Bioelectrochemical Response and Kinetics of Choline Oxidase Entrapped in Polyaniline-Polyacrylonitrile Composite Film

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A novel choline oxidase electrode was constructed by entrapping choline oxidase into polyaniline-polyacrylonitrile composite film. The enzyme film was prepared by *in situ* electropolymerization of aniline into porous polyacrylonitrile-coated platinum electrode in the presence of choline oxidase. The enzyme electrode exhibited sensitive and stable electrochemical response to choline. The kinetics analysis showed that the mass transport is partially rate-limiting. The influences of pH, applied potential and temperature on the response of the enzyme electrode were also described.

Keywords polyaniline-polyacrylonitrile composite, bioelectrochemical response, kinetics, enzyme electrode, choline oxidase, biosensor

Introduction

Enzyme immobilization technology has attracted much attention in connection with the design, fabrication and applications of biosensor. The catalytic characteristics of immobilized enzymes as well as the stability and sensitivity are influenced by immobilization methods and support materials. The main conventional approaches for enzyme immobilization on electrode surfaces, including adsorption, cross-linking, covalent binding and entrapment in polymeric gels, all exist somewhat disadvantages. So, exploring new immobilization methods and materials is always a significant work in the field of biosensor.

Recently, electropolymerization has considerable potential for the preparation of enzyme electrodes.[1-8] The precise control over the charge passed during polymer deposition allows control over the thickness of the deposited enzyme layers. The enzyme electrode prepared by electrochemical methods showed good reproducibility and stability, while the sensitivity was sometimes not satisfactory.[4] On the other hand, porous polymeric materials have been found wide applications as immobilization matrix of enzyme due to their high surface area.[9,10] In our laboratory, the polyacrylonitrile synthesized by single rare-earth catalyst-$Y(OAr)_3$ could be easily fabricated into microporous film with the pore size suitable for enzyme immobilization.[11] However, the enzyme electrode has lower long-term stability due to enzyme desorption from the film.[11] Combining the advantages of the two kinds of enzyme immobilization, we developed *in situ* electropolymerization and fabricated successfully a highly stable and sensitive polyphenol oxidase electrode.[12] In this paper, choline oxidase electrode was prepared by this method for determination of choline and acetylcholine (a neurotransmitter),[13-15] which is important in biological detection. The bioelectrochemical response to choline, operational parameters and kinetics of the enzyme electrode were described.

Experimental

Materials

Choline oxidase (ChO, EC1.1.3.17, from Alcaligenes species, 10 units/mg) was purchased from Sigma

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Received January 15, 2002; revised March 25, 2002; accepted April 4, 2002.
Project supported by the Special Funds for the Major State Basic Research Development Program (No. G1999064801), the National Natural Science Foundation of China (Nos. G29974024 and G20254001) and the Science and Technology Commission of Zhejiang Province (No. 11199600).

Company. Aniline was distilled before use. Polyacrylonitrile (PAN, molecular weight, M_η, is 28000) was synthesized by the polymerization of acrylonitrile with single rare-earth catalyst, $Y(OAr)_3$.[11] All the other chemicals were of analytical grade. Choline chloride solution in phosphate buffer solution (PBS, 0.1 mol·dm^{-3}) was daily prepared.

Construction of the enzyme electrode

A 10 μL of polyacrylonitrile solution (2 wt% polymer DMF solution) was coated on the platinum electrode (3 mm × 3 mm), and then was turned into porous membrane by phase inversion process.[16] The enzyme electrode was prepared by *in situ* electropolymerization. The electrolysis cell consisted of a PAN-coated Pt working electrode, a Pt counter electrode and a saturated calomel electrode (SCE). Polymerization was carried out in a PBS (pH 6.5, 0.1 mol·dm^{-3}) containing ChO (2 mg·mL^{-1}) and aniline (0.1 mol·dm^{-3}) at a constant potential of 0.48 V (vs. SCE). The consuming charge in the electrolysis was 0.92 mC. During the electrochemical oxidation, aniline was *in situ* polymerized in PAN matrix and the enzyme entrapped into polyaniline-polyacrylonitrile (PAn/PAN) composite matrix forming the PAn/PAN-ChO enzyme electrode.[12] The resulting enzyme electrode was washed thoroughly with PBS, and then stored in PBS (pH 8.0) at 4 ℃.

Measurements and apparatus

When being used for the determination of choline, the enzyme electrode reaction is as follows [Eq.(1)]:

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{Choline oxidase}} \text{betaine} + 2H_2O_2 \qquad (1)$$

The bioelectrode response is based on the oxidation current of generated hydrogen peroxide at a constant potential[2,4,8] [Eq.(2)]:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \qquad (2)$$

All electrochemical studies were performed with conventional three-electrode system. The equipment consisted of a PAR model 173 potentiostat-galvanostat with a model 179 digital coulometer and a PC-I precise potentiostat. All potentials given are referred to SCE.

Results and discussion

Effect of operational parameters on the current response of the enzyme electrode

The PAn/PAN-ChO electrode shows a fast bioelectrochemical response to choline (the response time is about 1 min), which is influenced by the pH value of solution, potential applied and temperature.

The pH dependence of the response of the enzyme electrode within the pH range of 6.0 to 9.2 in PBS (0.1 mol·dm^{-3}) containing choline (0.1 mmol·dm^{-3}) is illustrated in Fig. 1a. The current response of the enzyme electrode increases rapidly as the pH changes from 6.0 to 8.0, and then varies slightly between pH 8.0 and 9.2. A shift of the optimum pH toward higher values than free choline oxidase[17] may be caused by the interaction between the polymer and enzyme. On the other hand, the background current of the enzyme electrode increases with increasing pH. An optimum ratio of signal-to-background current (signal-to-noise, S/N) was observed at pH 8.0 (Fig. 1b), so the pH 8.0 was selected for the following experiments.

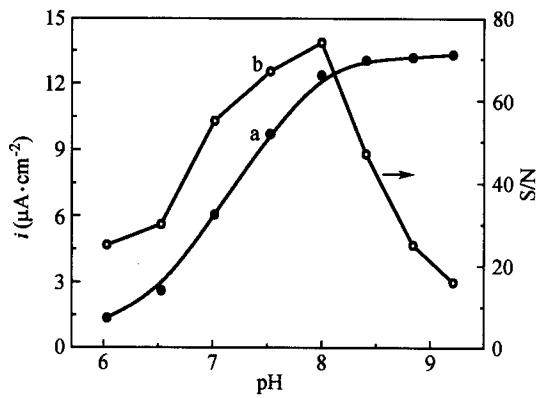

Fig. 1 Effect of pH on the current response (a) and signal-to-noise (b) of the enzyme electrode in PBS (0.1 mol·dm^{-3}) containing choline (0.1 mmol·dm^{-3}), at 25 ℃. Applied potential, 0.50 V.

The effect of applied potential, stepped from 0.25 V to 0.70 V, on the current response of the enzyme electrode in PBS (0.1 mol·dm^{-3}, pH 8.0) containing

choline (0.1 mmol·dm^{-3}) is shown in Fig. 2. The current response increases with increasing potential below 0.50 V, which indicates that the current response is mainly controlled by the enzyme kinetics. When the potential is over 0.50 V, the response changes slightly, which illustrates that the response is mainly controlled by the diffusion of substrate and product. The potential of 0.50 V was chosen as the working potential of the PAn/PAN-ChO electrode.

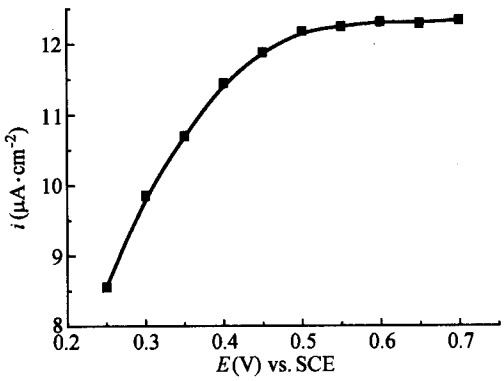

Fig. 2 Effect of applied potential on the response of the enzyme electrode in PBS (0.1 mol·dm^{-3}) containing choline (0.1 mmol·dm^{-3}) with pH 8.0, at 25 ℃.

Fig. 3a shows the effect of temperature on the response of the enzyme electrode. The current response of the enzyme electrode to choline (0.1 mmol·dm^{-3}) increases as temperature increases from 5 ℃ to 37 ℃, and then decreases as temperature increases further. The maximum response appears at about 37 ℃. According to the Arrhenius equation, The relationship of ln$i$ vs. T^{-1} in the temperature range of 5 ℃ to 25 ℃, based on the data in Fig. 3a, is a straight line (Fig. 3b). The apparent activation energy (E_a) of the enzyme electrode reaction calculated from the slope of the straight line is 21.4 kJ·mol^{-1}.

Kinetic characteristics of the enzyme electrode response

Fig. 4 shows the amperometric current response of the enzyme electrode as a function of choline concentration. The curve is linear with choline concentration up to 0.2 mmol·dm^{-3} and bends gradually at higher concentrations. The sensitivity of PAN/PAN-ChO electrode is 120 mA·mol^{-1}·dm^3·cm^{-2}, which is much higher than that reported in literatures.[13-15] The apparent Michaelis-Menten constant (K_M^{app}) of choline oxidase entrapped in PAn/PAN composite film was calculated to be 0.61 mmol·dm^{-3} (see inset in Fig. 4) according to the electrochemical Lineweaver-Burk form of the Michaelis-Menten equation.[18] The value is close to that reported (0.9 mmol·dm^{-3}) for the free enzyme,[19] illustrating the non-denaturating character of the procedure of enzyme anchoring.

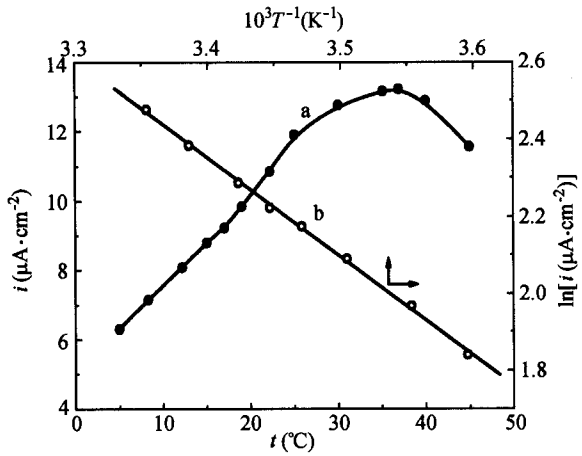

Fig. 3 (a) Influence of temperature on the response of the enzyme electrode in PBS (0.1 mol·dm^{-3}) containing choline (0.1 mmol·dm^{-3}) with pH 8.0; applied potential, 0.50 V. (b) Plots of ln$i$ against T^{-1} according to the data in curve a.

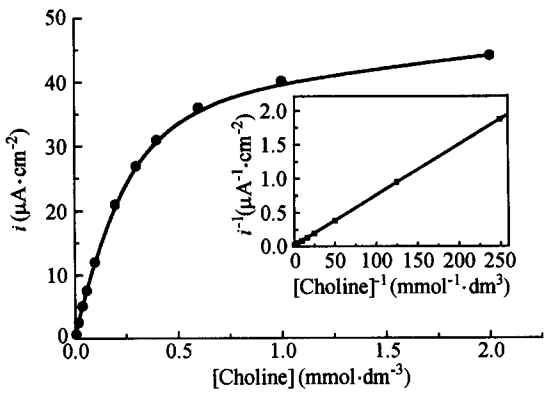

Fig. 4 Relationship between the electrode response and choline concentration in PBS (0.1 mol·dm^{-3}) with pH 8.0, at 25 ℃; applied potential, 0.50 V. Inset shows the determination of the apparent Michaelis-Menten constant.

The results in Fig. 4 were analyzed by the determination of the electrochemical kinetic constants, k_{ME}', K_{ME} and k_S'. Here, k_{ME}' represents the effective elec-

trochemical rate constant for the enzyme electrode at low substrate concentration, k_S' is the mass-transfer rate constant of substrate and the significance of K_{ME} is similar to that of the Michaelis constant in homogeneous enzyme kinetics. For substrate concentrations smaller than K_{ME} the system is unsaturated, and for substrate concentrations greater than K_{ME} the system becomes gradually saturated. The expressions for the determination of these kinetic constants were derived by Albery *et al*..[20] The first stage of the analysis is to find k_{ME}' by making a Hanes plot of [choline]/j against [choline] (here, $j = i/2F$, F is Faraday constant). These plots are shown in Fig. 5. k_{ME}' determined by the intercept of the Hanes plot at zero concentration is 6.9×10^{-6} m·s^{-1}. A further analysis of the results in Fig. 4 yields values of K_{ME} and k_S' by calculating values of the parameters ρ and y using the following equation[20] [Eq.(3)] and [Eq.(4)]:

$$\rho = j/(k_{ME}'[\text{choline}]) \quad (3)$$

$$y = (\rho^{-1} - 1)/[\text{choline}] = (1 - \rho\, k_{ME}'/k_S')/K_{ME} \quad (4)$$

Plots of y against ρ are shown as a good straight line (Fig. 6) for the PAn/PAN-ChO electrode. The values of K_{ME} and k_S', calculated from the slope and intercept of the straight line, are 0.34 mmol·dm^{-3} and 10.2×10^{-6} m·s^{-1}, respectively. The value of k_S'/K_{ME} is about 1.5, indicating that the transport of choline through the membrane is partially rate-limiting, mostly at low substrate concentration.[20]

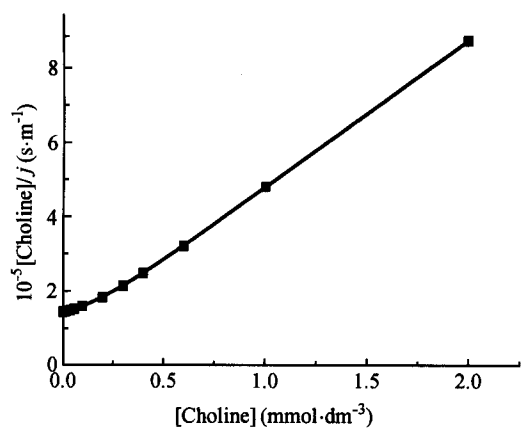

Fig. 5 Determination of the kinetic constant k_{ME}'.

Reproducibility and stability of the enzyme electrode

The reproducibility of bioelectrode construction was estimated from the response to choline (0.1 mmol·dm^{-3}) at six enzyme electrodes. The results reveal that the enzyme electrode has satisfactory reproducibility with a mean current response of 11.76 μA·cm^{-2} and a relative standard deviation of 7.4%. Good reproducibility can be ascribed to the controllable process of the electrode construction.

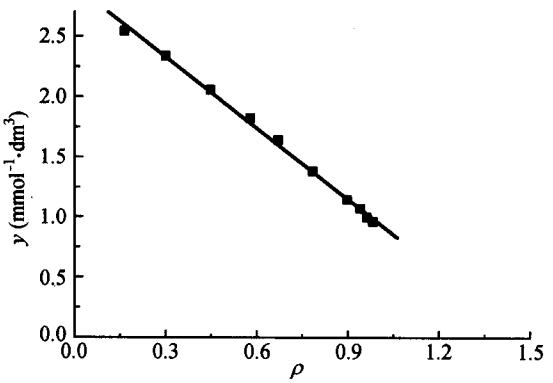

Fig. 6 Relationship between parameters y and ρ.

The operational stability of the PAn/PAN-ChO electrode was investigated by amperometric measurements in choline (0.1 mmol·dm^{-3}) at applied potential 0.5 V. No decrease of the current response was observed after the enzyme electrode was tested 30 times continuously. When the enzyme electrode was stored in PBS (0.1 mol·dm^{-3}, pH 8.0) at 4 ℃ and measured intermittently the response to choline (0.1 mmol·dm^{-3}), it kept the original response for one month. After two months, the enzyme electrode retained 68% of its original activity.

For comparison, other two kinds of choline oxidase electrodes under the same experimental conditions were prepared. One is PAN-ChO electrode, which was fabricated by adsorbing choline oxidase in porous polyacrylonitrile film. Another is PAn-ChO electrode, which was constructed by electrochemical doping choline oxidase in polyaniline film (similar to that reported in previous work[2,4,8]). The characteristics of the three kinds of electrodes are listed in Table 1. The results show that PAn/PAN-ChO electrode exhibits higher sensitivity than PAn-ChO electrode and better long-term stability than PAN-ChO electrode.

Table 1 Characteristics of three kinds of choline oxidase electrodes

Electrode	Initial sensitivity ($mA \cdot mol^{-1} \cdot dm^3 \cdot cm^{-2}$)[a]	Response time/min	Long-term stability
PAn/PAN-ChO	120	~1	Lose 32% activity after 2 months
PAn-ChO	11.5	~1	Lose 31% activity after 2 months
PAN-ChO	117	~1	Lose 68% activity after 1 month

[a]Determined by measurements in PBS (0.1 $mol \cdot dm^{-3}$, pH 8.0) containing choline (0.1 $mmol \cdot dm^{-3}$), at 25 ℃; applied potential, 0.50 V; assuming the linearity in the response.

Conclusions

A new choline oxidase electrode was developed by *in situ* electropolymerization method. The method combined the advantage of porous polymer materials and electropolymerization in bioelectrode construction. The PAn/PAN-ChO electrode prepared exhibits higher sensitivity than PAn-ChO electrode and better long-term stability than PAN-ChO electrode. This is probably a promising route to biosensor fabrication.

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(E0201158 ZHAO, X. J.; ZHENG, G. C.)