Development of an Amperometric Biosensor Based on a Redox-Mediator-Doped Polypyrrole Film

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ABSTRACT: An amperometric biosensor was developed for the quantitative estimation of phenolic compounds in aqueous media. The enzyme tyrosinase [poly(phenol oxidase) (PPO)] was adsorbed onto a hexacyanoferrate(II)-ion-doped conducting polypyrrole (PPY) film deposited on an indium tin oxide (ITO) coated glass-plate support. The PPO activity in the PPO/Fe^{2+}-PPY/ITO film was assayed as a function of the concentration of phenolic compounds. Cyclic voltammetric studies were carried out on this enzyme electrode, and the surface morphology of the enzyme-immobilized polymer film was studied with scanning electron microscopy. The results of the amperometric response of the PPO/Fe^{2+}-PPY/ITO film showed sensitivities of 0.14, 0.21, and 0.36 A $M^{-1}cm^{-2}$ and linear response ranges of 9.9–84.7, 6.7–72.6, and 3.9–48.8 μM for phenol, catechol, and *p*-chlorophenol, respectively. The PPO/Fe^{2+}-PPY/ITO electrode exhibited a response time of about 50 s and was stable for about 12 weeks at 4°C. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 93: 927–933, 2004

Key words: biocompatibility; biomaterials; electrochemistry; enzymes; films

INTRODUCTION

Phenol is one of the most important and widely used industrial chemicals in the manufacture of products ranging from plastic resins to plasticides. Studies have shown the existence of phenols as pollutants of air, water, and soil. Phenol is easily adsorbed in humans, regardless of the type of exposure. Besides this, high levels of phenols have been shown to have detrimental effects on animal health. Prolonged oral or subcutaneous exposure causes damage to the lungs, liver, kidney, and genitourinary tract. A number of phenol compounds are listed in European Community (EC) Directive 76/464/EEC, which concerns dangerous substances discharged into aquatic environments,[1] and in the U.S. Environmental Protection Agency list of priority pollutants because of their toxicity and persistence in the environment.[2,3] According to the U.S. Environmental Protection Agency, a phenol concentration of 4.0 mg L^{-1} in domestic drinking water is not expected to cause any adverse noncarcinogenic effects in a 70-kg adult over a lifetime of exposure, with a margin of safety.[4] Because of the concern regarding the toxicity and chemical importance of these compounds, there is a growing need for innovative devices to monitor phenols in complex environments and food, pharmaceutical, and industrial matrices. Amperometric biosensors that combine the high specificity of biological catalysts such as enzymes with the sensitivity and accuracy of electrochemical indicator reactions play an important role in the field of biomedicine and environmental science.

Electrochemically polymerized conducting polymers have received considerable attention over the last 2 decades. The remarkable switching capability of these materials between the conducting oxidized (doped) state and insulating reduced (undoped) state is the basis of many applications. Among others, polyconjugated conducting polymers have been recently proposed for biosensing applications because of a number of favorable characteristics, including (1) direct and easy deposition on sensor electrodes by the electrochemical oxidation of the monomer, (2) control of the thickness by deposition charge, and (3) redox conductivity and polyelectrolyte characteristics useful for sensor applications. These requirements are met with polypyrrole (PPY), which is the most commonly used polymer because of its easy oxidation, the low cost of the monomer, and the chemical stability of the polymer.[5] Ferrocene–pyrrole conjugates are efficient oxidants of reduced glucose oxidase, which upon anodic polymerization form redox active films to construct a reagentless glucose electrode.[6] The enzyme glucose oxidase and a mediator have been simultaneously immobilized in PPY films to provide a simple biosensor capable of detecting glucose rapidly over a

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wide concentration range without the use of any mediator in solution.[7] Cholesterol oxidase and a mediator have been incorporated into PPY films by electropolymerization and physical adsorption techniques, respectively, for biosensor applications to cholesterol.[8,9] Several enzyme electrodes have already proven useful for the task of environmental monitoring. For examples, several groups have reported sensitive amperometric biosensors for phenolic compounds.[10,11] Such devices rely on the immobilization of tyrosinase onto carbon or platinum transducers and the low potential detection of the liberated quinone product. Assays of industrial wastes and natural water have been documented,[12,13] including possible remote phenol sensing and single-use on-site sensing.[12]

Keeping in mind the importance of phenolic compounds and the applications of conducting polymers in the field of biosensing, we propose the development of an amperometric biosensor through the immobilization of enzyme tyrosinase, also known as poly(phenol oxidase) (PPO), in an electrochemically synthesized redox-electron-mediator-doped conducting PPY (Fe^{2+}-PPY) film on indium tin oxide (ITO) coated glass plates for the quantitative estimation of phenolic compounds in aqueous media.

EXPERIMENTAL

Materials

Mushroom tyrosinase (EC 1.14.18.1, 2500 U/mg from mushrooms) and L-tyrosine were obtained from Sigma. The pyrrole monomer and potassium hexacyanoferrate(II) were procured from Wako (Japan). Pyrrole was distilled three times, and a potassium hexacyanoferrate(II) solution was freshly prepared before use. All other chemicals were analytical-grade and were used without further purification.

Apparatus

Ultraviolet–visible (UV–vis) absorbance data were collected with a Jasco V-570 spectrophotometer. Fourier transform infrared (FTIR) spectra of the films were recorded on a Jasco 230 instrument. Scanning electron micrographs were obtained with a Shimadzu SS-550 super scan at an acceleration voltage of 12.0 kV. Cyclic voltammetry (CV) was carried out on a Hakuto Denko HSV-100 cyclic voltammetry instrument in a three-electrode-cell configuration consisting of a working electrode (PPO/Fe^{2+}-PPY/ITO film), a Ag/AgCl reference electrode, and a platinum wire as a counter electrode. A stirring bar and magnetic stirrer provided convective transport during the amperometric measurements.

Preparation of the redox-mediator-doped PPY film

The PPY films were electrochemically prepared from freshly distilled 0.1*M* pyrrole and 0.1*M* potassium hexacyanoferrate(II) aqueous solutions on a 0.5 cm $\times$ 0.5 cm (0.25 cm^2) ITO-coated glass plate at a fixed voltage of 0.8 V versus a saturated calomel electrode. The thickness of the films was about 1.6 μm, as calculated from the injected charge.[14]

Fabrication and characterization of the enzyme electrode

A phosphate buffer solution (10 μL) containing 20 units of tyrosinase was deposited onto a 0.5 cm $\times$ 0.5 cm (0.25 cm^2) electrochemically prepared thin hexacyanoferrate(II)-ion-doped PPY (Fe^{2+}-PPY) film. The films were washed three times with a buffer solution, dried at 10°C, and finally stored at 4°C in an incubator.

FTIR and scanning electron microscopy (SEM) were used to characterize the enzyme electrodes to determine the nature of the enzyme entrapment within the polymer matrix and the surface morphology of the film, respectively. Amperometric measurements were performed in a conventional three-electrode-cell configuration consisting of a working electrode (PPO/Fe^{2+}-PPY/ITO), a Ag/AgCl reference electrode, and a platinum wire as a counter electrode. A stirring bar and magnetic stirrer provided convective transport. Amperometric measurements were carried out on a Hokuto Denko HSV-100 cyclic voltammetry apparatus. All amperometric measurements were performed at about 25°C in an air-saturated phosphate buffer solution (pH 7.0).

The operational stability of the biosensor was tested by repetitive amperometric measurements conducted on a 50 μM phenol solution. The long-term storage stability was studied over 3 months via the monitoring of its current to the 50 μM phenol standard solution with intermittent usage (every 2–3 days) and storage in a refrigerator at 4°C in the dry state.

Enzyme activity measurements

PPO oxidizes L-tyrosine to dihydroxyphenylalanine, which in turn is oxidized to *o*-quinone. The latter is accompanied by an increase in absorbance at 280 nm (A_{280}). The rate of this increase is proportional to the enzyme concentration and is linear for 5–10 min after an initial lag. One unit causes a change in A_{280} of 0.001/min at 25°C and pH 6.5. One milliliter of 0.001*M* L-tyrosine, 1.0 mL of a 0.5*M* phosphate buffer (pH 6.5), and 0.9 mL of reagent-grade water were mixed and placed into cuvettes. This reaction mixture was oxygenated through the bubbling of air into the cuvettes through a capillary tube for 4–5 min. Afterward, the

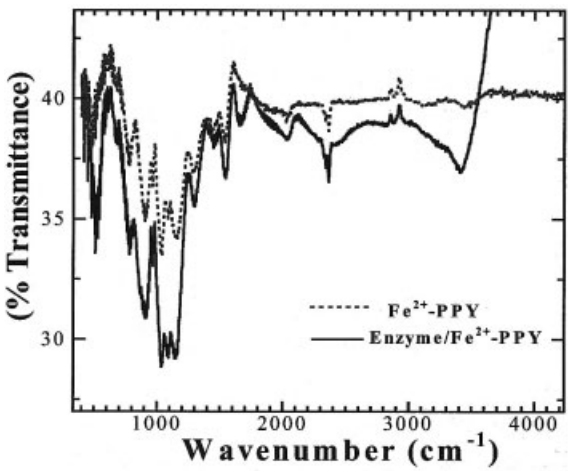


Figure 1 FTIR spectra of Fe^{2+}-PPY/ITO and PPO/Fe^{2+}-PPY/ITO at room temperature.

cuvettes were transferred to the spectrophotometer, and A_{280} was recorded for 10–15 min to achieve temperature equilibrium and to establish the blank rate. A PPO/Fe^{2+}-PPY/ITO electrode was immersed for 20 min in a phosphate buffer solution. The electrode was taken out from the buffer solution, and 0.1 mL of the buffer solution was added to the reaction mixture in a cuvette; A_{280} was recorded for 10–15 min to determine the possible leaching of the enzyme from the PPO/Fe^{2+}-PPY/ITO electrode. ΔA_{280} was determined from the linear portion of the curve, and the enzyme activity was calculated as follows:[15]

$$\text{Units(mL)} = \Delta A_{280} \times 1000 \text{ (mL of enzyme in reaction)}$$

It was found that 22% of the immobilized (adsorbed) enzyme leached out from the surface of the electrode within 10 min of the film being placed in a buffer solution.

RESULTS AND DISCUSSION

Characterization of enzyme-immobilized hexacyanoferrate(II)-ion-doped PPY films

The entrapment of the enzyme within the polymer matrix was confirmed with FTIR and UV–vis spectrophotometry. The FTIR spectra of Fe^{2+}-PPY and PPO/Fe^{2+}-PPY are shown in Figure 1. Sharp peaks at 1533 and 1000–1100 cm^{-1} have been attributed to the C═C stretching mode and C—C stretching, respectively.[16] The characteristic new peak at 3421 cm^{-1} in PPO/Fe^{2+}-PPY is assigned to N—H stretching of free amide, indicating the presence of immobilized tyrosinase enzyme. The peak at 1533 cm^{-1} in the Fe^{2+}-PPY spectrum is about 10 cm^{-1} positively shifted to 1544 cm^{-1}, and two new peaks arise at 1648 and 1080 cm^{-1} in the PPO/Fe^{2+}-PPY spectra. These results show that there might be electrostatic interactions between the positively charged doped Fe^{2+} and the negatively charged tyrosinase enzyme (isoelectric point = 4.2) within the polymer matrix.[17] The adsorption of the enzyme over the polymer matrix was also confirmed with UV–vis spectroscopy. When the PPO/Fe^{2+}-PPY/ITO electrode was immersed in a phosphate buffer containing L-tyrosine, the observed increase in A_{280} was characteristic of L-3,4-dihydroxyphenyl alanine produced by an enzymatic reaction. The results indicate that the enzyme (PPO) was incorporated into the hexacyanoferrate(II)-doped PPY film. However, from the enzyme activity test, it was found that 22% of the immobilized enzyme leached out within 10 min after the film was placed in the buffer solution.

The morphologies of Fe^{2+}-PPY/ITO and PPO/Fe^{2+}-PPY/ITO electrodes were characterized with SEM. Figure 2 shows SEM micrographs of Fe^{2+}-PPY/ITO and PPO/Fe^{2+}-PPY/ITO obtained with 2.5 × 500, 2.5 × 2000, and 2.5 × 7000 magnifications. Two types of dots (light dark and white) can be seen in the SEM micrograph of Fe^{2+}-PPY/ITO [Fig. 2(A)]; they become even more evident at the higher magnification of 2.5 × 2000, clearly indicating the presence of white doped $[Fe^{2+}(CN)_6]^{4-}$ domains over the porous, light dark granules of the pyrrole polymer. Moreover, two different sizes of the white dot particles can be seen uniformly distributed on the surface of PPO/Fe^{2+}-PPY/ITO [Fig. 2(C)]; this also becomes more evident at a higher magnification [Fig. 2(D)]. These brightly colored larger particles can be attributed to protein molecular chains, which become entrapped within the porous granular matrix of the doped polymer [Fig. 2(D)].

Electrochemical studies of enzyme-immobilized hexacyanoferrate(II)-ion-doped PPY films

The enzyme electrode tends to be more stable than the direct electrochemical oxidation of phenol to quinone. The enzyme electrode has an advantage over the direct electrochemical oxidation of phenol in its low potential detection (0 to −0.2 V vs Ag/AgCl, in contrast to +0.80 to +0.95 V vs Ag/AgCl) because at higher voltages, the enzymatically produced quinone is polymerized and is responsible for the fouling of the electrode.[17] Potassium hexacyanoferrate(II) has been used as a reducing agent for quinone species in aqueous phenol sensors,[18] and it has been suggested that the reaction between the quinone and hexacyanoferrate(II) reduces the possibility of enzyme inactivation by quinone. This being the case, hexacyanoferrate(II) may improve the lifetime and response of a bioelectrochemical sensor. In this approach, a conducting PPY film doped with hexacyanoferrate(II), a redox mediator, was used as a suitable matrix for enzyme (PPO) immobilization to construct an enzyme elec-

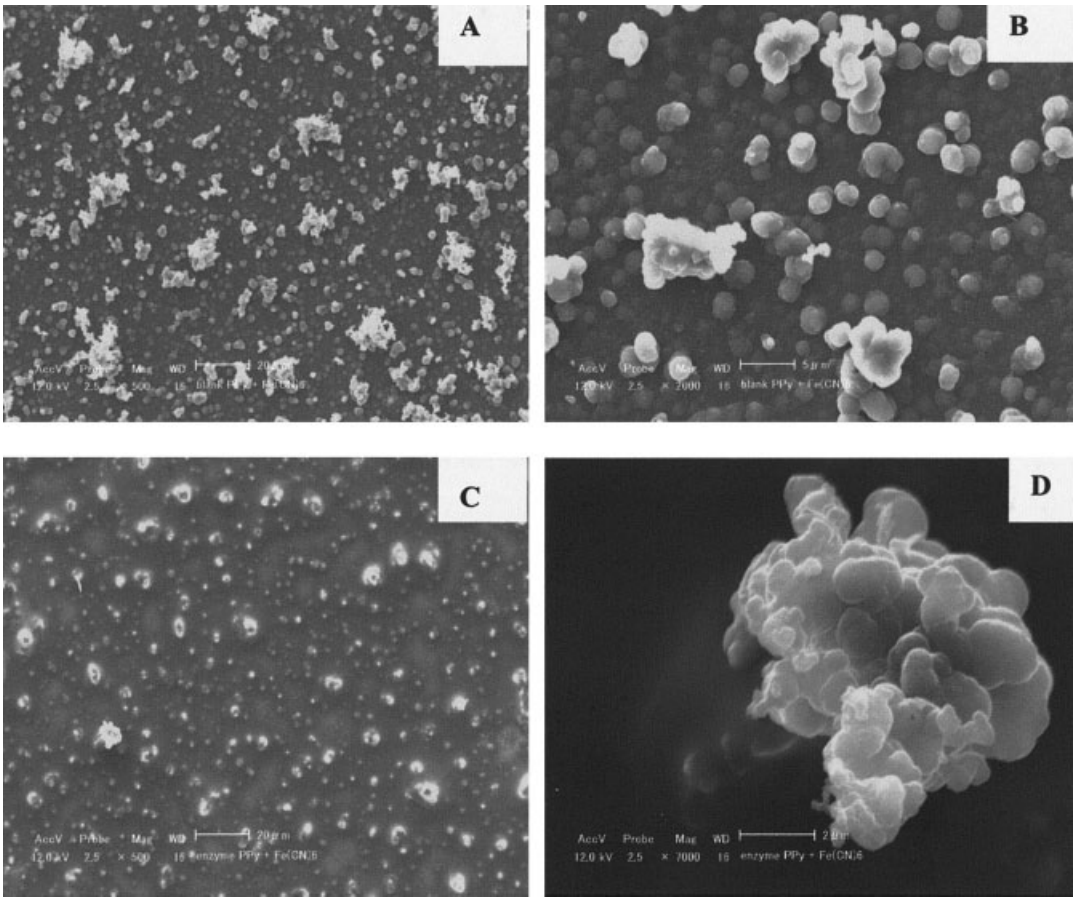
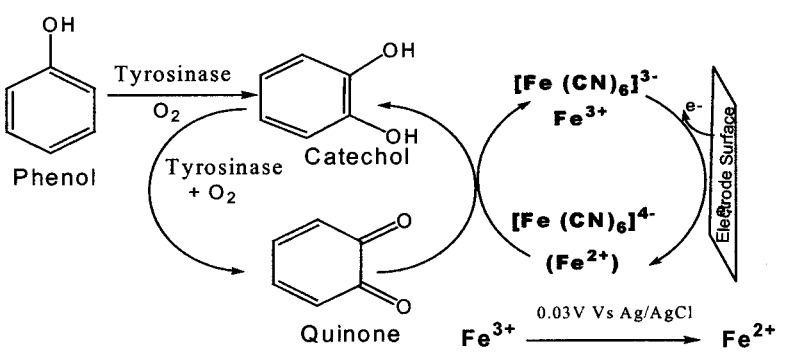


Figure 2 SEM micrographs of (A) Fe^{2+}-PPY/ITO at 2.5 × 500, (B) Fe^{2+}-PPY/ITO at 2.5 × 2000, (C) PPO/Fe^{2+}-PPY/ITO at 2.5 × 500, and (D) PPO/Fe^{2+}-PPY/ITO at 2.5 × 7000.

trode and to provide fast and convenient detection of phenolic compounds in an aqueous medium at a low potential range of about 0.0–0.1 V versus Ag/AgCl. The enzyme-catalyzed reaction is shown in Scheme 1.

The electrochemical studies were carried out on these mediator-doped polymer-based enzyme electrodes (PPO/Fe^{2+}-PPY/ITO) toward a phenolic concentration in aqueous media. A three-electrode cell was used for the electrochemical studies with the PPO/Fe^{2+}-PPY/ITO electrode as the working electrode, platinum foil as the counter electrode, and Ag/AgCl as the reference electrode. A 0.2*M* ammonium phosphate buffer solution was used as the electrolyte. The reduction peak current increased as the phenol concentration increased, as shown in Figure 3. At the same time, the oxidation peak decreased as the phenol concentration increased; this demonstrated a fast enzymatic reaction between the enzymatically produced quinone and $[Fe^{2+}(CN)_6]^{4-}$ species at the electrode surface. With each repeated electrochemical cycle for a



Scheme 1

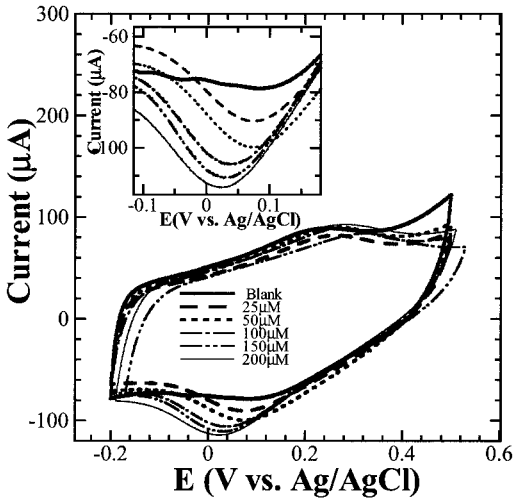


Figure 3 Cyclic voltammograms of PPO/Fe^{2+}-PPY/ITO electrodes in a 0.2*M* phosphate buffer (pH 7.0) with different concentrations of phenol (μM). The scanning rate was 5 mV/s.

fixed concentration of phenol, no significant change in the peak current was found, and this revealed that the enzyme activity lasted for a longer time. Even after similar experiments were repeated with the same PPO/Fe^{2+}-PPY/ITO electrode at least 10 times, no significant decrement in the reduction peak was observed; this demonstrated a high stability of enzyme activity within the polymer matrix and high reproducibility. The cyclic voltammetry study was carried out with a PPO/Fe^{2+}-PPY/ITO electrode at a fixed phenol concentration of 50 μM at different scanning rates of 5–25 mV/s (Fig. 4). The inset of Figure 4 shows the resulting plot of the reduction peak current versus the square root of the scanning rate in the range of 5–25 mV/s, indicating the condition of linear diffusion.

Amperometric response studies

A three-electrode-cell configuration similar to the one used in cyclic voltammetric experiments was used for the amperometric response studies of phenolic compounds (phenol, catechol, and *p*-chlorophenol) in an air-saturated phosphate buffer (pH 7.0). Chronoamperometric response monitoring was performed through measurements at 1-min intervals in agreement with the chronoamperometric protocol, and the results are shown in Figure 5(a–c). The amperometric response was measured at PPO/Fe^{2+}-PPY/ITO after the decay of the transient current for these phenolic compounds in aqueous solutions at a fixed bias voltage of +0.03 V versus Ag/AgCl under slow stirring at room temperature. An increase in the reduction current, that is, Fe^{3+} to Fe^{2+} reduction at the electrode surface, was due to the increasing oxidation of Fe^{2+} to Fe^{3+} by the enzymatically produced quinone species with the increasing concentration of the phenolic solution. Figure 6 illustrates the steady-state current dependence calibration curve on the concentration of phenolic compounds (phenol, catechol, and chlorophenol). Rapid enzyme electrode responses to the substrates and 95% of the transient current decay were obtained within 50 s. This short response of the PPO/Fe^{2+}-PPY/ITO electrode revealed that the faster electronic exchange occurred between the enzyme (PPO)-produced quinone and hexacyanoferrate(II). Linearity was observed within the ranges of 9.9–84.7 μM (regression, $r = 0.999$), 6.7–72.6 μM ($r = 0.999$), and 3.9–48.8 μM ($r = 0.999$) for phenol, catechol, and chlorophenol, respectively. The sensitivities of the enzyme electrode toward phenol, catechol, and *p*-chlorophenol were 0.14, 0.21, and 0.36 A M^{-1} cm^{-2}, respectively. The detection limits were calculated according to the formula $3\sigma_b/m$,[19] where m is the slope of the calibration graph and σ_b is the standard deviation of the blank signal. The detection limits for phenol, catechol, and *p*-chlorophenol were found to be 2.90, 2.03, and 1.19 μM, respectively. The sensitivity was in the following order: *p*-chlorophenol > catechol > phenol. The linearity was lost at higher concentrations in the reverse order; this was similar to a trend found earlier with other biosensors.[12,20] The loss of linearity at higher concentrations of the phenolic compounds was caused by diffusion limitations. However, no significant decrease in the response was observed after at least 10 uses in testing, and good reproducibility was displayed. The relative standard deviation determined by 10 successive analyses of a 50 μM phenol standard with a single PPO/Fe^{2+}-PPY/

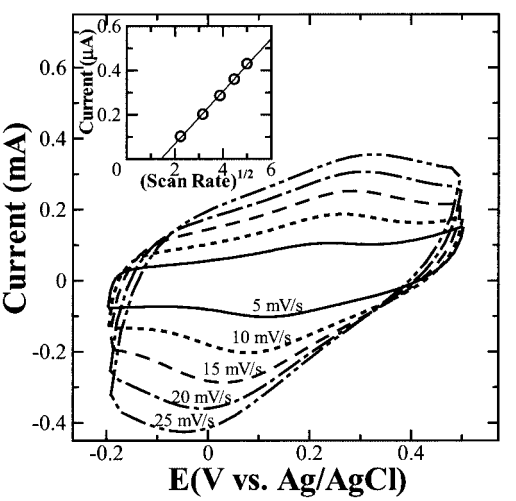


Figure 4 Cyclic voltammograms of PPO/Fe^{2+}-PPY/ITO electrodes in a 0.2*M* phosphate buffer (pH 7.0) with a 50 μM aqueous phenol solution at different scanning rates ranging from 5 to 25 mV/s.

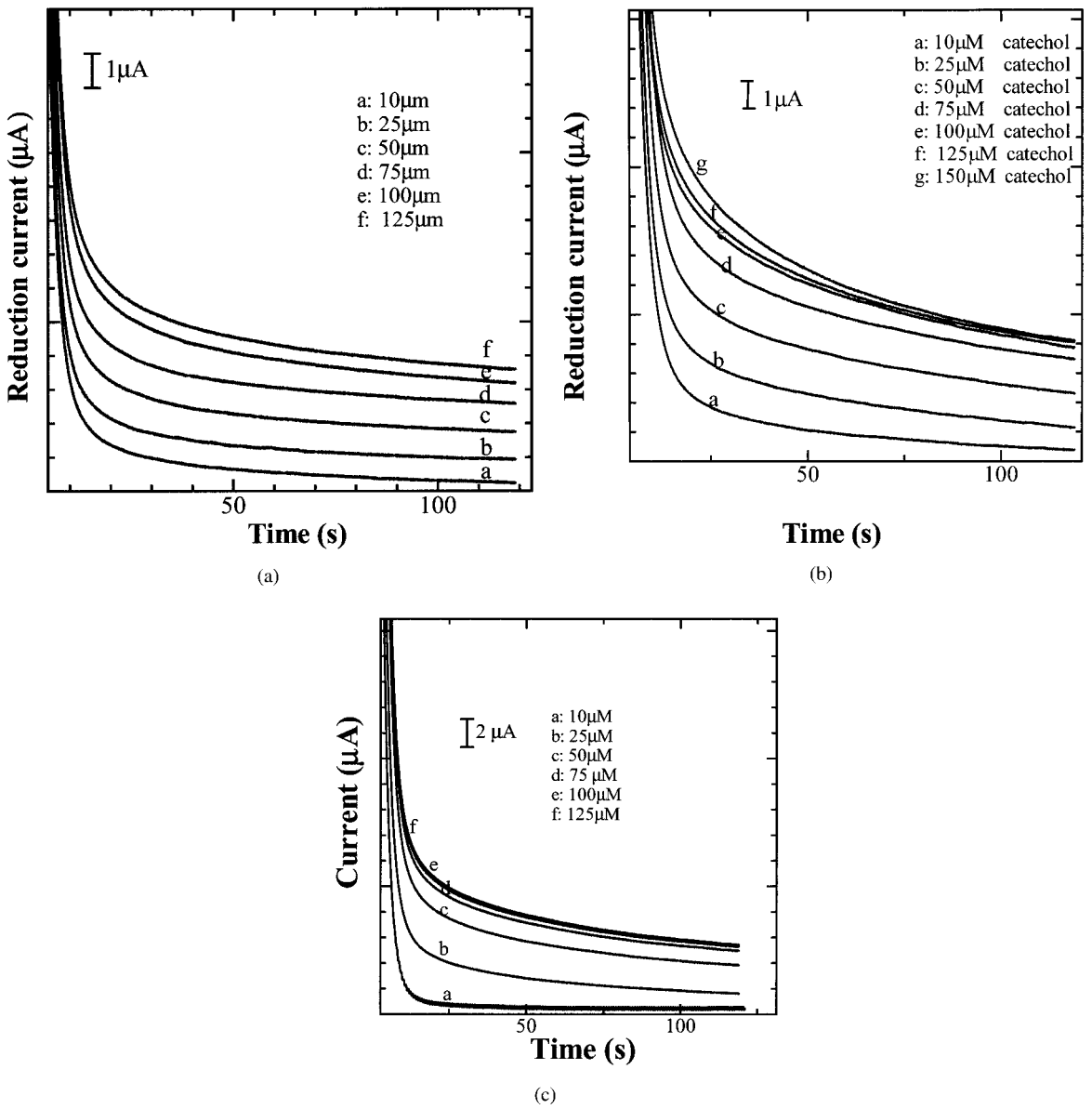


Figure 5 (a) Typical steady-state current response of a biosensor (PPO/Fe^{2+}-PPY/ITO) to an increasing concentration of phenol, (b) typical steady-state current (chronoamperogram) response of a biosensor (PPO/Fe^{2+}-PPY/ITO) to an increasing concentration of catechol, and (c) typical steady-state current (chronoamperogram) response of a biosensor (PPO/Fe^{2+}-PPY/ITO) to an increasing concentration of *p*-chlorophenol.

ITO electrode was found to be about 5%. In a series of 10 PPO/Fe^{2+}-PPY/ITO electrode sensors, a relative standard deviation of about 8% was obtained for the individual current response for the same sample (50 μM phenol).

The PPO/Fe^{2+}-PPY/ITO electrodes were also studied for enzyme stability at both room temperature and 4°C in an incubator. The stability of the enzyme was monitored with time in a sample of a 50-μM standard phenol solution. A very slow decrement in the response current was obtained up to 3 months. It was observed that the PPO/Fe^{2+}-PPY/ITO electrode retained 80% of the initial enzyme activity for 3 months when stored at 4°C in a refrigerator. This long-term stability of the PPO/Fe^{2+}-PPY/ITO electrode was more than that of the recently reported conventional biosensors (<15 days).[21–23] At room temperature, however, the electrode was stable for approximately 4 weeks and thereafter showed a rapid enzyme inactivation.

CONCLUSIONS

PPO can be physically adsorbed onto redox-mediator [hexacyanoferrate(II) ion]-doped PPY films on ITO glass plates. A PPO/Fe^{2+}-PPY/ITO electrode (biosen-

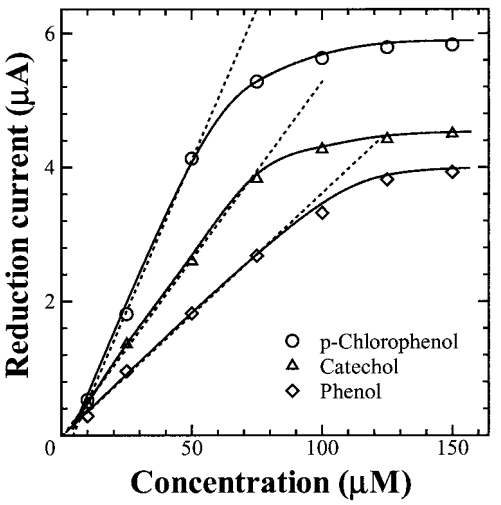


Figure 6 Calibration plots of a biosensor (PPO/Fe^{2+}-PPY/ITO) to (○) phenol, (△) catechol, and (◇) *p*-chlorophenol. The supporting electrolyte was a 0.2*M* phosphate buffer, and the applied potential was +0.03 V vs Ag/AgCl.

sor) showed sensitivities of 0.14, 0.21, and 0.36 A M^{-1} cm^{-2} and linear response ranges of 9.9–84.7, 6.7–72.6, and 3.9–48.8 μM for phenol, catechol, and *p*-chlorophenol, respectively. The long-term stability of the PPO/Fe^{2+}-PPY/ITO electrode was more than that of recently reported conventional biosensors (<15 days).[21–23] The longer stability, short response time (~50 s), and observed lowest detection limits of 2.90, 2.03, and 1.19 μM for phenol, catechol, and chlorophenol, respectively, indicated that these electrodes could be useful for the quantitative estimation of phenolic compounds in aqueous media. Further experiments are in progress with these PPO/Fe^{2+}-PPY/ITO electrodes to monitor quantitatively the other phenolic compounds and mixtures thereof in aqueous media.

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