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Improvement in selectivity and storage stability of a choline biosensor fabricated from poly(aniline-co-*o*-aminophenol)

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1. ABSTRACT

A choline biosensor was fabricated by using electrochemical doping to immobilize choline oxidase in poly(aniline-co-*o*-aminophenol) film that exhibits a good electric activity over a wide pH range. Using cyclic voltammetry, impedance measurement and scanning electron microscopy characterized the poly(aniline-co-*o*-aminophenol) film doped with choline oxidase. The amperometric detection of choline is based on the oxidation of the H_2O_2 enzymatically produced on the choline biosensor. The choline biosensor has a lower potential dependence. Thus, its operational potential was controlled at a low potential of 0.40 V(vs.SCE). The response current of the choline biosensor increases with increasing temperature from 277.1 to 308.1 K. An apparent activation energy of 30.8 kJ mol^{-1} was obtained. The choline biosensor has a wide linear response range from 1×10^{-7} to 1×10^{-4} M choline with a correlation coefficient of 0.9999 and has a high sensitivity of 127 μA cm^{-2}, at 0.40 V and pH 8.0. The response time of the biosensor is 15-25 s, depending on the applied potentials. An apparent Michaelis constant and an optimum pH for the immobilized enzyme are 1.8 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. Together, these findings show that the choline biosensor exhibits a better selectivity to interfering species and a better storage stability.

2. INTRODUCTION

Biosensors have become of increasing interest in recent years. This is due to the fact that enzyme catalysis at modified electrodes provides selective molecular recognition and transduction of biochemical information into electrical signals. The latter can be determined rapidly and accurately. Among selective molecular recognition, biomolecular recognition is particularly important in biochemistry, such as DNA molecule. DNA can be detected using biosensors, which has been extensively reviewed by Ju and Zhao (1).

In both the peripheral and central nervous systems of mammals, choline is the precursor and metabolite of the important neurotransmitter acetylcholine. Choline is often used as a marker of cholinergic activity in brain tissue. It is clear that choline plays an important role in biochemistry, which has been described in more details elsewhere (2).

Thus, the determination of choline is very important in the biochemistry field. A number of amperometric sensors, with immobilized choline oxidase (ChO) alone (3-5) and bi-enzyme of ChO and horseradish peroxidase (HRP) (2,5-9), have been reported. The measurement of the response current for the ChO biosensor is based on the oxidation of H_2O_2 at higher potentials, such as at 0.70 V (vs.SCE); on the contrary, the measurement of

the response current for the ChO/HRP biosensor is based on the reduction of H_2O_2 at lower potentials, such as at –0.1 V (vs.SCE). The ChO biosensor has advantages of a very low detection limit (2.5×10^{-9} M) and a wide linear range 1×10^{-5} to 1.75×10^{-3} M choline (3,4). However, its drawback is that some biological compounds, such as glucose, ascorbic acids and uric acid, may interfere with the detection of choline, because the operational potential of the ChO biosensor is rather high as mentioned above. The advantage of the ChO/HRP biosensor is a low operational potential, which can reduce interference. But the biosensors with HRP are most sensitive for a great number of phenolic compounds since phenols can be act as electron donors for peroxidase (10). Thus, interference of phenolic compounds may occur at the ChO/HRP biosensor. A choline biosensor, fabricated using a zinc oxide sol-gel membrane on a poly(N-acetylaniline) modified Pt electrode, also gave a wide linear response range of 1.0×10^{-6} to 1.6×10^{-3} M choline at a constant potential of 0.6 V (vs.SCE) (11).

Conducting polymers have been widely applied as the immobilization materials of enzymes. This is due to the fact that the conducting polymers can be directly polymerized on an electrode material, such as platinum and glassy carbon; the film is uniform and strongly adhesive to the electrode surface; and the thickness of the film can be readily controlled by the charge consumed during the electrochemical polymerization of the monomer. In addition, some conducting polymers can be used as an electron transfer mediator, because they can be oxidized and reduced reversibly.

There are three methods for the immobilization of enzymes using a conducting polymer. The first method is called electrochemical entrapment. In this manner, an enzyme electrode was prepared by the electrolysis of a monomer in a buffer solution containing an enzyme (12-14). The enzyme was entrapped into the conducting polymer film during the electrochemical polymerization of the monomer. A necessary condition for the buffer solution is that its pH value should be controlled at about 7 since an enzyme in a solution with high or low pH values will be denatured. The second one is called electrochemical doping, which is based on the conducting polymer doping and the isoelectric point of an enzyme. In this method, a conducting polymer film was first prepared under the conventional condition, followed by the electrochemical oxidation of the conducting polymer in an enzyme solution, whose pH value is higher than the isoelectric point of the enzyme. In this case, the enzyme with negative charges was doped into the conducting polymer film during the oxidation process to form an enzyme electrode (15). The biosensor fabricated using electrochemical doping is different from that prepared by using physical adsorption. The main difference between them is that the doping of the enzyme is caused by electrostatic interaction between enzyme molecules carried with negative charges and the polymer backbone carried with positive charges (16). Thus, the polyaniline glucose oxidase electrode fabricated by using electrochemical doping is much more stable than that prepared by physical adsorption (17). The third one is called covalent bonding, i.e. the covalent binding of the enzyme to the functional polymer is used to prepare the enzyme electrode (18). One major advantage of covalent bonding is the possibility to avoid leaching of enzyme from the immobilized layer. The choline biosensor and the ChO/HRP biosensor fabricated by using covalent bonding have a long-term storage stability (5). The sensitivity of the ChO/HRP biosensor retained almost 100% up to 55 days. And this biosensor has a good bioelectrochemical response to choline chloride and has a linear range from 1.0×10^{-6} to 8.0×10^{-5} M choline chloride and a fast response time (5).

The advantage of the enzyme electrode prepared by using electrochemical doping is that the conducting polymer, such as polyaniline or polypyrrole, was prepared under a conventional condition. Thus, the conducting polymer has a high conductivity and a good electrochemical property. Especially, the immobilized enzyme using electrochemical doping was carried out under a mild condition, which avoids the denaturation of the enzyme during the immobilization process. Strong evidence for this result is that the optimum pH of the immobilized enzyme, such as glucose oxidase immobilized in the polyaniline film (15) and xanthine oxidase immobilized in the polypyrrole film (19), is very close to that of the free enzyme in solution.

The information from Sigma Company shows that the determination of the choline oxidase activity was carried out at pH 8.0. And some of papers reported that the response current of the choline oxidase electrode was determined also at pH 8.0 (3,5). Thus, the optimum pH of choline oxidase is about pH 8.0. This pH value is not suitable to polyaniline. This is because polyaniline almost loses its electric activity at pH > 6. Fortunately, poly(aniline-co-*o*-aminophenol) still keeps a good electrochemical activity in the solution of Na_2SO_4 with pH 9.6 (20). Therefore, we employed poly(aniline-co-*o*-aminophenol) to immobilize choline oxidase. In this work, we reported the preparation of the choline oxidase electrode, characterization of the copolymer doped with the enzyme and effects of various factors on the properties of the choline biosensor.

3. MATERIALS AND METHODS

3.1. Materials and the immobilization of choline oxidase

Chemicals used were of reagent 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 water was used to prepare the solutions. An electrolytic cell for the synthesis of poly(aniline-co-*o*-aminophenol), the copolymer, 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 copolymerization was performed in a solution consisting of 0.2 M aniline, 0.01 M *o*-aminophenol, 0.3 M ferrocenesulfonic acid and 0.6 M H_2SO_4, using repeated potential cycling between –0.10 and 0.95 V. The electrochemical copolymerization behavior of aniline and *o*-aminophenol has been described in more details elsewhere (21).

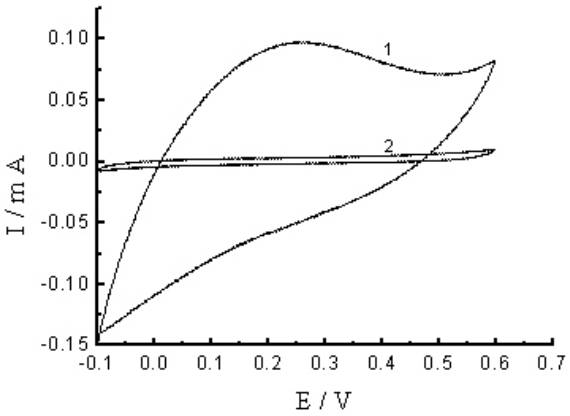

Figure 1. Cyclic voltammograms, (1) poly(aniline-co-*o*-aminophenol), (2) poly(aniline-co-*o*-aminophenol) doped with choline oxidase, in a solution of 0.1 M phosphate buffer with pH 8.0, at a scan rate of 60 mV s^{-1}

After the copolymerization, the copolymer was washed with 0.05 M H_2SO_4 solution to remove unreacted aniline, *o*-aminophenol and ferrocenesulfonic acid, and then was immersed in a 0.2 M H_2SO_4 solution to make cyclic voltammetry between –0.20 and 0.80 V for five cycles to remove further ferrocenesulfonic acid adsorbed on the copolymer surface. It is worth notice that the copolymers after such treatment will be used for cyclic voltammetry, impedance measurement, and the immobilization of choline oxidase. A copolymer film after above treatment was first reduced in 0.05 M H_2SO_4 solution at –0.25 V for 10 min, followed by washing with distilled water, and then was immersed in a solution of choline oxidase to be oxidized at 0.50 V for 10 min. 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. In this case, Choline oxidase with negative charges was doped into the copolymer film to form an enzyme electrode, because the isoelectric point of choline oxidase is 4.5 (22). The enzyme solution can be used repeatedly for preparing biosensors.

3.2. Electrochemical measurements

The pH values of the solutions were determined by using a PXD-12 pH meter. All electrochemical experiments were performed in a conventional three-electrode cell that consisted of a copolymer or a copolymer doped with choline oxidase working electrode, a platinum foil counter electrode and a saturated calomel reference electrode. Cyclic voltammetry was carried out on a CHI 407 electroanalysis apparatus. The scan rate was controlled at 60 mVs^{-1}. The impedance measurements were conducted with an Autolab PGSTAT 30 instrument. Frequency sweeps extended from 10^{4} to 0.01 Hz using a sinusoidal perturbance signal of 10 mV, peak-to-peak.

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 the digital meter, and the 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 morphology of a copolymer film doped with choline oxidase, which was washed with distilled water to remove choline oxidase adsorbed on the electrode surface prior to the measurement of morphology, was observed by using an XL-30 scanning electron microscopy (SEM). The experimental temperature was controlled at 293.1K, unless otherwise stated.

4. RESULTS AND DISCUSSION

4.1. Characterization of the copolymer doped with the enzyme

Using cyclic voltammetry, impedance measurement and scanning electron microscopy characterized the copolymer doped with choline oxidase. Figure 1 shows the cyclic voltammograms of the copolymer without (curve 1) and with choline oxidase (curve 2), respectively, in a solution of 0.1 M phosphate buffer with pH 8.0. It is obvious that the area of curve 2 is much smaller than that of curve 1, i.e., the electrochemical activity of the copolymer with choline oxidase is lower than that of the copolymer without one. This is caused by choline oxidase in the copolymer film, because the enzyme, i.e. protein, has an extremely low conductivity, which makes the conductivity of the copolymer decrease significantly.

Figure 2 (A) and 3(B) are the impedance plots of the copolymer without and with choline oxidase, respectively, in a solution of phosphate buffer with pH 8.0. The potential was set at 0.40 V. Figure 2 shows that the charge transfer resistance (R_{ct}) of the copolymer with choline oxidase is larger than that of the copolymer without choline oxidase. This difference is also caused by the enzyme doped into the copolymer film.

Figure 3 shows the morphology of the copolymer without (A) and with choline oxidase (B). The copolymer film is constructed by a reticulate structure consisting of fibers with different diameters. It is obvious that the copolymer film is porosity. The morphology of the copolymer film in Figure 3 (B) is quite different from that in Figure 3 (A). This difference is attributed to enzyme molecules that occupy the smaller holes between the fibers or cover on the fiber; and some of the enzyme molecules connected together on the copolymer surface (Figure 3B), which are caused by the electrostatic interaction between enzyme carried negative charges and the copolymer backbone with positive charges. However, some of larger holes (naked platinum) between fibers are empty, because the diameters of these holes are much larger than those of enzymes (10-100 nm). It seems that enzyme molecules hardly adsorbed on the naked platinum surface.

The above results give evidence that choline oxidase was doped into the copolymer film during the oxidation process of the copolymer in the enzyme solution.

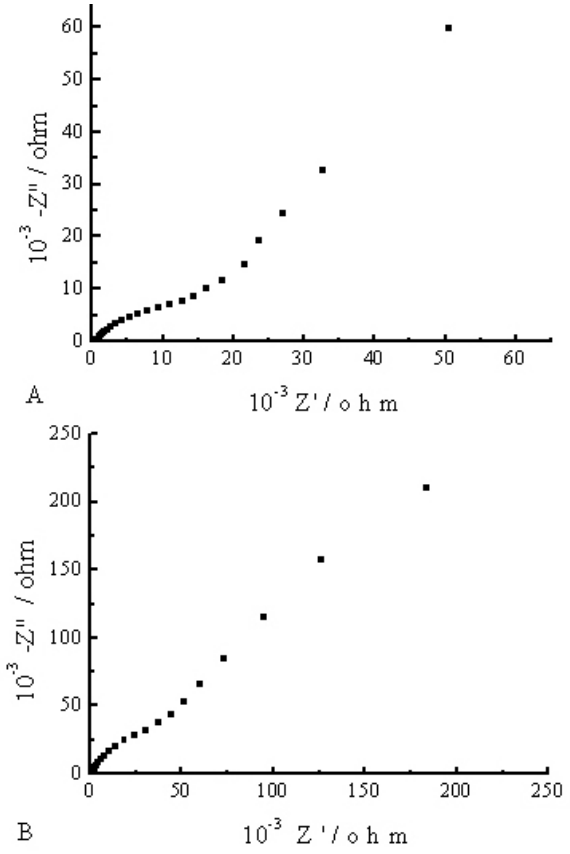

Figure 2. Impedance plots, (A) poly(aniline-co-*o*-aminophenol), (B) poly(aniline-co-*o*-aminophenol) doped with choline oxidase, in the 0.1 M phosphate buffer with pH 8.0, at a potential of 0.40 V.

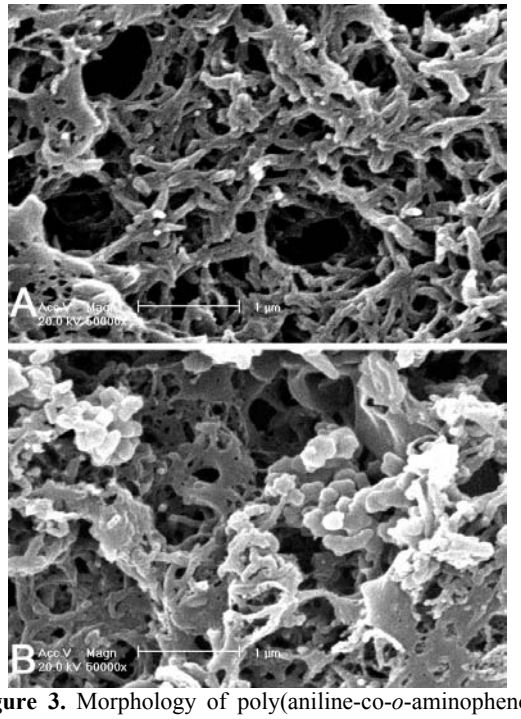

Figure 3. Morphology of poly(aniline-co-*o*-aminophenol) without (A) and with choline oxidase (B).

4.2. Effect of the applied potential on the response of the biosensor

The amperometric detection is based on the oxidation of the H_2O_2 generated enzymatically according to the following reactions:

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

$$H_2O_2 \xrightarrow[\text{(vs.SCE)}]{0.40\ V} O_2 + 2H^+ + 2e^-$$

The response currents of the choline biosensor in a solution containing 50 µM choline chloride and 0.1 M phosphate buffer with pH 8.0 were determined at 0.35, 0.40, 0.55 and 0.65 V. Figure 4 shows the change in the response current with time at different potentials. The currents on curves 1-4 increase quickly with time first, and then reach a maximum to form a peak, and finally decrease with time. The time necessary to reach a current maximum is the response time of the biosensor. It is obvious that the response time reduces with increasing potential, and is about 25 s at 0.35 V (curve 4) and 15 s at 0.65 V (curve 1). The formation of the current peak means that the rate of the enzyme-catalyzed reaction is faster than that of the electrode reaction, i.e., the H_2O_2 generated enzymatically can rapidly enough supply the needs of the following electrode reaction at 0.35 to 0.65 V. This 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, this effect is not large. In order to reduce interference of other organic compounds, the operational potential of the enzyme electrode was controlled at 0.40 V in the following experiments.

4.3. 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 5. A solution containing 50 µM choline in 0.1 M phosphate buffer was used in this experiment. The potential of the biosensor was set at 0.40 V. Figure 5 shows that the response current increases with increasing pH from 6.4 to 8.4. A maximum 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 5 are very close to the sensor fabricated by using physical immobilization of bi-enzyme of ChO and HRH onto the Prussian blue-based carbon paste electrode in the pH range of 6.0-9.0 (8), and also very close to that of choline oxidase in solution, in which the response increases with pH up to a maximum plateau of pH 8-9 (8). Each enzyme has an optimum pH that is characteristic of an enzyme. The above result indicates that the property of choline oxidase immobilized in the copolymer using electrochemical doping is not affected. However, the pH dependence of choline oxidase immobilized in the conducting polymer using covalent bonding shows that its response increases from pH 6.0 to 8.0, and then decreases quickly over pH 8.0 (5). This

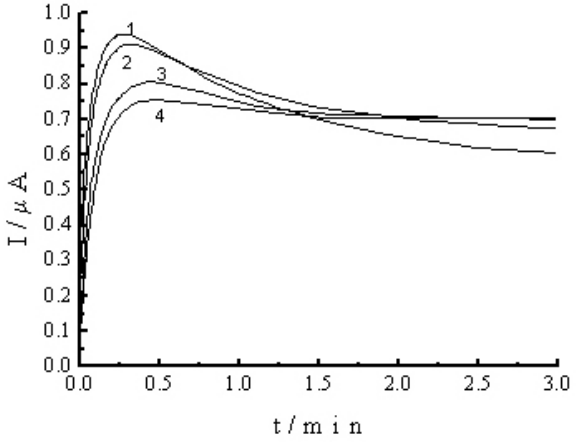

Figure 4. The change in the response current of the biosensor with applied potentials in a solution containing 50 μ M choline and 0.1 M phosphate buffer with pH 8.0. Curves: (1) 0.65 V, (2) 0.55 V, (3) 0.40 V, (4) 0.35 V.

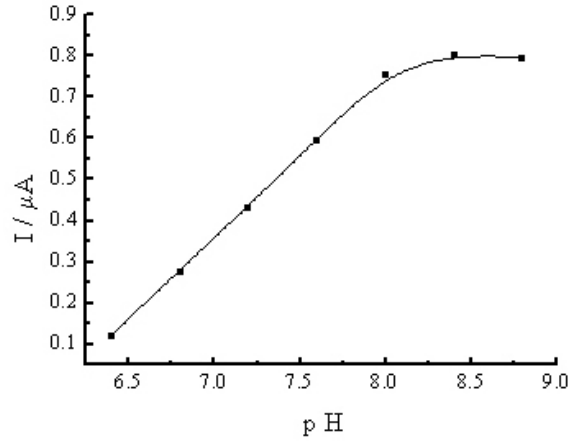

Figure 5. The response current of the biosensor at 0.40 V as a function of pH value, in a solution containing 50 μ M choline and 0.1 M phosphate buffer.

behavior is quite different from that of choline oxidase in solution as mentioned above. This difference is probably caused by the covalent bonding of choline oxidase and the conducting polymer.

4.4. Effect of the concentration of choline on the response current of the biosensor

Based on 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 0.40 V and the pH of the solution was controlled at 8.0. Considering both of the response current of the biosensor being a little higher at pH 8.4 than that at pH 8.0 and the effect of pH on the electric activity of the copolymer, the pH 8.0 of the solution is chosen in the following experiments. Figure 6 shows the change in the response current with the concentration of choline from 0.1 to 200 μM. The response current increases linearly with increasing choline concentration from 0.1 to 100 μM with a correlation coefficient of 0.9999 and then increases slowly with further increase in the concentration of choline. The linear range in Figure 6 (A) is larger than that of ChO/HRP biosensor fabricated by using covalent bonding (5). Figure 6 (B) is a typical enzyme-catalyzed reaction curve between the reaction rate and the substrate concentration.

The plot of I^{1}versus [choline]$^{-1}$, based on the experimental data in Figure 6, is shown in Figure 7. A maximum current response and an apparent Michaelis constant K_m were calculated from the intercept and the slope of the straight line to be 35.54 μA and1.8 mM, respectively. The latter is very 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 (8) and also close to that of choline oxidase in solution with pH 8 (22,23). Each enzyme has a characteristic K_m for a given substrate. Thus, the K_m determined here confirms again that the property of choline oxidase immobilized in the copolymer film is hardly affected by using electrochemical doping. Based on the maximum current and the area of electrode, the sensitivity of the biosensor is 127μA cm^{-2}, which is rather high.

4.5. 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 50 μM choline and 0.1 M phosphate buffer with pH 8.0 is shown in Figure 8. The potential was controlled at 0.40 V. The temperature was set between 277.1 and 308.1 K. Figure 8 shows that the response current increases with increasing temperature.

Based on the data shown in Figure 8, a plot of log I versus T^{-1} gives a straight line (omitted here). An apparent activation energy E_a was calculated from the slope of the line to be 30.8 kJ mol^{-1}.

4.6. Interference and stability of the biosensor

Ascorbic acid, uric acid, phenol and glucose were used for the study of interference with the determination of choline. The solutions used for this study consisted of 50 μM of each compound and 0.1 M phosphate buffer with pH 8.0. The potential of the biosensor was set at 0.40 V. Figure 9 shows the currents of the biosensor in each solution as a function of time. Curve 1 in Figure 9 is the change in the response current of the biosensor in choline solution with time. The maximum currents of the biosensor in the ascorbic acid solution (curve 2) and uric acid solution (curve 3) are 0.088 and 0.036 μA, which are about 10% and 4.5% of the response currents of the biosensor in the choline solution under the same condition, respectively. Thus, the effect of ascorbic acid and uric acid on the determination of choline is smaller, because of a lower operational potential. Curves 4 and 5 in Figure 9 are the change in the oxidation current of phenol and glucose with time. It is obvious that little oxidation current of phenol or glucose was detected on the biosensor at 0.40 V. This indicates that phenol and glucose were hardly oxidized on the copolymer film. The above results show that the choline biosensor has a good selectivity to interfering species.

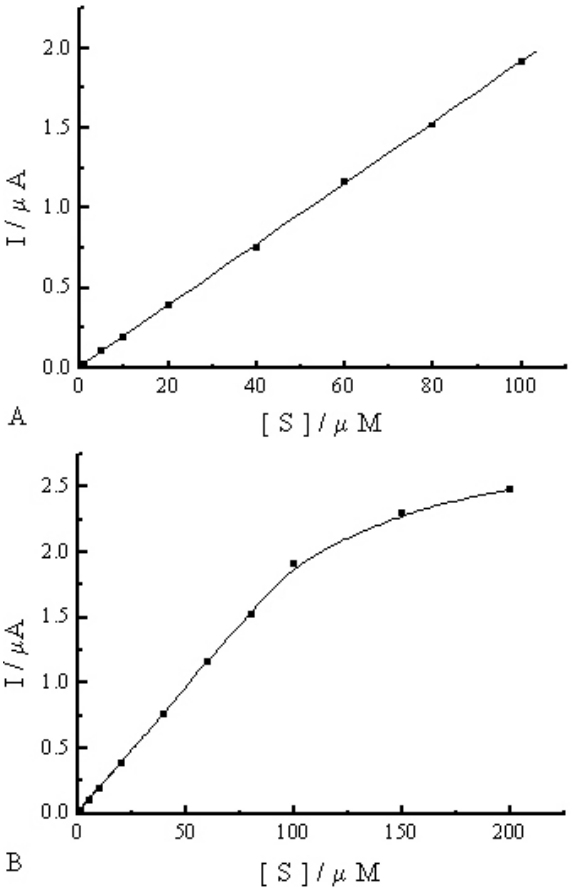

Figure 6. The relationship between the response current of the biosensor and the concentration of choline, pH 8.0, at a constant potential of 0.40 V.

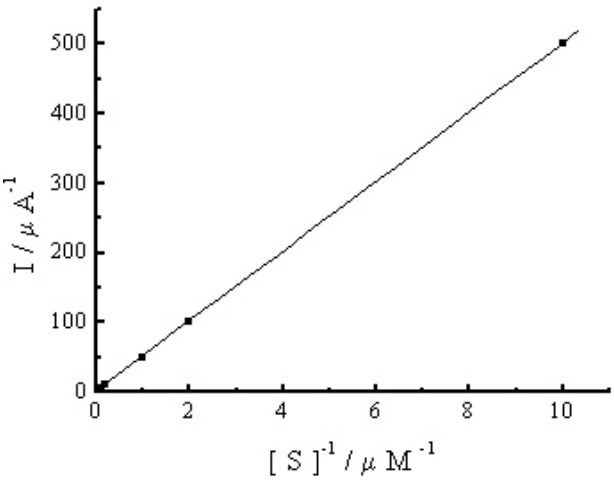

Figure 7. The plot of 1/I versus 1/[S] based on the data shown in Figure 6.

The stability of the biosensor was tested in a solution consisting of 60 μM choline and 0.1 M Na_2HPO_4 with pH 8.0 and at 0.40 V. The response current of the biosensor decreased from 0.503 to 0.257 and 0.229 μA after 40 and 52 days, i.e. 49% and 54% decay of the initial activity was observed. Thus, the biosensor has a better storage stability, which but is less than that of the choline biosensor fabricated by using covalent bonding (5). This may be caused by de-doping of choline oxidase and the activity of choline oxidase itself since the biosensor was kept in a 0.1 M Na_2HPO_4 solution of pH 8.0 at 277.1K after each measurement and the enzyme solution for the preparation of this biosensor has been stored for 20 days.

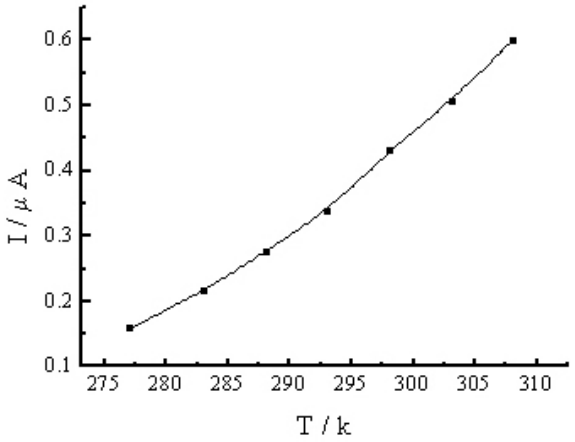

Figure 8. The change in the response current of the biosensor with temperature in a solution containing 50 μ M choline and 0.1 M phosphate buffer with pH 8.0, at a potential of 0.40 V.

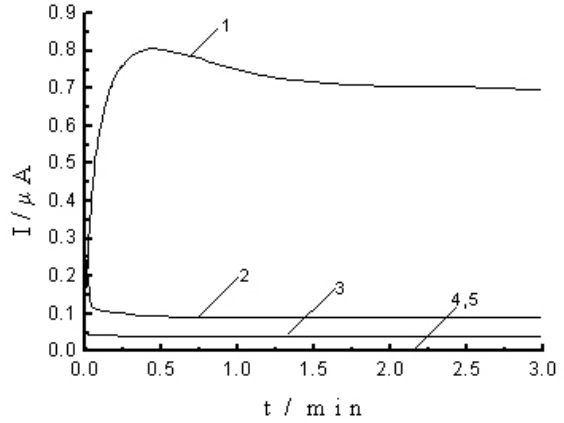

Figure 9. Effect of compounds on the response current of the biosensor, curves: (1) choline, (2) ascorbic acid, (3) uric acid, (4) phenol, (5) glucose, in a solution containing 50 μM of each compound and 0.1 M phosphate buffer with pH 8.0, at 0.40 V.

5. CONCLUSIONS

A choline biosensor has been prepared by using electrochemical doping. The biosensor has a wide linear range with a high sensitivity and a lower potential dependence. The latter is favorable to practical applications. The apparent Michaelis constant and the optimum pH of the immobilized choline oxidase are very close to those of choline oxidase in solution. This is strong evidence that choline oxidase immobilized in the copolymer film by using electrochemical doping was hardly denatured. Thus, poly(aniline-co-*o*-aminophenol) is a good material suitable to the immobilization of choline oxidase.

6. ACKNOWLEDGEMENTS

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