Polyaniline/Prussian Blue Composite Film Electrochemical Biosensors for Cholesterol Detection

LI, Jian-Ping[a,b](李建平) PENG, Tu-Zhi*,[a](彭图治)

[a] *Department of Chemistry, Xixi Campus, Zhejiang University, Hangzhou, Zhejiang 310028, China*

[b] *Department of Applied Chemistry, Guilin Institute of Technology, Guilin, Guangxi 541004, China*

An electrochemical biosensor fabricated by immobilization of cholesterol oxidase (ChOx) in a polyaniline (PAN)/prussian blue (PB) conductive layer of glassy carbon electrode has been prepared, based on the detection of hydrogen peroxide produced by ChOx at −0.05 V. The properties of the biosensor were investigated and the measurement conditions for cholesterol were optimized. A linear relationship between electrochemical signal and cholesterol concentration in a range of 1×10^{-6}—8×10^{-5} mol/L was observed. It is one of the most sensitive sensors for cholesterol determination, since a low detection limit of 1.8×10^{-7} mol/L was found. Good properties of the biosensor were attributed to high activity of ChOx and effective electro-catalysis of PB modifier in the composite layer on electrode surface.

Keywords biosensor, electrochemistry, polyaniline, prussian blue, cholesterol oxidase

Introduction

Cholesterol is a very important bioactive compound. Numerous attempts have been made to create sensitive, selective, reliable and low cost cholesterol sensors during the last decade because of the significance in clinical diagnosis of coronary heart diseases, arteriosclerosis, cerebral thrombosis and miscellaneous other disorders.[1] It is difficult to directly detect cholesterol by electrochemical methods. Only a few of stripping voltammetric measurements were reported after adsorption on mercury surface.[2,3] Electrochemical biosensors based on immobilized cholesterol oxidase (ChOx), a flavo-enzyme that produces hydrogen peroxide during cholesterol oxidation, have been the subject of many studies.[4-13]

Classical devices for cholesterol biosensors were based on monitoring either the enzymatic consumption of oxygen or the production of hydrogen peroxide. The electro-oxidization of hydrogen peroxide requires high anodic potential (about +0.6 V) and is affected by cooxidizable substances, *e. g.*, ascorbic acid, uric acid, bilirubin and acetaminophen which are usually present in bio-samples. It was expected that an additional electrocatalytic layer of prussian blue (PB) placed on surface of electrode prior to ChOx immobilization would be allowed to detect cholesterol at lower potential (about +0.1—−0.1 V) by measuring hydrogen peroxide reduction current and, thus, to avoid the influence of reductants. PB modified electrode exhibited high activity and selectivity in detection of hydrogen peroxide through its catalytic electro-reduction, thereby deposited PB film was called as "artificial enzyme peroxidase".[14] Recently, many enzyme-derived biosensors were fabricated, based on PB modified electrodes,[14-19] however no reference of PB enzymatic sensor for cholesterol measurement was reported.

For an electrochemical biosensor, the stability and activity of enzyme in media of electrode membrane are related not only to water-organic media, but also to enzyme microenvironment.[20] Various methods for immobilization of ChOx in matrixes have been tested, such as conducting polymers, carbon paste, liposome, natural material and sol-gel.[5-13] In these biosensors, the activity of ChOx and

* E-mail: tzp@emb.zju.edu.cn

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their lifetime are different.

This paper describes a cholesterol biosensor prepared by immobilization of ChOx in a composite film of polyaniline (PAN)/PB. The PAN/PB film is pre-deposited onto glassy carbon electrodes (GCE) by electro-polymerization technique. The PAN/PB film is not only a good carrier of ChOx, but also an effective catalytic-oxidation layer for H_2O_2. The reactions of the biosensor for cholesterol determination are shown in Fig. 1.

Experimental

Apparatus and reagents

Electroanalytical measurements were performed on a CHI660A electrochemical workstation (CH Instruments Inc., USA) with a conventional three-electrode system. The working electrode was a ChOx PAN/PB modified GCE; an Ag/AgCl electrode containing 1.0 mol/L KCl was used as the reference electrode, and a platinum wire as the counter electrode.

Cholesterol oxidase (ChOx, EC 1.1.3.6, C-8649, 18 U/mg), cholesterol and Triton X-100 were purchased from Sigma. A cholesterol stock solution (5 mmol/L) was prepared by dissolving 0.0967 g of cholesterol in a 50 mL of flask containing a mixture of isopropanol (1 mL) and Triton X-100 (1 mL) in a bath at 60 ℃, and then diluted with distilled water. The solution was stored at 4 ℃ in dark and stable for two weeks (until a slight turbidity was observed). Diluted cholesterol solutions were freshly prepared from the stock solution with phosphate (0.01 mol/L) buffer solution containing 1% (*V/V*) Triton X-100. All other chemicals used were of analytical grade and double distilled water was employed throughout. Hydrogen peroxide solution (0.1 mol/L) was prepared by diluting 100 μL of 30% hydrogen peroxide in 10 mL of double distilled water and stored at 4 ℃, and the exact concentration of hydrogen peroxide was determined by iodimetry. The working standard solutions were freshly prepared by dilution of the stock solution with water.

Preparation of PAN/PB *modified electrode and enzyme biosensor*

A GCE with 3 mm inner diameter was polished using the alumina powder (1 μm, diameter) on a polishing pad. Subsequently the polished electrode was rinsed to remove alumina with a sharp stream of water from a wash bottle, and then ultrasonically treated in a mixed solution of 2 mol/L HCl-alcohol (50%, *V/V*) for 15 min. Finally the electrode was washed with water, and dried at room temperature.

The PAN/PB composite film was prepared on clean surface of GCE by cyclic voltammetric method in a mixed solution with $K_3[Fe(CN)_6]$ (0.005 mol/L), $FeCl_3$ (0.005 mol/L), aniline (0.005 mol/L), H_2SO_4 (0.5 mol/L) and K_2SO_4 (0.05 mol/L) for 15 cycles at 0.4—−0.2 V. The PAN/PB modified electrode was electrochemically pretreated in phosphate (0.01 mol/L) buffer with K_2SO_4 (0.1 mol/L) (pH 7.3) by keeping at −0.05 V for 10 min and cycling between 0.36 and −0.05 V for 10 cycles. The treated electrode was washed with water and dried under infrared lamp for 1 h before enzyme immobilization.

The electrochemical doping for preparing ChOx electrodes was adopted in our experiment. The PAN/PB composite film was immersed in phosphate buffer (0.02 mol/L, pH 4.0) and reduced at −0.40 V for 20 min; then the reduced film was immediately moved into the phosphate buffer (0.02 mol/L, pH 6.2) containing ChOx and oxidized at 0.60 V for 20 min. Cholesterol oxidase which carries a negative charge in phosphate buffer was doped in the PAN/PB film to form enzyme electrodes during the oxidation process.

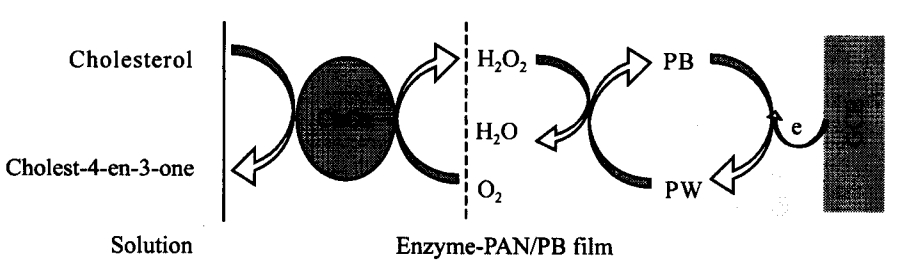

Fig. 1 Scheme of multi-step reactions at ChOx PAN/PB biosensors.

Determination of cholesterol

Cyclic voltammograms were recorded in a phosphate buffer (0.02 mol/L, pH 7.3) solution with 0.8% Triton X-100 (*V/V*) at a potential range of +0.3— −0.2 V, and the height of reductive peak was measured. Amperometric experiments were carried out at a potential of −0.05 V, and curves of *i-t* were recorded. In order to obtain stable response, currents should be measured after 1 min of sample injection.

Results and discussion

PAN/PB *deposition at* GCEs

A couple of well-defined cathodic and anodic peaks (E_c 0.14 V, E_a 0.27 V) were revealed in cyclic voltammograms for PAN/PB composite deposition at a GCE, which were corresponding to the reduction and oxidation of PB respectively (Fig. 2). PB modified electrodes have shown very promising properties for voltammetric and amperometric detection of hydrogen peroxide at low potentials. The selectivity and stability of these electrodes, however, depend on how the electropolymerization of PB is performed.[21] In our experiments, an excess of K_2SO_4 was added to the solution to keep PB stable during potential scan. Since the redox process of PB is accompanied by the participation of cations in electrolyte solution , and these cations can freely penetrate PAN/PB film duringthis redox process. K_2SO_4 was chosen for this purpose. The results indicate that K^+ accelerates the process as long as its concentration is higher than 0.01 mol/L. Therefore, 0.05 mol/L K_2SO_4 was added in buffer solutions in our experiments.

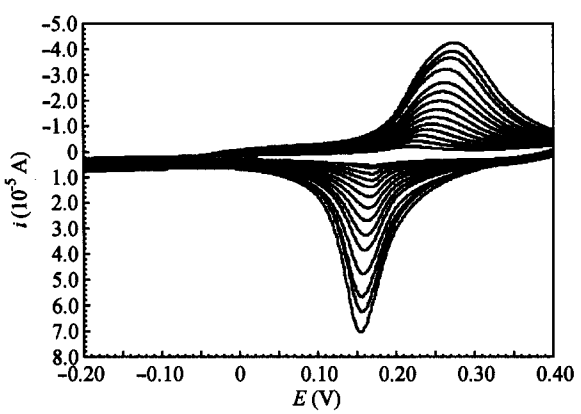

Fig. 2 Cyclic voltammograms of electrochemical deposition of PAN/PB film on a GCE in a mixed solution with $K_3[Fe(CN)_6]$ (0.005 mol/L), $FeCl_3$ (0.005 mol/L), aniline (0.005 mol/L), H_2SO_4 (0.5 mol/L) and K_2SO_4 (0.05 mol/L); scan rate 50 mV/s.

Buffer composition and pH

In order to investigate the effect of buffer composition on the performance of ChOx PAN/PB derived biosensors, following buffers (0.01 mol/L, pH 7.3) were tested: phosphate, citrate and Brinton-Robinson solutions with K_2SO_4 (0.1 mol/L) as supporting electrolyte. A well-defined wave of PB in cyclic voltammograms and good response to cholesterol were observed in phosphate solution (Fig. 3). The phosphate buffer was chosen in further experiments.

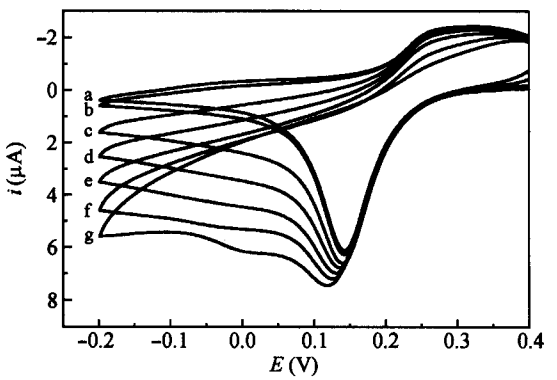

Fig. 3 Cyclic voltammograms of ChOx PAN/PB modified GCE in phosphate solution (pH 7.3, 30 ℃) with K_2SO_4 (0.05 mol/L) and 0.8% Triton X-100 (a); adding cholesterol of 2×10^{-6}, 1.2×10^{-5}, 2.0×10^{-5}, 3.0×10^{-5}, 4.0×10^{-5}, 5.0×10^{-5} mol/L, respectively (b—g); scan rate 50 mV/s.

The effect of pH of buffer on the response for cholesterol was investigated and results showed that the optimized pH range of phosphate buffer was 7.2—7.5. Buffer solution of pH 7.3 was chosen for further experiments.

In general, Triton X-100, a non-ionic surfactant as effective lipid solubilizing agent, is contained in buffer solutions for solubilizing cholesterol, which is a sparingly soluble compound. The increase of Triton X-100 results in the increase of cholesterol solubility. At the same time, high concentration of Triton X-100 also activates

ChOx. The effect of Triton X-100 concentration on peak current was examined and an optimized concentration range of 0.7%—1.1% was found.

Response time, stability and reproducibility

Usually, the response of enzyme electrodes is slow and a response time of a few minutes is needed. However, the maximum current of ChOx PAN/PB biosensors was obtained only in 20—30 s in our experiments. Obviously, the diffusion of cholesterol to the enzyme entrapped in PAN/PB layer does not be obstructed and the electron transfer is fast in this conductive film. It means that PAN/PB films could benefit the response of the biosensor.

The stability of the sensor was evaluated by lifetime experiment. The sensor was used for 3 h each day and the calibration graphs involving $n = 7$ cholesterol determinations were carried out at hourly intervals. The sensor was stored at 4 ℃ after use. The means of daily values of slopes were compared. The results showed that the slope at the seventh day is 96% of that at the first day. To the 16th day, the slope only declined to 92% and the half-life period of these enzyme sensors was nearly 30 d. This weak decrease does not seem to be ascribed to the catalytic activity of enzyme, but probably to the decreasing dissolve of PB. It demonstrates that ChOx entrapped in PAN/PB layer is stable and makes the biosensor to keep a longer lifetime. The enzyme entrapped in PAN/PB matrix is more stable than one entrapped in other films.[4-10]

The reproducibility of the sensor was evaluated by the precision, which was obtained by repeat determination of cholesterol (1×10^{-5} mol/L). The cathodic current responses (represented by i-i_0) were 4.22, 4.17, 4.29, 4.31, 4.26, 4.16, 4.10, 4.18, 4.32 μA respectively, and the relative standard division (RSD) was 2.8% ($n = 9$).

Determination of K'_m(ChOx)

In order to examine enzyme situation in biosensors, the apparent Michaelis-Menten constant (K'_m) of ChOx was determined. A Lineweaver-Burk double reciprocal plot based on the experimental data is shown in Fig. 4. The maximum current response (i_{max}) and K'_m was calculated from the intercept and slope of the curve. i_{max} and K'_m were found to be 7.5 μA and 0.54 mmol/L. The value of K'_m obtained in cholesterol biosensors is very similar to K'_m values (0.45—0.9 mmol/L) reported for native ChOx in a homogeneous air-saturated solution.[22] This suggests that there are no restrictions or conformational changes of the immobilized enzyme in PAN/PB layers of biosensors.

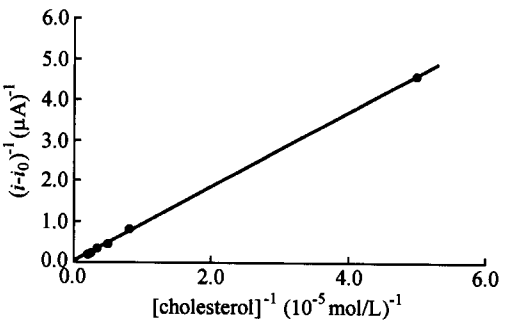

Fig. 4 Determination of apparent Michaelis-Menten constant K'_m of ChOx in biosensors.

Interference

The influence of some possible interfering species on the response of cholesterol was investigated when the biosensor was used in sample solutions. In particular, ascorbic acid and uric acid were focused on, since these compounds were easily oxidized at bare electrodes. It was found that ascorbic acid (1×10^{-5} mol/L), whose concentration was higher than the normal level (2×10^{-6} mol/L) in serum, only resulted in a relative division of 4.8% on the current for detecting cholesterol (1×10^{-5} mol/L). While, uric acid (1.2×10^{-5} mol/L) produced a similar division (<5%). It can be asserted that ascorbic acid and uric acid do not weaken the potentialities of the system for a successful application in biosensor area. Other compounds, such as glucose (5×10^{-3} mol/L), lactic acid (5×10^{-4} mol/L), cystein (2×10^{-4} mol/L) and albumin (1×10^{-3} mol/L), had also be examined and negligible effects were found for determinations of cholesterol either (1×10^{-5} mol/L). It seems that the modified layer on electrode surface protects the biosensor from interference in sample solutions.

Voltammetric determination

The ChOx PAN/PB derived biosensor exhibited a

linear response relationship between cathodic peak height and concentration of free cholesterol in the range of 1×10^{-6}—4×10^{-5} mol/L. The linear regression equation was: $i\ (\mu A) = 4.261 \times 10^5\ c + 0.286$, with a correlation coefficient of 0.9991. Fig. 5 exhibited that the sensor allowed detecting cholesterol in the range of 0—8×10^{-5} mol/L. The detect limit was determined according to the definition of IUPAC, *i.e.*, the expression of 3 s/*K*, where sensitivity (*K*) is the slope of calibration curve. And the result was 1.8×10^{-7} mol/L. In the literature, detect limits of most cholesterol sensors were in the order of magnitude of 10^{-5}—10^{-6} mol/L.[1,4-7] It means that the ChOx PAN/PB sensor is one of the most sensitive sensors for cholesterol determination.

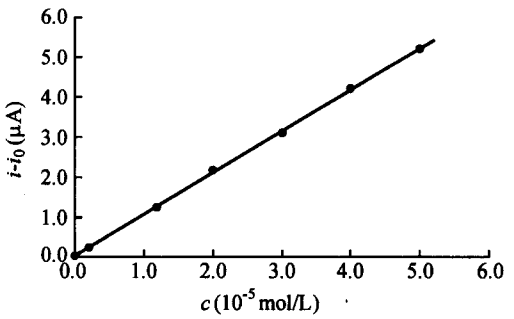

Fig. 5 Calibration curve of ChOx PAN/PN biosensor for cholesterol determination in concentration range of 2×10^{-6}—2×10^{-4} mol/L.

Amperometric determination

Amperometric determinations for cholesterol have been carried out in a three-electrode cell configuration similar to the one used in cyclic voltammetry experiments. Chronoamperometric curves for detecting cholesterol in phosphate buffer (pH 7.3, 30 ℃) were recorded by polarizing the working electrode (ChOx PAN/PB GCE) at −0.05 V vs. Ag/AgCl in a stirring solution. The chronoamperograms of ChOx biosensor are presented in Fig. 6, in which typical calibration curves are obtained. The cholesterol in a concentration range of 0—2×10^{-4} mol/L could be determined by amperometric method. It implies that the biosensor is suitable for electrochemical detection in flow injection analysis and liquid chromatography.

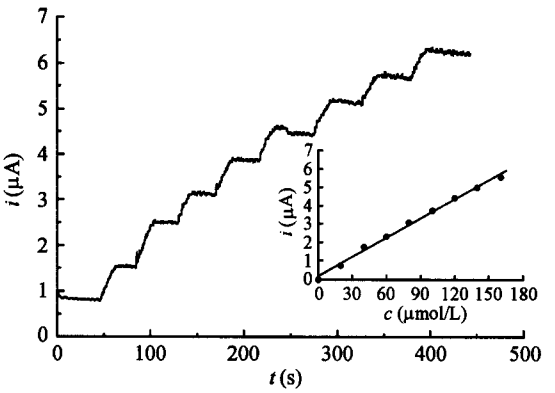

Fig. 6 Chronoamperomtric response of ChOx PAN/PB biosensor on cholesterol (20 μmol/L) (nine additions) in phosphate buffer (0.1 mol/L) with K_2SO_4 (0.05 mol/L) and 0.8% Triton X-100 (pH 7.3, 30 ℃); polarizing potential −0.05 V. The inserted figure is a calibration graph.

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