

Planar Amperometric Glucose Sensor Based on Glucose Oxidase Immobilized by Chitosan Film on Prussian Blue Layer

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Abstract: A planar amperometric glucose microsensor based on glucose oxidase immobilized by chitosan film on Prussian Blue layer has been developed. The experimental results show that the optimum detection potential is 50 mV (versus Ag/AgCl) and the optimum pH is 6.5. Under the selective conditions the sensor exhibits excellent sensitivity of 98 nA/M and a linear range of 0.1-6.0 mM. The apparent Michaelis-Menten constant of the sensor is 21 mM. The response time is less than 60 seconds. No apparent change in the response to glucose was observed during one month. Foremost, the interference of ascorbic and uric acids can be avoided due to selective permeability of chitosan film and electrocatalysis of PB layer to H₂O₂. The sensor has been applied to detect glucose in human blood serum.

Keywords: Prussian Blue, Electrocatalysis, Chitosan, Selective permeability, Glucose sensor

Introduction

Amperometric biosensors for glucose are of great importance in bioanalysis. A great deal of attention has been paid in recent years to avoid the effects of electrochemically active interferences, such as ascorbic acid, acetaminophen and uric acid, usually present in biological fluids (1-3). Two different

approaches to avoid interference, reported in recent years, seem to be of a great importance in creating biosensor based on detection of hydrogen peroxide formed in the course of an enzyme-catalysed oxidation of glucose.

One approach is related to the use of selective membrane, which is permeable for hydrogen peroxide but impermeable for interference species. When placed between a sensing element, usually a platinum electrode, and an enzyme-containing layer, the selective membrane suppresses the penetration of interference species to the electrode surface. However, many of the known selective membranes, e.g., cellulose acetate membrane, do not ensure a full avoidance of interference (4-7). In our previous work, a double-functional chitosan membrane was first introduced to immobilize enzyme on the working electrode and to prevent the penetration of interferents such as uric and ascorbic acids (8).

Other approach to avoid the interference is selective electrocatalysis. When placed on an electrode surface, an electrocatalyst is able to diminish the overpotential of the anodic oxidation of hydrogen peroxide. As a result, hydrogen peroxide can be determined amperometrically at substantially lower electrode potential. Thus, a current response due to the presence of interferents can be almost eliminated.

In recent years, some metal hexacyanoferrate complexes were reported to be suitable electrocatalyst which can reduce the operating potential of glucose oxidase (GOD)-based sensor (9). An electrochemically deposited film of Prussian Blue (PB) has been found to be an excellent catalyst for H_2O_2 electroreduction exhibiting better properties than others such as Pt or horseradish peroxidase (10). Itaya et al. first found PB electrocatalytic properties for oxygen and H_2O_2 electrochemical reduction (11). The thin polycrystalline electrodeposited PB film has been intensively investigated (12-14) and applied as a mediator for biosensors design (15-18).

In the present paper, both approaches above mention were used. An amperometric glucose microbiosensor based on GOD immobilized by chitosan film on PB layer has been developed. The experimental conditions related to the preparation and characterization of the sensor have been studied in detail. The sensor exhibits excellent performances, in particular the interference of ascorbic acid and uric acid can be avoided due to selective permeability of chitosan film and electrocatalysis of PB film. The sensor has been applied to detect the glucose in human blood serum.

Experimental

Chemicals

All reagents were of analytic grade. All solutions were prepared with deionized water. GOD (23800 u/g, Sigma) and chitosan (Sigma) was used as received.

Microelectrode preparation

A 2-inch Si wafer with SiO_2 (1 μm) was used as the substrate. Each structure consists of a Ti (50 nm) layer, either Pt (200 nm) for working electrode (WE) and counter electrode (CE) or a 400-nm Ag layer for the reference electrode (RE) and finally a Ti (50 nm) layer. The two thin layer of titanium

served as adhesion promoters to the silicon dioxide and the following layer of silicon nitride. Metallic layers were evaporated by an electron gun at a substrate temperature of 25°C. Electrode geometry was patterned using the lift-off technique. Silicon nitride was chosen as the top insulator because it is known to be less permeable to water vapor and alkali ions than silicon dioxide. Reactive ion etching was used to etch away the silicon nitride in the areas of WE, CE, RE and their pads (Fig. 1). The wafer was immersed in a titanium etchant ($\text{H}_2\text{SO}_4/\text{H}_2\text{O} = 1/1$, 80°C) to ensure that there was no titanium left on the free surfaces. Partial chlorination of the Ag layer was performed at wafer level in 0.25 mol/L FeCl_3 solution.

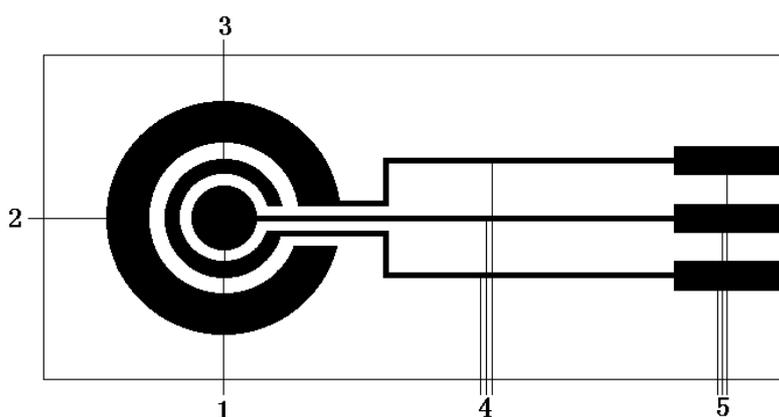


Figure 1. Schematic of the chip: 1) WE, 2) CE, 3) Ag/AgCl RE, 4) Conductive lines, 5) Pads.

After dicing the wafer, the individual chips were mounted on printed circuit boards (PCB) and wire bonding was done. An epoxy resin was used to encapsulate the sensor, leaving only the active area exposed.

PB layer electropolymerization

PB layer was prepared by cyclic voltammetric method. Before electrodeposition PB film, Pt disk electrode was chemically cleaned and rinsed in order of 2 mol/L of KOH, sulfuric acid, ethanol and deionized water. Then PB was electrodeposited on the Pt electrode by cyclic scan within the limits of 0 to 0.5 V in a fresh solution containing 1.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 2 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.1 M KCl and 2 mM HCl (pH 2.0). Before being used, the PB/Pt electrode was processed in 1 M KCl solution (pH 4) by cyclic scan in a rate of 50 mV/s from -0.2 to 0.5 V until stable response was established.

GOD immobilization

One gram of chitosan was added into 4 ml of acetic acid and stirred for 20 minutes until complete dissolution. Transfer one microliter of chitosan solution on the WE and naturally dried. Then it was rinsed several times with deionized water to remove residual acetic acid in the chitosan membrane and dried. Next it was immersed into 2.5% glutaraldehyde solution for 5 minutes. One microliter of 250

U/ml GOD was transferred on the WE. After drying, they were rinsed several times with deionized water to remove the unimmobilized enzyme, followed by natural drying before being used.

Measurements

The PCB was connected to a CHI 600A Electrochemical Analyzer (China) throughout a ribbon connector. All electrochemical measurements were performed in the three-electrode mode. For biosensors testing the applied WE potential was set to +50mV versus the Ag/AgCl RE. After their background currents stabilized, their steady-state current values were measured when either glucose solution or one of two different interference species solution was added to the phosphate buffer solution.

Results and Discussion

Electrocatalytic properties to H_2O_2 for PB/Pt electrode

Fig. 2 shows the electrochemical behaviors of both the bare Pt electrode and the PB/Pt electrode in a 5 mM of H_2O_2 solution. There was no current peak in the cyclic voltammogram of the bare Pt electrode and the response current is small, while a reductive peak appear at +150 mV in one of the PB/Pt electrode. The current of the reductive peak is about 18 μA , which is very larger than the current for the bare electrode of Pt at the same potential. The potential of the peak starts at 400 mV, positive going 200mV compared with one of the bare Pt electrode. It demonstrated that PB film play obvious catalysis role to electrochemical reduction for H_2O_2 .

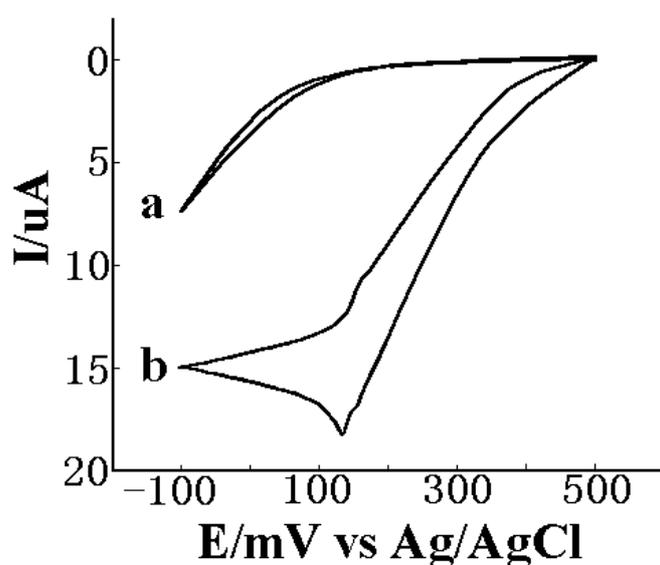


Figure 2. Cyclic voltammograms of H_2O_2 recorded by (a) the bare Pt electrode and (b) the PB/Pt electrode.

Effects of experimental parameter on performances of the sensor

Effect of PB film thickness

The thickness for PB film may be controlled by the concentration of the deposition solution and scan time. The experimental results show that the thicker PB film, the longer response time, the lower response sensitivity and the wider linear range. The appropriate deposition condition can be chosen according to requirement for sensitivity, response time and linear range. In consequent experiments, PB film was deposited by a single potential cycling in solution containing 5 mM $K_3Fe(CN)_6$ and $FeCl_3$ in 0.1 M KCl and 0.1 M HCl (pH 2.0) in a rate of 20 mV/s from 0 to 0.5 V.

Effect of enzyme quantity

Large dependence of response of the sensor on enzyme quantity immobilized was observed, as shown in Fig. 3. The larger quantity of the enzyme immobilized, the higher sensitivity of the sensor and the narrower linear range are.

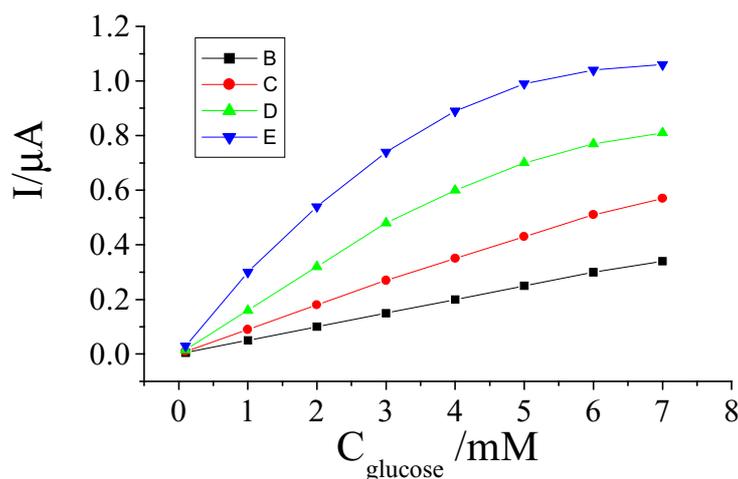


Figure 3. Effect of various GOD immobilized quantities on response to glucose. (B) 0.1 U, (C) 0.05 U, (D) 0.025 U, (E) 0.0125 U.

Selection of WE potential

Fig. 2 shows that large response current can be obtained within the range of -0.1 to $+0.1$ V of the WE potential. In general, the lower WE potential of the sensor, the lower susceptibility to interference species, resulting in the increase of selectivity of the sensor. With a decrease in WE potential, however, the background current will increase and the time reached steady-state current will prolong, resulting in an increase in the detection error. Thus, 0.05V was compromisingly chosen as WE potential of the sensor.

Selection of pH value

Actually, the effect of pH on the sensor involves two factors: (1) the effect of pH on the electrocatalytic property of the PB layer (Fig.4 (a)); (2) the effect of pH on the activity of GOD (Fig. 4(b)). Fig. 4(c) shows the combined relation between the response current of the sensor and the pH values of the glucose solution. The sensor exhibits that larger response current can be obtained within the range of pH 6.0 to 7.0. However, the higher value of pH in the glucose solution, the lower stability of the PB. Thus, pH6.8 phosphate buffer solution was chosen as bulk solution.

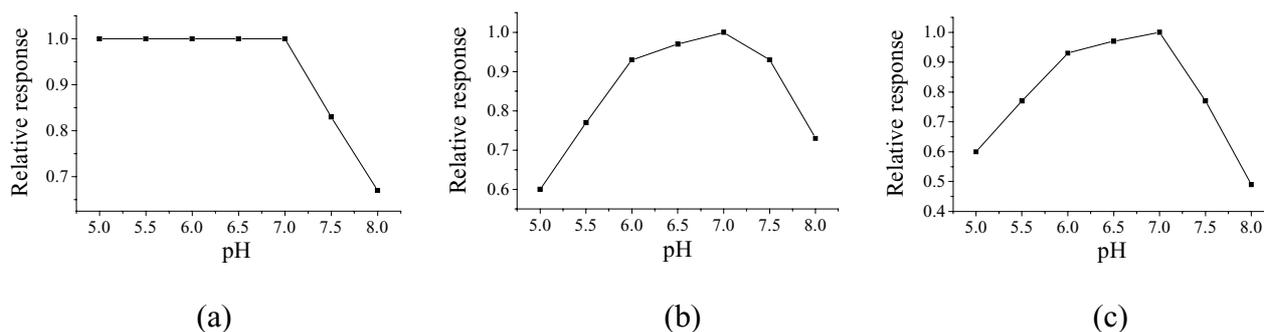


Figure 4. Effect of pH on the responses for (a) PB/Pt electrode, (b) GOD/Pt electrode and (c) GOD/PB/Pt electrode.

Effect of temperature on response

Fig. 5 shows the effect of temperature on the response current of the sensor (concentration of glucose, 3.0mM). The current at certain concentration increased with temperature until 40°C and then decreased. The lower temperature, the lower activity of GOD is. When the temperature is higher than 40°C, the GOD on the WE will partially lost its activity. Hence, it is necessary to control seriously environmental temperature during measurement or to make compensation for temperature.

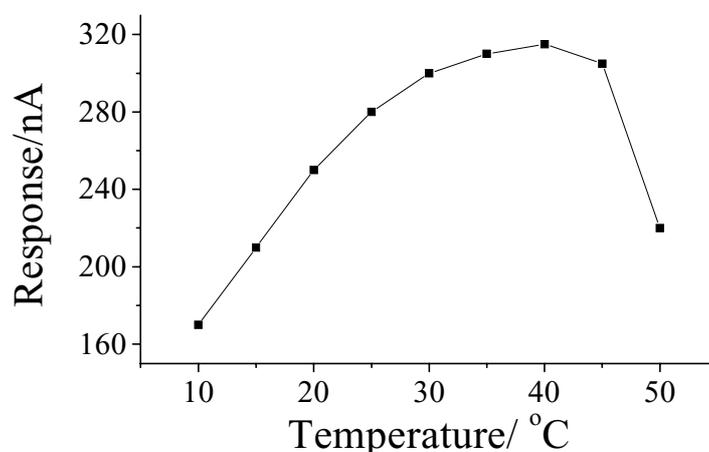


Figure 5. Effect of temperature on response current of the sensor.

Performances of the sensor

Response time

The response times of the glucose sensor were 42s for concentration change from 0 to 6 mmol/L and 60 s for reversion, respectively. They were estimated from the response-time curve using a potentiostat set at +50 mV (Fig. 6) indicating the establishment of stable mass transport within 60 s.

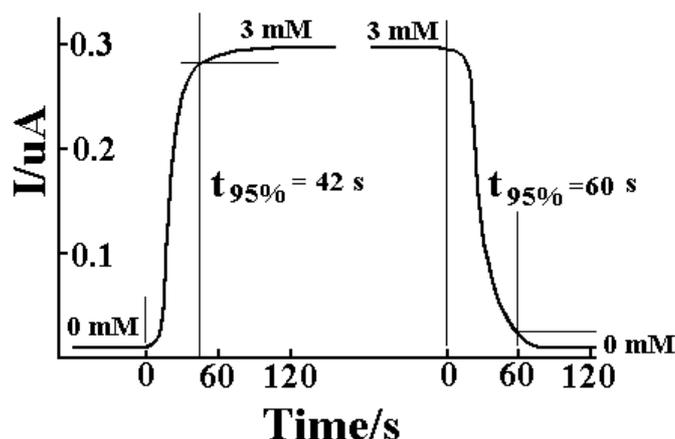


Figure 6. The response time curves of the sensor.

Precision

Good precision was confirmed by 11 continuous measurements in pH 6.86 phosphate buffer solutions containing 3.0 mM of glucose. The experiment values were listed in Table 1.

Table 1. The precision of the sensors.

No.	1	2	3	4	5	6	7	8	9	10	11
I/nA	291	294	298	292	297	295	290	289	299	295	294
AV	294										
RSD	3.0%										

AV: Average value, RSD: Relative standard deviation.

Calibration curve

The calibration curve of the glucose sensor with experimental condition chosen above is shown in Fig. 7. Linear range, detection limit, sensitivity and response time are 0.1 – 6.0 mM, 0.06 mM (signal-to-noise ratio, 3), 98 nA/M and 60 sec, respectively. Measurement range may be expanded up to 20 mM. The apparent Michaelis – Mentent constant of the sensor is 21 mM calculated according to Michaelis – Mentent equation.

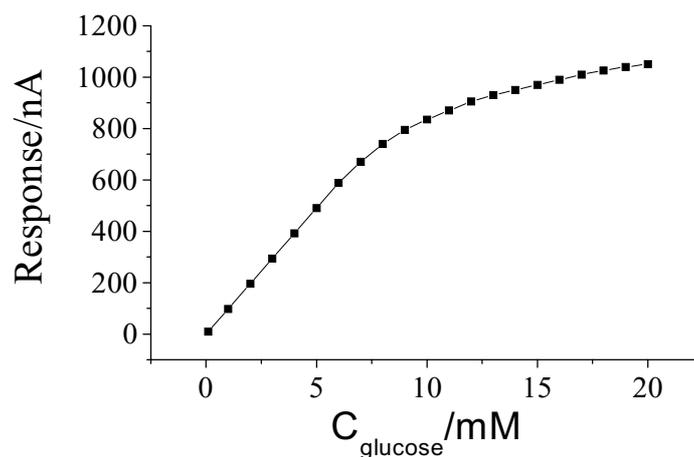


Figure 7. Calibration curve of the sensor.

Testing of anti-interference

Fig. 8 shows the effect of ascorbic and uric acids on the glucose sensor response. The arrows indicate the peaks for different solution and their concentrations. The peaks for 0.06 mM of ascorbic acid, 0.3 mM of uric acids and their mixture are 3,1 and 4 nA higher than the average of background current, respectively while the peak for 3.0 mM of glucose with two interferent species is only 4 nA higher than one for pure glucose solution. The difference is within 3.0 % of RSD.

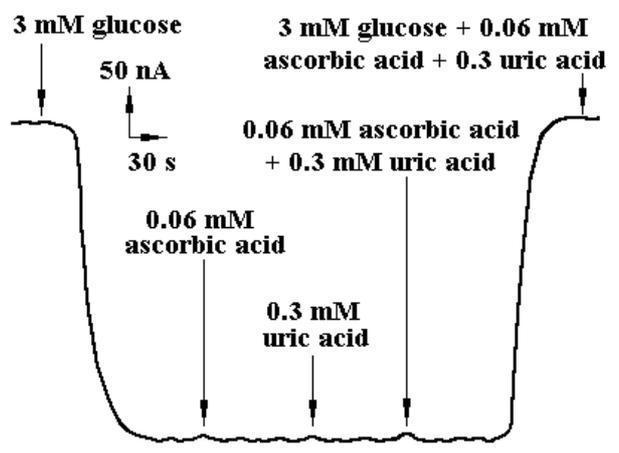


Figure 8. Response of the sensor in the presence of ascorbic and uric acids.

Long-term stability

The GOD-PB/Pt sensor response for 4.0mM glucose was measured 10 times a day (storage in refrigerator during nonuse). The average value for the 10 measurements is plotted against the number of days after the preparation of the sensor. No apparent change in the response of the sensors was

observed during the 30 days from Fig.9. Good long-term stability obtained can be attributed to the use of double functional chitosan membrane, which is permeable for H_2O_2 but impermeable for larger molecules such as Prussian Blue resulting in no loss of it from the electrode surface (9).

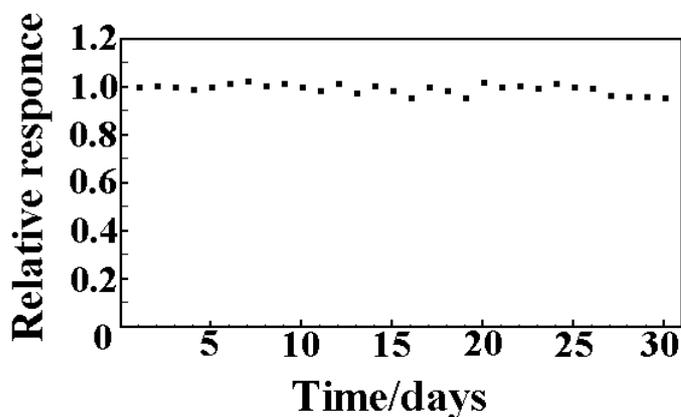


Figure 9. Long-term stability of the sensor.

Accuracy

Measurement of accuracy of the glucose sensor was taken in a standard serum with composition similar to that of normal human blood serum. The measured result of glucose was 6.1 mM, which are almost identical to the recommended values of 6.0.

Results obtained from the glucose sensor directly in human blood serum were compared with ones obtained by spectrophotometry, as shown in Fig. 10. On testing 100 samples of serum, the correlation coefficient reaches 0.996.

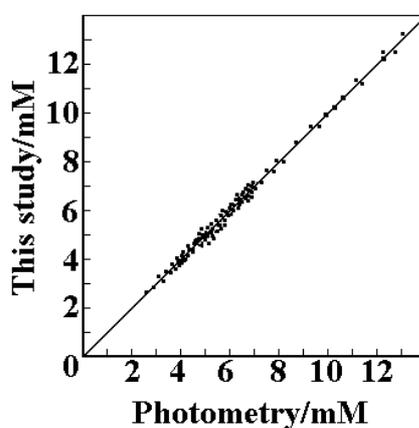


Figure 10. Correlation and regression line for measurements of 100 human serum samples between spectrophotometry and our sensors.

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Sample Availability: Available from the authors.