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In situ electropolymerized polyaniline–polyacrylonitrile composite film for the construction of a polyphenol oxidase-based biosensor

H. XUE, Z. SHEN* and H. ZHENG
Department of Polymer Science & Engineering, Zhejiang University, Hangzhou 310027, China
*(*author for correspondence, e-mail: zqshen@163.net)*

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Abstract

Polyaniline–polyacrylonitrile composite films prepared by *in situ* electropolymerization of aniline on polyacrylonitrile-coated platinum electrode were used to immobilize polyphenol oxidase forming an enzyme biosensor. The novel polyphenol oxidase-based biosensor exhibited high sensitivity, low background and excellent stability, which showed no loss of activity after 100 consecutive measurements and intermittent usage for six months with storage in a phosphate buffer at 4 °C. The construction parameters of the enzyme electrode were optimized. The influences of pH and temperature on the biosensor response were explored. The sensitivities of the enzyme electrode for catechol, phenol, *p*-cresol and *m*-cresol were 2.03, 0.96, 1.38 and 1.5 A M^{-1} cm^{-2} respectively.

1. Introduction

The environmental control of organic pollutants such as phenolic compounds in industrial effluents has initiated the development of analytical techniques for fast, sensitive and selective monitoring of these compounds. Polyphenol oxidase-based biosensors may provide a promising method for their simple and fast determination [1–5].

Polyphenol oxidase (PPO) can catalyse oxidation of phenolic compounds. Oxygen takes part in the biocatalytic reaction to *o*-hydroxylate the phenolic compounds to catechols, with subsequent dehydrogenation to *o*-quinones. Some amperometric PPO biosensors based on the detection of electroreduction of the liberated *o*-quinones of the enzyme catalytic reaction have been developed recently [3, 6–14], but many of them have limited lifetime due to desorption of enzyme, electrode fouling and enzyme inactivation by the generated *o*-quinones [15]. Thus, development of a good immobilization method and material to improve the biosensor stability is required.

Recently, electropolymerized polyaniline has received attention in biosensor fabrication [16–20]. The enzyme can be intercalated directly into a polyaniline matrix by electrochemical doping, and the resultant biosensors have good stability and fast response. But the electroactive polyaniline exhibits a large reduction background current at low potential, so it was not directly applied to the biosensors that are operated at low potential, such as the PPO biosensor. In this work, we developed a novel amperometric PPO biosensor based on an *in situ* electropolymerized polyaniline–polyacrylonitrile composite film with low background current. The optimum parameters for the construction of the biosensor, and the influences of pH and temperature on the response were explored.

2. Experimental details

2.1. *Materials*

Polyphenol oxidase (PPO, EC1.14.18.1, from mushroom, 600 units mg^{-1}) was purchased from Amresco. Aniline was distilled before use. Polyacrylonitrile (molecular weight M_η 28000) was synthesized by the polymerization of acrylonitrile with single rare earth catalyst, $Y(OAr)_3$ [21]. All other chemicals were obtained commercially and were of analytical grade. Phenolic solutions in 0.1 M phosphate buffer (PBS) were prepared daily.

2.2. *Preparation of polyaniline–polyacrylonitrile composite film*

Polyacrylonitrile (PAN) was dissolved in DMF and coated on the platinum electrode and then was fabricated into a porous membrane by the phase inversion process [22]. The amount of PAN was controlled by the concentration and volume of the polymer solution. Polyaniline–polyacrylonitrile (PAn–PAN) composite

film was prepared by electropolymerization of aniline on a PAN-coated Pt electrode. The electrolysis cell consisted of a PAN-coated Pt working electrode, a Pt counter electrode and a saturated calomel electrode (SCE). Electropolymerization was carried out in an aqueous solution containing 0.1 M aniline and 0.1 M PBS (pH 6.5) at a constant potential of 0.48 V vs SCE. The amount of polyaniline was controlled by the charge passed during electrolysis.

2.3. *Construction of the enzyme electrode*

The enzyme electrode was prepared by electropolymerization of aniline on PAN-coated Pt in solution containing polyphenol oxidase. Polymerization was carried out in 0.1 M PBS (pH 6.5) containing 2 mg ml^{-1} PPO and 0.1 M aniline at a constant potential of 0.48 V vs SCE. During the electrochemical oxidation, aniline was *in situ* polymerized in the PAN matrix and the enzyme co-intercalated into the PAn–PAN composite matrix forming a PAn–PAN/PPO electrode. The resulting enzyme electrode was washed thoroughly with 0.1 M phosphate buffer solution, and then stored in PBS (pH 6.52) at 4 °C.

2.4. *Measurements and apparatus*

When being used for the determination of phenol, the enzyme catalytic reaction is as follows:

$$\text{phenol} \xrightarrow[O_2]{\text{PPO}} \text{catechol} \xrightarrow[O_2]{\text{PPO}} o\text{-quinone}$$

The enzyme electrode response is based on the amperometric detection of generated *o*-quinone.

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, a PC-I potentiostat, a YEW 3036 X–Y recorder and a YEW 3066 pen recorder. All potentials are referred to SCE.

3. Results and discussion

3.1. *Optimization of the biosensor fabrication parameters*

During the electrochemical polymerization of aniline on a PAN-coated Pt electrode, a brown PAn–PAN composite film was obtained rapidly within 2 min at a constant potential of 0.48 V. But, polyaniline film cannot form on bare Pt electrode under the same condition. This phenomenon is in accordance with the cyclic voltammograms of the polymerization of aniline on bare and PAN-coated Pt electrodes (Figure 1). The initial oxidation potential of aniline on bare and PAN-coated Pt is 0.44 V (curve (a)) and 0.50 V (curve (b)), respectively. The peak current of aniline oxidation on PAN-coated Pt is twice as large as that on bare Pt. These results show that the polymerization of aniline on PAN-coated Pt is easier than on bare Pt. This is probably due to the fact that the aniline monomer is absorbed by porous polyacrylonitrile and then *in situ* polymerized in the polyacrylonitrile matrix.

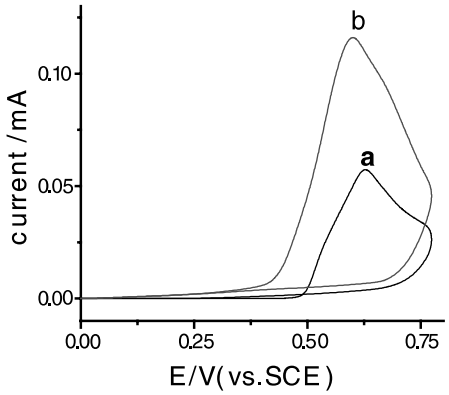

Fig. 1. Cyclic voltammograms of the polymerization of aniline on (a) bare Pt electrode and (b) PAN-coated Pt electrode; electrolysis solution, 0.1 M phosphate buffer (pH 6.5) containing 0.1 M aniline; scan rate, 10 mV s^{-1}.

Table 1 shows the effect of polyaniline content in the PAn/PAN composite film on the biosensor characteristics. The initial sensitivity of the biosensor to catechol decreases with increasing polyaniline content. However, the maximum sensitivity was observed at 24% polyaniline content after the enzyme electrode was stored in PBS at 4 °C for a week. On the other hand, an optimum ratio of signal-to-background current was also observed under the same condition. Considering the stability and measurement accuracy of the biosensor, 24% polyaniline content in the composite film was selected for fabricating the enzyme electrode in the following experiments.

The effect of the polymerization potential for fabricating the enzyme electrode on the sensitivity and ratio of signal-to-background current of the biosensor was tested over the range 0.48 to 0.60 V. As seen in Table 2, biosensors obtained under different polymerization potentials show similar sensitivities, but the ratio of signal-to-background current decreases with increasing polymerization potential. Therefore, the polymerization potential of 0.48 V was set for the construction of the enzyme electrode.

Table 1. Effect of polyaniline content in PAn/PAN composite film on the biosensor characteristics

PAn /%	Initial sensitivity* /A M^{-1} cm^{-2}	Ratio of signal to background	Sensitivity after a week[a] /A M^{-1} cm^{-2}
0	2.67	3.2	1.15
11.2	2.17	17	1.43
24.0	2.08	100	2.06
51.3	1.17	93	0.96
100	0.087	21	0.065

* Determined by measurements in 0.1 M PBS (pH 6.52) containing 20 μM catechol, at 25 °C; applied potential, −0.05 V; assuming the linearity in the response.

Table 2. Effect of the polymerization potential on the sensitivity and ratio of signal-to-background current of the biosensor

Polymerization potential /V	Sensitivity* /A M^{-1} cm^{-2}	Ratio of signal to background
0.48	2.08	100
0.5	1.94	80
0.52	2.24	71
0.55	2.14	57
0.60	2.01	40

* Condition as in Table 1.

3.2. *Effect of pH and temperature on the biosensor response*

The PAn–PAN film showed no response to phenols at an applied potential −0.05 V, while PAn–PAN/PPO film exhibited sensitive and stable response. The response of the enzyme electrode was influenced by pH and temperature.

The pH dependence of the biosensor over the pH range 4.7 to 8 in 0.1 M PBS containing 20 μM catechol is illustrated in Figure 2. The current response of the sensor varies slightly between pH 4.7 and 6.5, and then decreases rapidly as the pH change from 6.5 to 8. A wide optimum pH of 5–8 was reported for free enzyme [23]. It seems that PAn–PAN composite has not altered the optimum pH of polyphenol oxidase significantly.

The effect of temperature on the response of the biosensor is shown in Figure 3. The response to 20 μM catechol increases with temperature increase from 6.8 to 35 °C, and then decreases as temperature increases further. The maximum response appears at about 35 °C. According to the Arrhenius equation, the ln i against T^{-1} relationship in the temperature range 6.8 to 25 °C, based on the data in Figure 3, is a straight line (Figure 4). The apparent activation energy (E_a) of the enzyme electrode reaction, calculated from the slope of the straight line was 25.4 kJ mol^{-1}. The value is smaller than that (35.7 kJ mol^{-1}) of the enzyme immobilized cyro-hydrogel [10], which indicates that the reaction of the PAn–PAN/PPO electrode with catechol is faster.

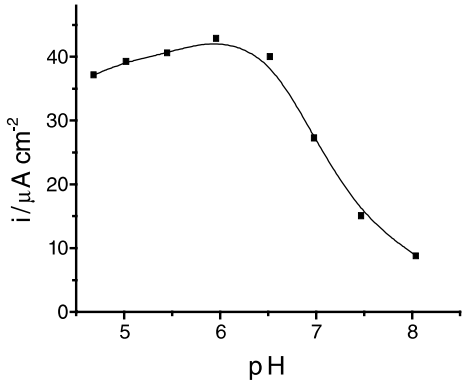

Fig. 2. Effect of pH on the response of the enzyme electrode in 0.1 M PBS containing 20 μM catechol, at 25 °C. Applied potential, −0.05 V.

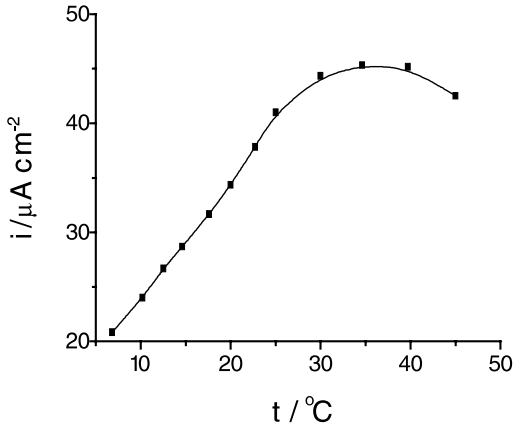

Fig. 3. Influence of temperature on the response of the biosensor in 0.1 M PBS containing 20 μM catechol with pH 6.52, at 25 °C. Applied potential, −0.05 V.

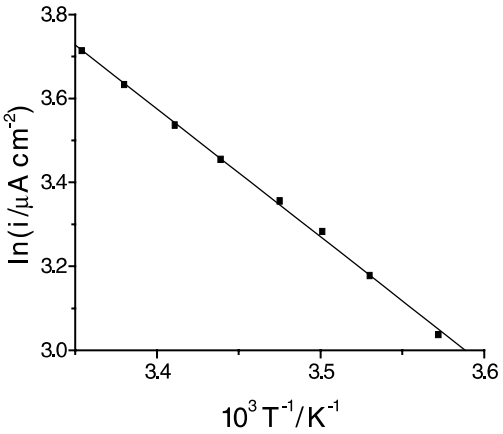

Fig. 4. Plots of log i against T^{-1} according to the data in Figure 3.

3.3. *Response characteristics of the enzyme electrode*

Figure 5 shows the amperometric current response of the sensor as a function of catechol concentration. The calibration curve is linear with catechol concentration up to 75 μM and curves gradually at higher concentrations. The sensitivity of the biosensor to catechol is 2.03 A M^{-1} cm^{-2}. The apparent Michaelis–Menten constant (K_M^{app}) for catechol was calculated to be 0.22 mM according to the electrochemical Lineweaver–Burk form of the Michaelis–Menten equation [24]. This value agrees with that reported (0.24 mM) for the free enzyme [25], illustrating the non-denaturating character of the procedure of enzyme anchoring.

Five phenolic compounds (catechol, phenol, *p*-cresol, *m*-cresol and paradioxybenzene) were monitored by the enzyme electrode. Table 3 presents the response characteristics of the enzyme electrode, including linear range, correlation coefficient, sensitivity and K_M^{app} for the different phenolic compounds. Among the five phenolic compounds, paradioxybenzene gives no response at the enzyme electrode, while the other four compounds show a sensitive response. The sensitivity follows the sequence: catechol > *m*-cresol > *p*-cresol > phenol. The

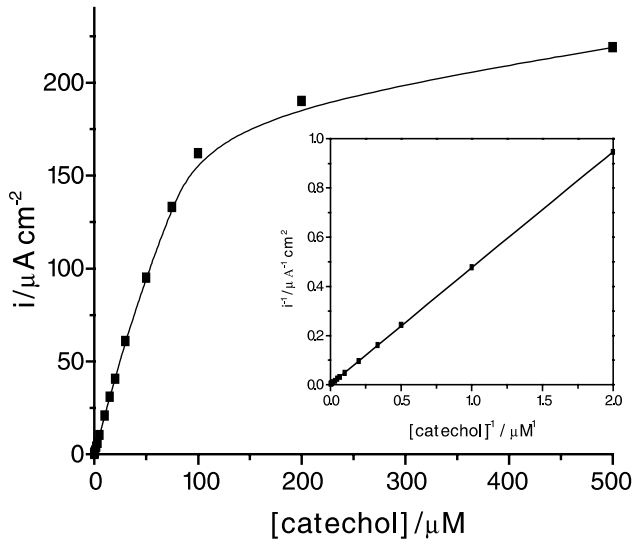

Fig. 5. Calibration curve of the biosensor for catechol in 0.1 M PBS with pH 6.52, at 25 °C; applied potential, −0.05 V. Inset shows determination of apparent Michaelis–Menten constant (K_M^{app}).

Table 3. Response characteristics of the PAn–PAN/PPO electrode to various phenolic compounds

Phenolic compound	Linear range /M	Correlation coefficient	Sensitivity /A M^{-1} cm^{-2}	K_M^{app} /μM
catechol	5×10^{-8} –7.5×10^{-5}	0.998	2.03	220
phenol	1×10^{-7} –7.5×10^{-5}	0.999	0.96	254
p-cresol	2×10^{-7} –5×10^{-5}	0.999	1.38	104
m-cresol	2×10^{-7} –4×10^{-5}	0.999	1.5	77
paradioxy-benzene	/	/	0	/

K_M^{app} are 220, 254, 104 and 77 μM for catechol, phenol, *p*-cresol and *m*-cresol respectively.

For commercialization of the biosensor to be feasible, it must have good operational stability and long lifetime. The operational stability of the PAn–PAN/PPO electrode was investigated by consecutive measurements of its response to 5 μM catechol samples within 8 h (Figure 6). After 100 measurements, the enzyme electrode retained about 97% of its initial activity. This is beneficial to the accurate determination for phenolic compounds. When the enzyme electrode was stored in 0.1 M PBS (pH 6.52) at 4 °C and measured intermittently, the response to 20 μM catechol remained unchanged for six months. This lifetime is markedly longer than that (in general less than three months) reported [6–14]. On the other hand, the sensor in the absence of PAn was unstable, which kept only 57% initial activity after a week (Table 1). These results illuminate that PPO was probably entrapped by polyaniline during *in situ* electropolymerization so that the enzyme is not easily desorbed from the polymer composite film.

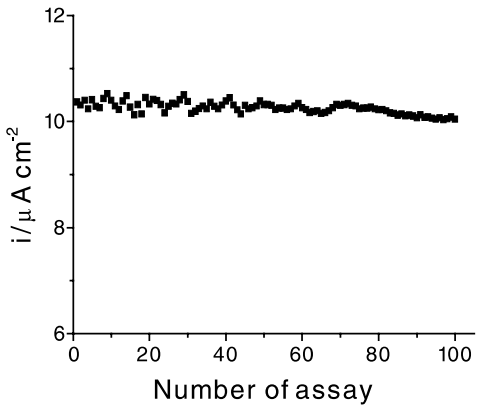

Fig. 6. The operational stability of the biosensor. Experimental condition as in Figure 5.

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