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Bioelectrochemical response of a choline biosensor fabricated by using polyaniline

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On the basis of the isoelectric point of an enzyme and the doping principle of conducting polymers, choline oxidase was doped in a polyaniline film to form a biosensor. The amperometric detection of choline is based on the oxidation of the H_2O_2 enzymatically produced on the choline biosensor. The response current of the biosensor as a function of temperature was determined from 3 to 40℃. An apparent activation energy of 22.8 kJ·mol^{-1} was obtained. The biosensor had a wide linear response range from 5×10^{-7} to 1×10^{-4} M choline with a correlation coefficient of 0.9999 and a detection limit of 0.2 μM, and had a high sensitivity of 61.9 mA·M^{-1}·cm^{-2} at 0.50 V and at pH 8.0. The apparent Michaelis constant and the optimum pH for the immobilized enzyme are 1.4 mM choline and 8.4, respectively, which are very close to those of choline oxidase in solution. The effect of selected organic compounds on the response of the choline biosensor was studied.

choline, choline oxidase, biosensor, polyaniline matrix

1 Introduction

Choline is the precursor and metabolite of the important neurotransmitter acetylcholine. Choline is often used as a marker of cholinergic activity in brain tissue. In addition, under special circumstances dietary choline serves as a supplementary vitamin. It is clear that choline plays an important role in the biochemical process. Thus, the detection of choline is very important in clinical diagnosis and food analysis.

In recent years, amperometric biosensors have received a great deal of attention due to high selectivity and sensitivity, fast response, and low costs. A number of amperometric sensors, with immobilized choline oxidase (ChO) alone[1−7] and bi-enzyme of ChO and horseradish peroxidase (HRP)[8−11], have been reported. The measurement of the response current for the ChO biosensor is based on the oxidation of H_2O_2 generated enzymatically. The enzyme-catalyzed reaction and electrochemical reaction are as follows:

$$\underset{\text{choline}}{(CH_3)_3-N^+-CH_2-CH_2OH}+2O_2+H_2O\xrightarrow{ChO}\underset{\text{betaine}}{(CH_3)_3-N^+-CH_2-COOH}+2H_2O_2$$

$$H_2O_2\rightarrow O_2+2H^++2e^-$$

The detection of the electrode reaction was carried out at higher potentials, for example, at 0.70 V (vs. SCE). At such high potential, the detection of choline readily underwent interference with electroactive species in the tested solution. This is a drawback of the ChO biosensor. However, the ChO biosensor has advantages with a low detection limit of 2.5×10^{-9} M[1] and a wide linear range of 1×10^{-5} to 1.75×10^{-3} M choline[2].

On the contrary, the measurement of the response

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current for the ChO/HRP biosensor is based on the reduction of H_2O_2 in the presence of HRP and the redox mediator. The latter plays an important role in shuttling the electron transfer between the immobilized HRP and the electrode. The reactions are as follows:

$$H_2O_2 + 2H^+ + 2\mathrm{Med(red)} \xrightarrow{\mathrm{HRP}} 2H_2O + 2\mathrm{Med(ox)}$$

$$\mathrm{Med(ox)} + e^- \rightarrow \mathrm{Med(red)}$$

In general, the operational potential for the reduction of H_2O_2 in the presence of a mediator is controlled at lower potentials, for example, at – 0.10 V (vs. SCE). Thus, the advantage of the ChO/HRP biosensor is that it can reduce interference. However, this biosensor needs two kinds of enzymes and a mediator.

Among conducting polymers, polyaniline has a rather high conductivity and good reversible redox characteristics. Therefore, polyaniline could function as "molecule-wires" to relay electron transfer[12], which is in favor of the electron transfer in the enzyme-catalyzed reactions. Such properties of polyaniline offer a possibility for the oxidation of H_2O_2 at lower potentials. Therefore, we used polyaniline to immobilize choline oxidase and studied the effect of various factors on the bioelectrochemical response of the ChO biosensor.

2 Methods

2.1 Materials and the immobilization of choline oxidase

Chemicals used were of regent grade. Aniline was distilled before use. Choline oxidase (EC.1.1.3.17, 14 units/mg solid) from Alcaligenes species was purchased from Sigma Company. Doubly distilled water was used to prepare the solutions.

An electrolytic cell for the synthesis of polyaniline consisted of two platinum foils and a saturated calomel reference electrode (SCE). The area of a working electrode was 3.5 mm × 4.0 mm. The electrochemical polymerization of aniline was carried out in a solution consisting of 0.2 M aniline and 0.6 M H_2SO_4 at a constant potential of 0.75 V. A PAR Model 173 potentiostat/galvanostat with a Model 179 digital coulometer was used for the electrochemical polymerization of aniline. The electrolytic solution was stirred with a magnetic bar during the electrolytic process. A cohesive and uniform film was formed on the working electrode. The thickness of the film was controlled by the charge consumed in the electrolysis, which was 6.8×10^{-2} C. We must point out here that the thickness of the polyaniline film was controlled more easily by using the electrolysis of a constant potential than by using that of repeated potential cycling, because in the former method charges during the electrolysis of aniline were monitored via a digital coulometer. After the electrolysis, the polyaniline film was washed with 0.05 M H_2SO_4 solution to remove un-reacted aniline.

An enzyme solution containing 4.0 mg choline oxidase in 3 mL of 0.1 M phosphate buffer with pH 8.0 was used for preparing enzyme electrodes. The polyaniline film was first reduced in 0.05 M H_2SO_4 solution at –0.30 V for 10 min, followed by washing with distilled water, and then was immediately immersed in a solution of choline oxidase to be oxidized at 0.50 V for 12 min. Under the electrostatic interaction, choline oxidase with negative charges was doped into the polyaniline film carrying positive charges to form an enzyme electrode (Figure 1), because the isoelectric point of choline oxidase is 4.5[13].

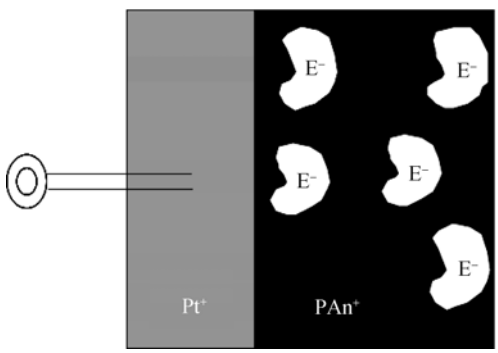

Figure 1 Scheme of choline oxidase (E^-) doped in polyaniline film (PAn^+).

The experimental result shows that the quantity of electricity was -1.060×10^{-2} C for the reduction of the polyaniline film at –0.30 V, and was 9.643×10^{-3} C for the oxidation of the polyaniline film in the enzyme solution of pH 8.0. This indicates that polyaniline in the enzyme solution of pH 8.0 was oxidized well, which made it possible to immobilize choline oxidase. Like the redox process of organic compounds, the reduction of polyaniline is strongly dependent on the pH value of the solution, because of requiring protons to supply polyaniline; however, polyaniline releases protons to the solution during the oxidation process, which results in a slight dependence of pH. Thus, polyaniline is still electroactive

during the oxidation process, but a necessary condition is that polyaniline has to be reduced completely in a strongly acidic solution prior to the immobilization of choline oxidase.

2.2 Electrochemical measurements

A PC-1 potentiostat with a digital meter was used for the determination of the response current of the biosensor. The response current value can be directly read off from a digital meter, and a plot of the response current I as a function of time t was simultaneously recorded on a YEW 3066 pen recorder. The response current of the enzyme electrode was measured in a quiescent solution containing choline chloride. The method for determining the response current was described in detail elsewhere[14]. The solutions used for the determination of choline consisted of 0.1 M phosphate buffer with various concentrations of choline and were prepared freshly prior to each measurement. The experimental temperature was controlled at 20℃, unless otherwise stated.

3 Results and discussion

3.1 Cyclic voltammetric measurements of choline at the PAN doped ChO platinum electrode

An initial cyclic voltammetric study was performed to establish whether choline could be measured using the the PAN-modified platinum electrode doped ChO. The resulting cyclic voltammorgrams (Figure 2) demonstrated the catalytic oxidation response for choline at ChO/PAN/Pt electrode (b) is characteristic of electrocatalytic oxidation of choline.

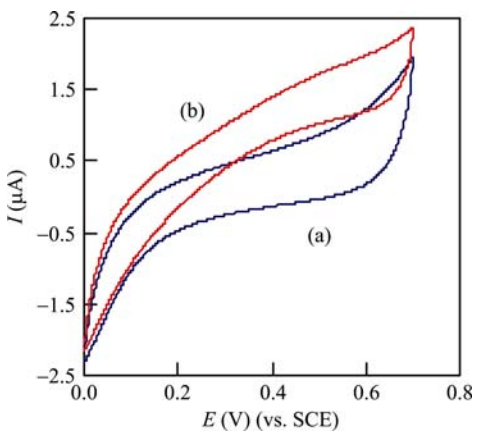

Figure 2 Cyclic voltammogram of the PAN-modified Pt electrode doped with ChO, in 0.1 M PBS (pH 8.0) (a) in the absence and (b) in the presence of 200 μM choline. Scan rate: 10 mV·s^{-1}.

3.2 Effect of the applied potential on the response of the biosensor

The response currents of the choline biosensor in a solution containing 60 μM choline and 0.1 M phosphate buffer with pH 8.0 were determined at 0.35, 0.40, 0.45, 0.50, and 0.55 V. Figure 3 shows the change in the response current with time at different potentials.

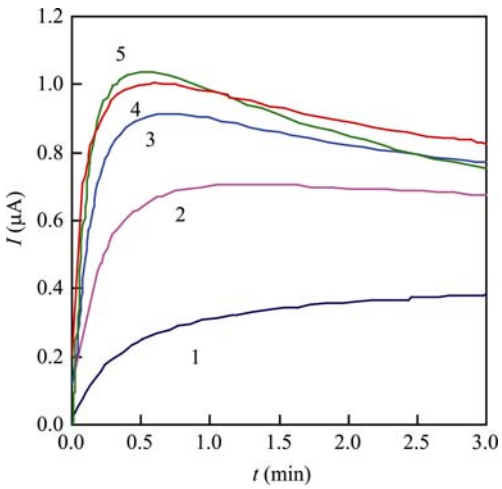

Figure 3 The change in the response current of the biosensor with applied potentials in a 0.1 M phosphate buffer solution containing 60 μM choline with pH 8.0. Curve 1, 0.35 V; curve 2, 0.40 V; curve 3, 0.45 V; curve 4, 0.50 V; curve 5, 0.55 V.

Curve 1 in Figure 3 shows that the response current increases quickly at the very onset and then increases slowly with time, indicating that the enzyme catalyzed reaction rate is faster than that of the electrochemical oxidation of H_2O_2 at 0.35 V. This means that the formation rate of H_2O_2 in the enzyme-catalyzed reaction is fast enough, which not only meets the needs of the electrode reaction at 0.35 V, but also makes H_2O_2 at the electrode surface accumulate. The latter results in the continuous increase of the current with time in the experimental period of 3 min. As the applied potential increases from 0.40 to 0.55 V, the response current of the biosensor increases with increasing applied potential (curves 2—5). This result is expected because this is an electrochemical oxidation reaction. Furthermore, as can be seen from curves 2—5 that the currents increase quickly first, then reach a maximum to form a peak, and finally decrease with time. The time necessary for reaching a current maximum is defined as the response time of the biosensor. It is obvious that the response time reduces with increasing potential, and is about 40 s at 0.40 V (curve 2) and 30 s at 0.55 V (curve 5). The formation of the current

peak ensures that the biosensor output is choline dependent, which is the basis for the quantitative determination of choline. The peak current on *I-t* curve was taken as the measured value of the response current in this work. Even though the response current of the biosensor increases with applied potentials, the difference between the response currents at 0.50 and 0.55 V is not large. To reduce interference of other electroactive species, the operational potential of the enzyme electrode was controlled at 0.50 V in the following experiments. This operational potential is lower than that of other choline biosensors[1,4].

3.3 Effect of pH on the response current of the biosensor

The response current of the biosensor as a function of pH is shown in Figure 4. A solution containing 60 μM choline in 0.1 M phosphate buffer was used in this experiment. The potential of the biosensor was set at 0.50 V. As can be seen in Figure 3, the response current increases with increasing pH from 6.4 to 8.4. A maximal current occurs at pH 8.4, and then the response current decreases a little as the pH increases up to 8.8. Thus, an optimum pH value for the immobilized choline oxidase is 8.4.

The pH dependence and the optimum pH in Figure 4 are very close to those of the sensor fabricated by using physical immobilization of bi-enzyme of ChO and HRP onto Prussian Blue-based carbon paste electrode in the pH range of 6.0—9.0[10], and also close to those of choline oxidase in solution, in which the response increases with pH up to a maximal plateau of pH 8.9[10].

However, the optimum pH in Figure 4 is the same as that of choline oxidase immobilized in poly(aniline-co-o-aminophenol) film[15]. The latter immobilization method is the same as what is reported here, i.e., using electrochemical doping. Each enzyme has an optimum pH that is characteristic of an enzyme. It is clear that enzyme immobilization using electrochemical doping has no influence on the nature of the enzyme, because it was carried out under a mild condition.

3.4 Effect of the concentration of choline on the response current of the biosensor

On the basis of the above results, the conditions for the determination of the relationship between the response current and the concentration of choline are that the operational potential of the biosensor was set at 0.50 V and the pH of the solution was controlled at 8.0. Considering the response current of the biosensor is a little higher at pH 8.4 than at pH 8.0 and considering the effect of pH on the electric activity of polyaniline, we chose pH 8.0 for the solutions in the following experiments.

Figure 5 shows the change in the response current with the concentration of choline from 0.5 to 250 μM. The response current increases linearly with increasing choline concentration from 0.5 to 100 μM, which is expressed by a linear regression equation, I (μA) = 8.6614 × [Choline] + 0.0026, R^2 = 0.9999. The biosensor has a detection limit of 0.2 μM choline. The sensitivity was calculated to be 61.9 mA·M^{-1}·cm^{-2}.

The linear range is larger than that of ChO/HRP biosensor fabricated by using covalent bonding[4]. The calibration curve follows the Michaelis-Menten kinetics. Inset of Figure 4 shows the plot of I^{-1} vs. [choline]$^{-1}$, on the basis of the experimental data in Figure 5. A maximal response current and an apparent Michaelis

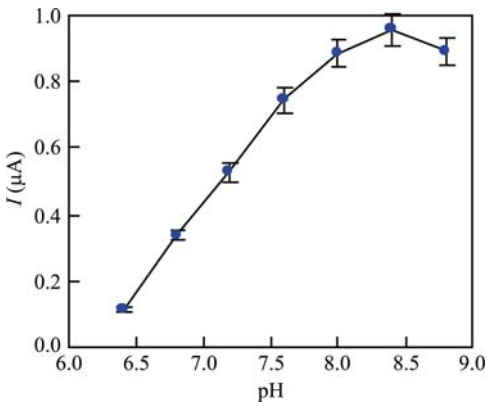

Figure 4 The response current of the biosensor at 0.50 V as a function of pH value, in a 0.1 M phosphate buffer solution containing 60 μM choline.

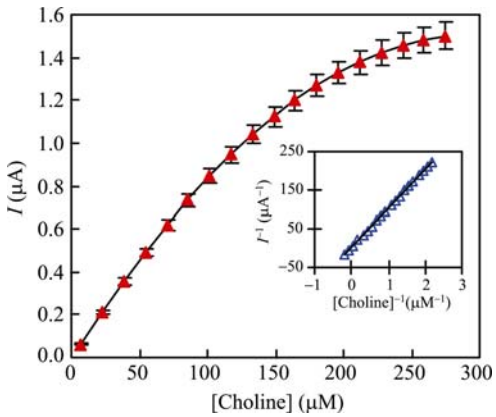

Figure 5 The calibration curve of the biosensor to choline. Inset shows the plot of I^{-1} vs. [Choline]$^{-1}$.

constant K_M^{app} were calculated from the intercept and the slope of the straight line to be 12.656 μA and 1.4 mM, respectively. The latter is close to 2 mM for the biosensor fabricated by using the physical immobilization of bi-enzyme of ChO and HRP on the Prussian Blue-based carbon paste electrode[10] and also close to that of choline oxidase in a solution with pH 8[13,16]. Each enzyme has a characteristic K_M^{app} for a given substrate. Thus, the K_M^{app} determined here confirms that the property of choline oxidase immobilized in the polyaniline film is hardly affected by using electrochemical doping.

3.5 Effect of temperature on the response current of the biosensor

The relationship between temperature and the response current of the biosensor in a solution containing 60 μM choline and 0.1 M phosphate buffer with pH 8.0 is shown in Figure 6. The potential was set at 0.50 V. The temperature was controlled between 3 and 40℃. As can be seen in Figure 6, the maximal response current occurs around 35℃.

In general, for enzyme-catalyzed reaction, the reaction rate increases with temperature until a maximal rate is achieved, but at temperatures above the maximum, decreased rates are observed because of thermal denaturalization of the enzyme. Thus, Figure 6 indicates that a typical relationship between an enzyme reaction rate and temperature exists in choline oxidase immobilized in the polyaniline film. On the basis of the data shown in Figure 6, a plot of log I vs. T^{-1} gives a straight line (the linear regression equation: $\log I = -1242.9\ T^{-1} + 4.7839$, $R^2 = 0.9996$). An apparent activation energy Ea was calculated from the slope of the line to be 22.8 kJ·mol^{-1}, which is smaller compared with the activation energy for enzyme-catalyzed reactions (20—85 kJ·mol^{-1}). One of the reasons for the small apparent activation energy is probably the catalytic effect of polyaniline on the oxidation of H_2O_2[12]. Also, this is why the operational potential of the biosensor can be controlled lower than that of other choline biosensors[1].

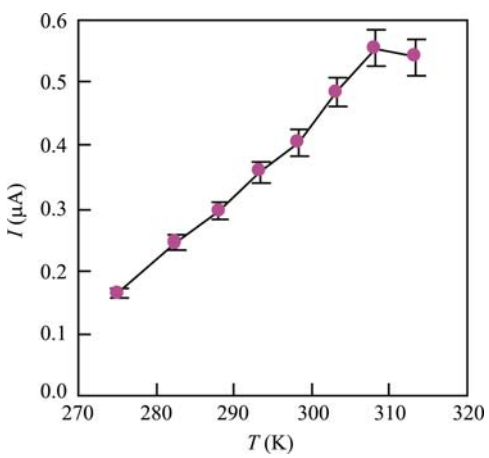

Figure 6 The change in the response current of the biosensor with temperature in a 0.1 M phosphate buffer solution containing 60 μM choline with pH 8.0, at 0.50 V.

3.6 Interference and stability of the biosensor

Uric acid, phenol, and glucose were selected for the study of interference with the determination of choline. The solutions used in this study were 60 μM choline, 60 μM phenol, 0.238 mM uric acid, and 5 mM glucose in 0.1 M phosphate buffer with pH 8.0, respectively. The concentrations of glucose and uric acid used here are the same as their concentrations in the blood plasma of human. The potential of the biosensor was set at 0.50 V. Uric acid has a marked interference, with the response being 42.8% of the biosensor's response to the standard choline solution. However, glucose has little interference with the determination of choline.

The operational stability and the storage stability of the biosensor were exemplified in a solution containing 60 μM choline and 0.1 M phosphate buffer with pH 8.0 at 0.50 V. The data from five consecutive measurements show that maximal and minimal current values are 0.623 and 0.604 μA, respectively. The error in measurement is 3.1%. The long-term storage stability of the biosensor was tested on every four days. After each measurement, the biosensor was kept in a phosphate buffer solution with pH 8.0 at 4℃. The result shows that the response current of the biosensor from 0.771 to 0.464 and 0.398 μA after 40 and 52 days, i.e., 39.8% and 48.4% decay of the initial activity were observed, respectively (Figure 7). Thus, the biosensor has a better storage stability than the choline biosensor fabricated by using poly(aniline-co-o-aminophenol) does[15], but the storage stability of the choline biosensor fabricated by using covalent bonding is better than that of the former[2,4]. Covalent linkage plays an important role in protecting the leakage of the enzyme from the immobilized layer, which results in preserving the catalytic activity[17]. However, the decrease in the catalytic activity of the biosensor with time may be caused by hydrogen peroxide generated enzymatically and de-doping of choline oxidase, since hydrogen

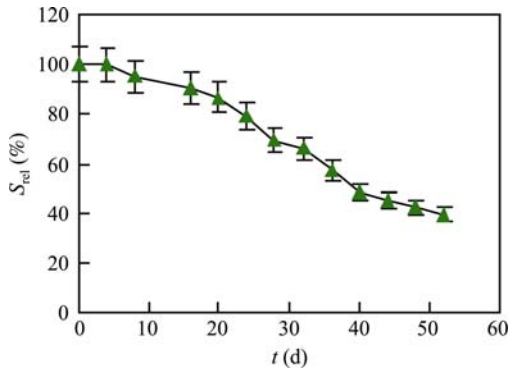

Figure 7 The long-term stability of the proposed choline sensor.

peroxide is harmful to the enzyme, and the latter is due to the fact that the biosensor was stored in the phosphate buffer with pH 8.0.

3.7 Comparison with other choline sensors based on polymer

In the literature, a choline biosensor constructed by poly(2-hydroxyethyl methacrylate)-grafted telfon film, had a linear range of 52－348 μM and an average response time of 22 s[18]. In another study, for ChO entrapped in photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinum, the linear range was determined as 2.5－150 μM, and the response time was 2 min[19]. Most recently, Shimomura reported a choline sensor based on a hybrid mesoporous membrane F127. The determination range and the response time were 5.0－800 mM and approximately 2 min, respectively, and the sensitivity was about 1.25 $mA \cdot M^{-1} \cdot cm^{-2}$. Their sensor was stable and 85% of the initial response was maintained even after storage for 80 days[20]. Such improved analytical performance reflects the host matrix of PAN as well as the efficient biosensing method with quiescent solutions. However, the analytical performance can be further improved by the polymer based on PAN-derivates[15].

4 Conclusions

A choline biosensor was prepared by using polyaniline film as an immobilized matrix. The immobilization of choline oxidase was performed under a mild condition, which ensures the enzyme against denaturalization. Evidence for this is that the apparent Michaelis constant and the optimum pH for immobilized choline oxidase are very close to those of the one in solution. The biosensor has a wide linear response range with a detection limit of 0.2 μM choline. Therefore, this biosensor can be used for the determination of choline.

1 Leca B, Morelis R M, Coulet P R. Design of a choline sensor via direct coating of the transducer by photo polymerization of the sensing layer. Sens Actuators B, 1995, 26-27: 436－439[DOI]
2 Doretti L, Ferrara D. Covalently immobilized enzymes on biocompatible polymers for amperometric sensor applications. Biosens Bioelectron, 1996, 11: 365－373
3 Ben Rejeb I, Arduini F, Arvinte A, Amine A, Gargouri M, Micheli L, Bala C, Moscone D, Palleschi G. Development of a bio-electrochemical assay of AFB(1) detection in olive oil. Biosens Bioelectron, 2009, 24: 1962－1968[DOI]
4 Bai Y H, Zhang H, Xu J J, Chen H Y. Relationship between nanostructure and electrochemical/biosensing properties of MnO_2 nanomaterials for H_2O_2/choline. J Phys Chem C, 2008, 112: 18984－18990
5 Langer J J, Filipiak M, Kęcińska J, Jasnowska J, Włodarczak J, Buładowski B. Polyaniline biosensor for choline determination. Surface Sci, 2004, 573: 140－145[DOI]
6 Shimomura T, Itoh T, Sumiya T, Mizukami F, Ono M. Amperometric determination of choline with enzyme immobilzed in a hybrid mesoporous membrane. Talanta, 2009, 78: 217－220[DOI]
7 Lopez M S P, Perez J P, Lopez-Cabarcos E, Lopez-Ruiz R. Amperometric biosensors based on choline oxidase entrapped in polyacrylamide microgels. Electroanalysis, 2007, 19: 370－378[DOI]
8 Wang J, Liu G D, Lin Y H. Amperometric choline biosensor fabricated through electrostatic assembly of bienzyme/polyelectrolyte hybrid layers on carbon nanotubes. Analyst, 2006, 131: 477－483[DOI]
9 Watanabe J, Ishihara K. Sequential enzymatic reactions and stability of biomolecules immobilized onto phospholipid polymer nanoparticles. Biomacromolecules, 2006, 7: 171－175[DOI]
10 Moscone D, D'ottavi D, Compagnone D, Palleschi G. Construction and analytical characterization of Prussian blue-based carbon paste electrodes and their assembly as oxidase enzyme sensors. Anal Chem, 2001, 73: 2529－2535[DOI]
11 Schuvailo O N, Dzyadevych S V, El'skaya A, Gautier-Sauvigne S, Csoregi E, Cespuglio R, Soldatkin A P. Carbon fibre-based microbiosensors for *in vivo* measurements of acetylcholine and choline. Biosens Bioelectron, 2005, 21: 87－94[DOI]
12 Zhou B, Sun R G, Hu X F, Wang L H, Wu H P, Song S P, Fan C H. Facile interfacial electron transfer of hemoglobin mediated by conjugated polymers. Int J Mol Sci, 2005, 6: 303－310[DOI]
13 Ikuta S, Imamura S, Misaky H, Horiuti Y. Purification and characterization of choline oxidase from *Arthrobacter globiformis*. J Biochem (Tokyo), 1977, 82: 1741－1749
14 Wang H Y, Mu S L. Bioelectrochemical characteristics of cholesterol oxidase immobilized in a polyaniline film. Sens Actuator B, 1999, 56: 22－30[DOI]
15 Zhang J, Shan D, Mu S L. Improvement in selectivity and storage stability of a choline biosensor fabricated from poly(aniline-co-o-aminophenol). Frontiers in Biosci, 2007, 12: 783－790[DOI]
16 Bergmeyer H U, Grassi M, Walter H E. In Methods of Enzymatic Analysis, 3rd ed., Bergmeyer, HU Ed, Germany: Verlag Chemie Weinheim. 1983, 2: 172－173
17 Cosnier S. Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. A review. Biosens Bioelectron, 1999, 14: 443－456[DOI]
18 Yucel D, Ozer N, Hasirci V. Construction of a choline biosensor through enzyme immobilization on a poly(2-hydroxyethyl methacrylate)-grafted Teflon film. J Appl Polym Sci, 2007, 104: 3469－3477[DOI]
19 Marty J L, Sode K, Karube I. Amperometric determination of choline and acetylcholine with enzymes immobilized in a photocross-linkable polymer. Anal Chim Acta, 1990, 228: 49－53[DOI]
20 Shimomura T, Itoh T, Sumiya T, Mizukami F, Ono M. Amperometric determination of choline with enzyme immobilized in a hybrid mesoporous membrane. Talanta, 2009, 78: 217－220[DOI]