the enzyme layer. Considering the stability of the sensor, we chose pH 6.5 as the optimum pH value which is also the pH of physiological fluids.

Optimisation of potential. The cyclic voltammetry of hydrogen peroxide obtained at a PB modified graphite electrode (Fig. 1) shows the reduction of H_2O_2 in the range from +200 mV to −200 mV *vs*. Ag/AgCl. The oxidation of H_2O_2 starts at a potential of +700 mV *vs*. Ag/AgCl. The calibration curves of a typical glucose sensor carried out in pH 6.5 potassium buffer applying different potentials at −50 mV, +200 mV and +900 mV are shown in Fig. 3. The sensitivity of the sensor measured at +900 mV is lower than that at −50 mV. Applying a potential at +200 mV causes the lowest sensitivity.

The general catalysis mechanism presented in Fig. 4 points out that several reactions can limit the electrode response. Here, the stirring was assumed to be fast enough so that the diffusion of glucose and oxygen from the bulk solution was not the rate limiting step. The oxidation of β-D-glucose and the reduction of O_2 by GOD–$FADH_2$ is related to the enzyme activity. The reduction or oxidation of H_2O_2 is an electrocatalytic reaction. The enzyme activity within the enzyme layer is high enough to be not rate-limiting. Therefore, simple Michaelis–Menten enzyme kinetics with glucose as the substrate were assumed. At steady-state conditions, based on the equilibrium of the flux, the following equation is reasonable.

$$\frac{V_{max}[S]}{K_M^{app}+[S]} = k_{d,s}([S]-[S_0]) + k_s[H_2O_2]$$

$$k_{d,s}([S]-[S_0]) = k_{d,s}[H_2O_2]$$

$$v = k_s[H_2O_2]$$

Then

$$I = -\frac{I}{[S]}K_M^{app} + \frac{I_{max}}{\frac{k_{d,s}}{k_s}+1}$$

Here, $k_{d,s}$ is the transport rate constant of glucose ($k_{d,s}$ = 0.62 $D_s^{2/3}\omega^{1/2}\nu^{-1/6}$); k_s is the heterogeneous electron transfer rate constant, whose value is related to the applied potential and the catalyst; [S] and [S_0] are the glucose concentration in the bulk solution and at the electrode surface. Higher response means a higher k_s value. Therefore, the catalytic ability of PB for the oxidation of H_2O_2 at +900 mV is lower than that for the reduction of H_2O_2 at −50 mV. At a more negative potential, the catalytic efficiency of reduction is higher. As a consequence, −50 mV was chosen as the detection potential.

Under optimum conditions, current–time responses of a typical biosensor obtained by successive addition of a stock glucose solution to an air-saturated stirred potassium buffer solution are shown in Fig. 5. The response time was within 20 s. The calibration curve was linear from 5 μM to 4.5 mM glucose and the lowest detection limit was 0.5 μM based on a response/noise ratio higher than 3. The apparent Michaelis–Menten constant, K_m^{app} is 18 ± 0.2 mM, the maximum kinetic current I_{max} was 22 ± 0.5 μA mM^{-1}. These constants are calculated by using the Eadie–Hofstee graphical representation of duplicate reactions with linear-regression analysis and are verified by the Wilkinson statistical method. The corresponding maximum current density was 1.14 μA mM^{-1} cm^{-2}.

The stability of the glucose sensor was investigated by discontinuous monitoring for 60 d. The sensor was stored dry at 4 °C. The sensitivity of the sensor decreased by 25% within the first 2 d. Afterwards the decrease slowed down and the sensitivity remained constant. A total loss of 10% was observed after 2 months. We think the decrease in the first 2 d is caused by washing out the unstable part of the PB membrane and the adsorbed enzyme on the surface of the enzyme layer.

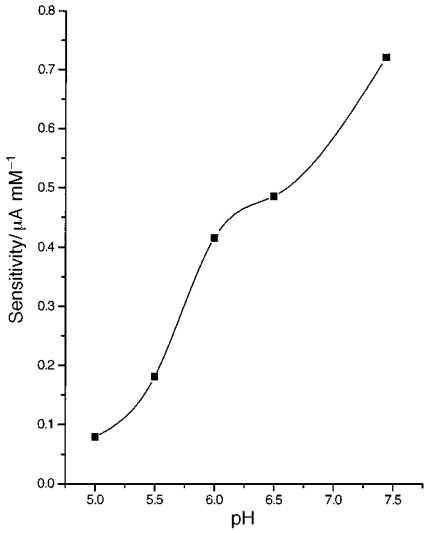

Fig. 2 Glucose sensitivities of a typical biosensor as a function of pH.

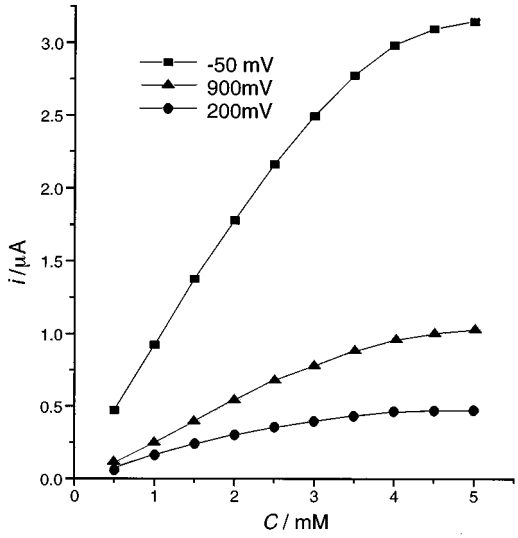

Fig. 3 Calibration curves of one typical sensor for glucose at different applied potentials.

Interference. The detection of H_2O_2 oxidation is always seriously interfered with by some components of blood. However, in some mediator type glucose sensors, the applied potential can be reduced to +200 mV and the interfence will be decreased. In our system the reduction of H_2O_2 can be detected at +200 mV, unfortunately the oxidation of ascorbic acid (AA) occurs at the same potential because the PB membrane is a good

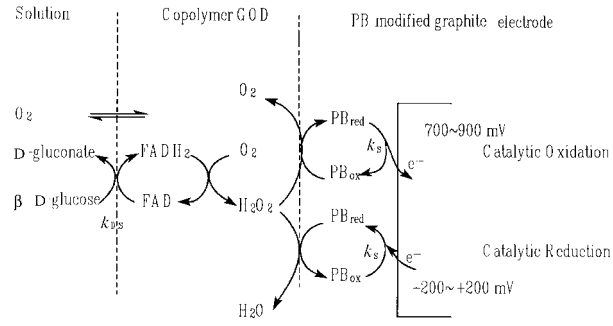

Fig. 4 Mechanism of a PB-modified copolymer immobilized GOD sensor.

catalyst for the oxidation of AA.[14] At a potential of −50 mV, no response of AA on pure PB electrode was observed, but when injecting AA after injecting H_2O_2, the response to H_2O_2 was reduced (Fig. 6B). The response of the sensor to glucose was interfered with by equal amounts of AA (Fig. 6C). The result of the same experiment done with a Nafion covered PB-copolymer GOD electrode was different (Fig. 6D). After the injection of AA, the response increased a little. The reason is that, when AA was injected, there were two reactions taking place in this system. One was the oxidation of AA by oxygen, leading to dehydroascorbic acid (DAA) and H_2O_2, and the other was the consecutive oxidation between AA and H_2O_2.

$$AA + O_2 \rightarrow DAA + H_2O_2 \quad (1)$$

$$AA + H_2O_2 \rightarrow DAA + 2\ H_2O \quad (2)$$

Without the Nafion film, the AA reacted with the enzymatic product H_2O_2 at the electrode surface. The Fe(III) in the PB membrane catalyzed this reaction,[15] which decreased the response of the sensor (Fig. 6B and C). When the enzyme sensor was covered with Nafion, the diffusion of AA was hindered by the Nafion layer, so that reaction (1) took place. The H_2O_2 produced according to reaction (1) catalytically reduced at the PB enzyme electrode surface, consequently increasing the response of the sensor. However, reaction (1) can not be catalyzed by the Fe from the PB membrane. The effect was weaker than that of reaction (2). This is the reason for the following result. When the concentration of AA was in 10-fold excess of that of glucose, the response of the Nafion covered sensor will also be reduced.[19] However, in blood, the concentration of AA is far lower than that of glucose. At the potential of −50 mV, the interfering currents caused by some common interferents such as ascorbic acid, acetaminophenol and uric acid can not be observed at concentrations above the respective physiological concentration ranges (Fig. 6A). The sensitivity of the sensor was significantly decreased by the Nafion layer. Therefore, we used the uncovered electrode in the real sample determination.

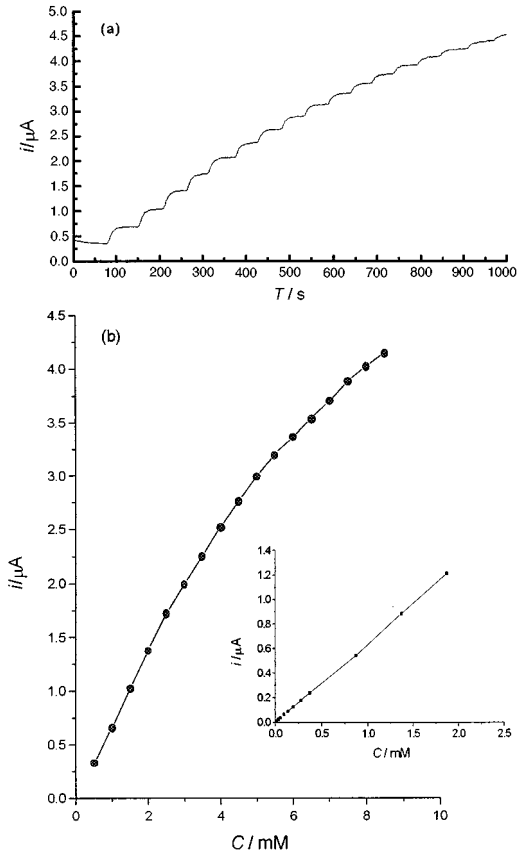

Fig. 5 (a) Current–time responses of a typical sensor for successive additions of a glucose standard solution to an air-saturated, stirred buffer solution (pH 6.5). Potential −50 mV. (b) Relevant calibration curve.

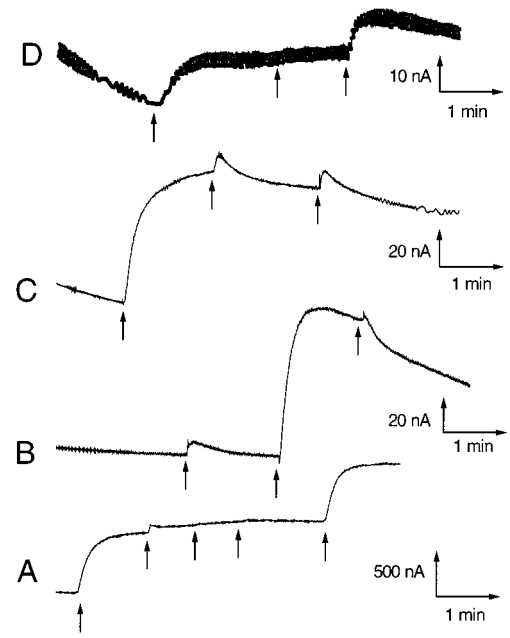

Fig. 6 Current–time curves of some interferents on the sensor. A, on GOD/PB graphite electrode 0.5 mM glucose, 0.1 mM AA, 0.2 mM acetaminophenol, 0.5 mM uric acid, 0.5 mM glucose; B, on BSA/PB electrode, 0.5 mM AA, 0.1 mM H_2O_2, 0.5 mM AA; C, on GOD/PB electrode, 0.1 mM glucose, 0.1 mM AA, 0.1 mM AA; D, Nafion/GOD/PB electrode, 0.1 mM glucose, 0.1 mM AA, 0.5 mM AA. Experimental parameters are the same as in Fig. 5.

Real sample determination. The glucose concentrations of serum samples from both healthy and diabetic persons were determined with the developed sensor at −50 mV. A volume of 100 μl untreated serum was injected into 5 ml of potassium phosphate buffer. The results are presented in Table 1. The determinations were also performed at +900 mV. The background currents from the interferents were eliminated by detecting the serum samples using another PB-copolymer-BSA electrode without GOD. The results from the detection at −50 mV compared well with those supplied by the hospital using a spectroscopy method.

Conclusion

In this study a new copolymer immobilized GOD sensor was fabricated by detecting the reduction of the enzymatic product on a PB modified graphite electrode. The stable, high activity copolymer immobilized enzyme layer adhered strongly to the inorganic PB membrane modified graphite electrode. The reason that the sensitivity changed with potential is discussed and the interference from high concentrations of AA at −50 mV

Table 1 Glucose concentrations of three untreated serum samples by different methods (mM)

	Serum 1	Serum 2	Serum 3
Hospital (spectroscopy)	5.0	17.3	7.6
Sensor 1 (−50 mV)	4.94	17.13	7.26
Sensor 2 (−50 mV)	5.16	17.08	7.42
Sensor 2 (900 mV)	5.35	15.86	6.57

is clearly explained. The sensor provides a simple, sensitive and practical tool for the determination of serum glucose.

Acknowledgments

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