Immobilization of Cholesterol Oxidase and Potassium Ferricyanide on Dodecylbenzene Sulfonate Ion-Doped Polypyrrole Film

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ABSTRACT: :Dodecylbenzene sulfonate (DBS)-doped polypyrrole (PPY) conducting polymer films were electrochemically deposited onto the indium-tin-oxide (ITO)-coated glass plates in aqueous medium. The enzyme cholesterol oxidase (ChOx) was immobilized on these DBS–PPY films by a physical adsorption technique. These ChOx-immobilized DBS–PPY films were characterized by ultraviolet–visible and Fourier transform infrared spectroscopy. The enzyme activity studies indicate that ~40% of ChOx leaches out from the ChOx/DBS–PPY film. The ChOx activity in the ChOx/DBS–PPY film was assayed as a function of cholesterol concentration. The results of amperometric measurements conducted on ChOx/DBS–PPY/ITO film show linearity over the range 2–8 mM of cholesterol solution. The ChOx/DBS–PPY/ITO electrodes exhibit a response time of 30 s and are stable for ~3 months at 4 °C. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 3486–3491, 2001

Key words: immobilization; cholesterol oxidase; dodecylbenzene sulphonate; polypyrrole; biosensor

INTRODUCTION

Electrochemically polymerized conducting polymers have received considerable attention over the last two decades. The remarkable switching capability of these materials between the conducting oxidized (doped) and the insulating reduced (undoped) state is the basis of many applications. Among others, the polyconjugated conducting polymers (PCB) have been recently proposed for biosensing applications because of a number of favorable characteristics, such as (1) direct and easy deposition on sensor electrode by electrochemical oxidation of monomer, (2) control of thickness by deposition charge, and (3) redox conductivity and polyelectrolyte characteristics of the polymer useful for sensor application. These requirements are met with polypyrrole (PPY), which is the most commonly used polymer because of water solubility, easy oxidation, low cost of the monomer, and chemical stability of the polymer.[1]

Recently, an electrochemically synthesized PPY membrane with electroconductivity was reported, and it was shown that the electropolymerized PPY works as an efficient molecular interface for electron transfer. This result was clarified with a flavin adenine dinucleotide (FAD)-entrapped PPY membrane with a smooth and reversible oxidation and reduction of FAD, the pros-

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thetic group of glucose oxidase.[2] A unique method of electrochemical regeneration of nicotinamide adenine dinucleotide (NAD^+) has also been reported in a mediator-entrapped polymer membrane electrode.[3] It has been reported previously that the conductivity of thin film of PPY[4–6] deposited on textile from an aqueous solution of PPY undergoing oxidative polymerization by an *in situ* process is generally dependent on the dopant anion, such as anthraquinone-2-sulfonic acid, which gives the smallest surface resistance. It was suggested[5,6] that this planar anion induces a charge in the PPY molecular conformation, which results in increased electrical conductivity. Because ion exchange in PPY leads to morphological changes, one can therefore utilize this process as a tool for controlling the morphology. The incorporation of a large size dopant anion, such as *para*-toluene sulfonate (PTS), and ferricyanide into PPY films during electropolymerization makes PPY more porous and facile for immobilization of enzyme.[7] It was shown that redox species like phenazene, thionine, and ferricyanide work as good electron acceptors for cholesterol oxidase (ChOx) in place of O_2 and are commonly used as the electron mediators.[8–10] These redox active species therefore allow accurate electrochemical determination of cholesterol without any interference of several dissolved compounds in blood, such as ascorbic and uric acids.[11]

Cholesterol and its fatty acid esters are important compounds for human being because they are components of nerve and brain cells and are precursors of other biological materials, such as bile acid and steroid hormones.[12,13] However, accumulation of cholesterol in blood due to excessive ingestion results in fatal diseases such as arteriosclerosis, cerebral thrombosis and coronary diseases.[14,15] It is therefore highly desirable to develop techniques that allow conventional and rapid determination of cholesterol. Keeping this in view, we have immobilized the ChOx enzyme in an electrochemically synthesized dodecylebenzene sulfonate (DBS) ion-doped PPY conducting film on indium-tin-oxide (ITO)-coated glass plates for application to cholesterol biosensor.

EXPERIMENTAL

Materials

Pyrrole (E. Merck) was purified by fractional distillation at 130°C and stored at 4°C. Dodecylbenzene sulfonic acid (DBS) was procured from Aldrich (USA). Cholesterol oxidase (ChOx; E.C.1.1.3.6), with specific activity of 18 units mg^{-1} solid, and horseradish peroxidase (HRP; E.C.1.11.1.7), with a specific activity of 120 units mg^{-1} solid, were procured from Sigma Chemical Company (USA). All other chemicals were of analytical grade and were used as received without purification. Aqueous solutions were prepared in double-distilled deionized water (Millipore RO 10TS).

Apparatus

Ultraviolet–visible (UV–vis) absorbance data were collected with a Shimadzu (Model 160A) spectrophotometer. Fourier transform infrared (FTIR) spectra of the film were recorded on Nicolet (510P). Cyclic voltammetry was carried out with an Electrochemical Interface (Model SI 1286) with three-electrode configuration consisting of a working electrode (ChOx/Fe^{3+}/DBS–PPY/ITO film) Ag/AgCl , as a reference electrode and a platinum wire as a counter electrode. The output was recorded directly on a Hewlett-Packard color pro printer. Amperometric response measurements were conducted with a Keithley electrometer (Model EC617).

Polymer Preparation

DBS ion-doped PPY films were electrochemically prepared on ITO glass plates by taking 0.1 M monomer and 0.1 M DBS (1:1) in aqueous medium and passing a constant current of 2 mA through the solution, using platinum as reference electrode. The films thus prepared were then washed with doubled-distilled deionized water prior to being used.

Immobilization of Enzyme and Mediator

First, 10 μL of a solution of 0.1 M phosphate buffer (pH 7.0) containing 3 IU of ChOx (18 units mg^{-1} solids, 5 mg/0.3 mL) and 5 μL of 0.1 M potassium ferricyanide were physically deposited onto a 1 $\times$ 1 cm^2 DBS–PPY film. The ChOx/Fe^{3+}/DBS–PPY films were dried at room temperature for ~12 h and were stored in a refrigerator at ~4°C.

Preparation of Cholesterol Solution

Cholesterol solution was prepared as described by Trettnek and Wolfbeis.[16] The cholesterol (3.86 g) was dissolved in 12.8 mL of propane-2-ol and 3.85 mL of triton X-100, and the solution was mixed.

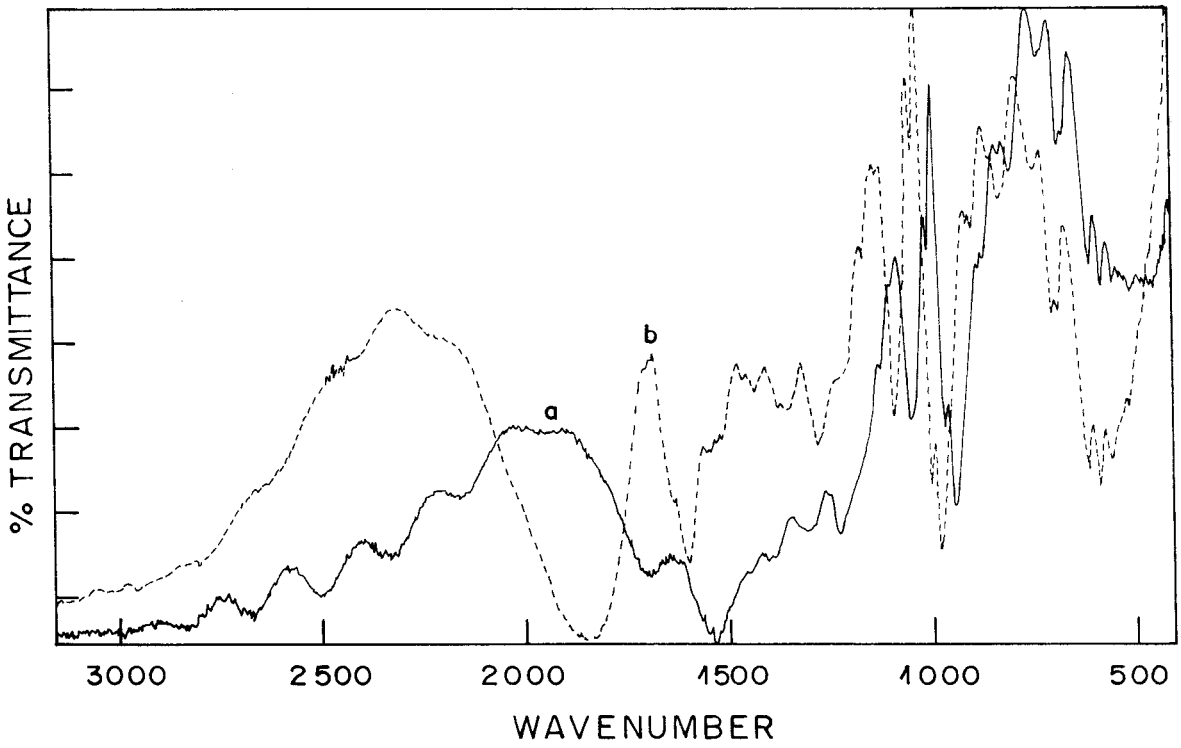

Figure 1 FTIR spectra of electrochemically prepared (a) DBS/PPY film and (b) ChOx immobilized on DBS–PPY film.

After homogenization, the volume was made up to 100 mL with 0.1 M phosphate buffer (pH 7.0) and thermostatted at 35°C. This standard solution was further diluted to make different cholesterol solutions.

Enzyme Activity Measurements

Cholesterol (0.05 cm^3 of 6 mmol dm^{-3} solution) was dissolved in 2-propanol, and 3 cm^3 of 0.1 mol dm^{-3} phosphate buffer (pH 7.0) were mixed and kept in a thermostat at ~35°C. The ChOx/DBS–PPY films were immersed in the presence of horseradish peroxidase (HRP) and incubated for ~2 min. Then, the ChOx/DBS–PPY films were removed, and the absorbance of the solution was measured at 240 nm with a double-beam spectrophotometer to determine the cholestenone produced by the enzymatic reaction. The apparent enzyme activity was evaluated by the following formula,[17,18] which is based on the difference in the absorbance before and after incubation of the ChOx/DBS–PPY film:

$$U\ \text{cm}^{-2} = AV/(\varepsilon ts) \quad (1)$$

where A is the difference in absorbance before and after incubation, V is the total volume (3.05 cm^3), ϵ is the millimolar extinction coefficient of cholestenone (12.2), t is the reaction time, and s is the film surface (1 × 1 cm^2). One unit of the enzyme activity is defined here as the activity that results in 1 μmol cholestenone per minute. The activity measurements were done on all the enzyme (ChOx)-immobilized DBS ion-doped PPY films. However, it has been observed that ~40% of the enzyme immobilized leaches out within ~20 min after the film was placed in cholesterol/buffer solution.

RESULTS AND DISCUSSION

Characterization of Enzyme-Immobilized Dodecylbenzene Sulfonate-Doped Polypyrrole Film

The FTIR spectra of electrochemically prepared ChOx/DBS–PPY film are shown in Figure 1. Sharp peaks seen at 1500–1400 cm^{-1} and1100–1000 cm^{-} have been attributed to C═C stretching mode and C—C stretching , respectively. The characteristic peaks at 3100 and 3500 cm^{-1} assigned to N—H linkage and N—H stretching of free amide indicated the presence of immobilized ChOx. The entrapment of enzyme (ChOx) was also confirmed with the help of UV–vis spectroscopy. When the ChOx/DBS–PPY film is

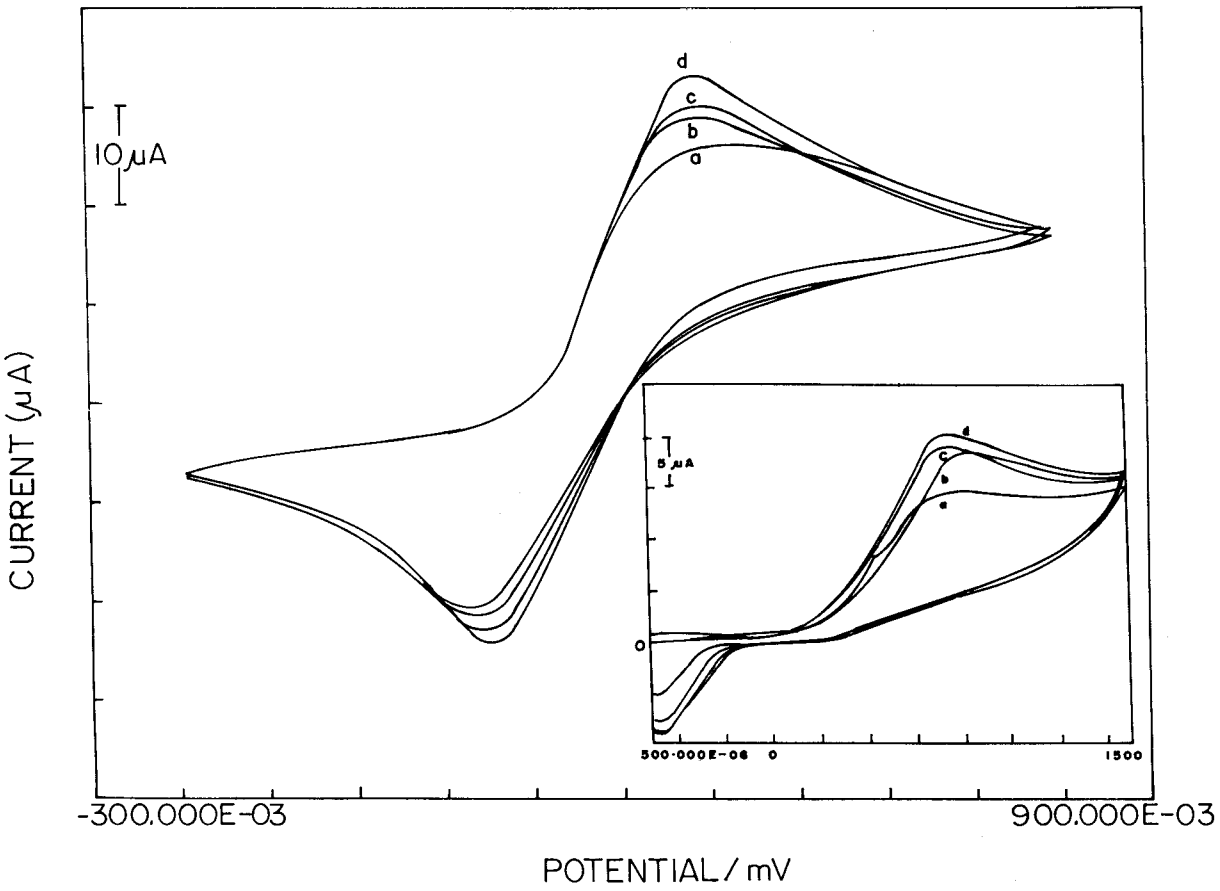

Figure 2 A cyclic voltammogram of ChOx/DBS–PPY film obtained as a function of cholesterol concentrations (2–8 mM) without potassium ferricyanide as a mediator: (a) 2 mM; (b) 4 mM; (c) 6 mM; (d) 8 mM. the inset shows cyclic voltammograms of the ChOx/Fe^{3+}/DBS–PPY/ITO electrode with potassium ferricyanide as a mediator as a function of cholesterol concentration: (a) 2 mM; (b) 4 mM; (c) 6 mM; (d) 8 mM.

immersed in phosphate buffer containing cholesterol, the observed increase in absorbance at 240 nm is characteristic of cholestenone produced by the enzymatic solution. This result indicates that the ChOx has been incorporated in the DBS ion-doped PPY film.[17] ChOx/DBS–PPY/ITO films were also studied for the stability at both room temperature as well as in refrigerated conditions. The film was stable for ~12 weeks when stored at 4–5°C in refrigerated conditions. At room temperature, however, the film was stable for ~8 weeks.

Electrochemical Studies of Polypyrrole Film Containing Immobilized Cholesterol Oxidase and Potassium Ferricyanide

The cyclic voltammetry experiments were performed in 0.1 M phosphate buffer (pH 7.0) using enzyme (ChOx)-immobilized DBS–PPY/ITO film with and without ferricyanide ion mediator as a working electrode, Ag/AgCl as a reference electrode, and platinum (Pt) wire as a counter electrode.

The reaction scheme is as follows:

$$\text{Cholesterol} + \text{ChOx} \longrightarrow \text{Cholestenone} + \text{ChOx}_{\text{red}} \quad (2)$$

$$\text{ChOx}_{\text{red}} + \text{Fe}^{3+}\ (\text{ferricyanide}) \longrightarrow \text{ChOx} + \text{Fe}^{2+}\ (\text{Ferrocyanide}) \quad (3)$$

$$\text{Fe}^{2+}\ (\text{Ferrocyanide}) \xrightarrow{0.4\ \text{V}} \text{Fe}^{3+}\ (\text{ferricyanide}) + \text{e}^{-}\ (\text{at electrode}) \quad (4)$$

Potassium ferrocynate acts as a mediator for cholesterol oxidase in PPY film.

The cyclic voltammograms obtained using ChOx/DBS–PPY electrode for different cholesterol solutions (2–8 mM)with and without potassium ferricyanide (mediator) are shown in Figure 2. An oxidation peak of enzymatically produced H_2O_2 is observed at 0.75 V versus Ag/AgCl, using ChOx/DBS–PPY/ITO as a working electrode, which increases with an increase in cholesterol concentration (2–8 mM). A high voltage of 750 mV may cause other species to be oxidized, which may finally alter the ultimate anodic current. We have therefore conducted a similar experiment using potassium ferricyanide as a mediator and immobilized ChOx/Fe^{3+}/DBS–PPY/ITO as a working electrode polarized at a low voltage potential for amperometric response of cholesterol in phosphate buffer. An oxidation peak obtained at 0.75 V is shifted cathodically by ~350 mV and is observed at 0.40 V when ChOx/Fe^{3+}/DBS–PPY/ITO electrode is used with potassium ferricyanide as a mediator. The inset of Figure 2 shows cyclic voltammogram of cholesterol using ChOx/Fe^{3+}/DBS–PPY/ITO electrode, with an increase in cholesterol concentration (2–8 mM).

Amperometric Response Studies

The three electrode cell configuration similar to the one used in cyclic voltammetric experiments has been used for the amperometric detection of cholesterol in phosphate buffer (pH 7.0). The results are shown in Figure 3. The working electrode ChOx/DBS–PPY/ITO was polarized with mediator (potassium ferricyanide) at 0.4 V and without mediator at 0.75 V with respect to an Ag/AgCl electrode. The anodic current measured in 6 mM cholesterol solution (1 mL) at ChOx/DBS–PPY/ITO electrode (without potassium ferricyanide) polarized at 0.75 V yields the steady state in ~60 s. This increase in the anodic current is attributed to the direct oxidation of H_2O_2 on the surface of the DBS–PPY electrode. However, it has been found that when a similar amperometric experiment was conducted with ChOx/Fe^{3+}/DBS–PPY/ITO electrode polarized at 0.40 V, the electrode reveals a response time of ~30 s when measured in a 6mM cholesterol solution (1 mL). The short response of ChOx/Fe^{3+}/DBS–PPY/ITO electrode to cholesterol solution reveals that the faster electronic exchange occurs between ChOx and potassium ferricyanide mediator.

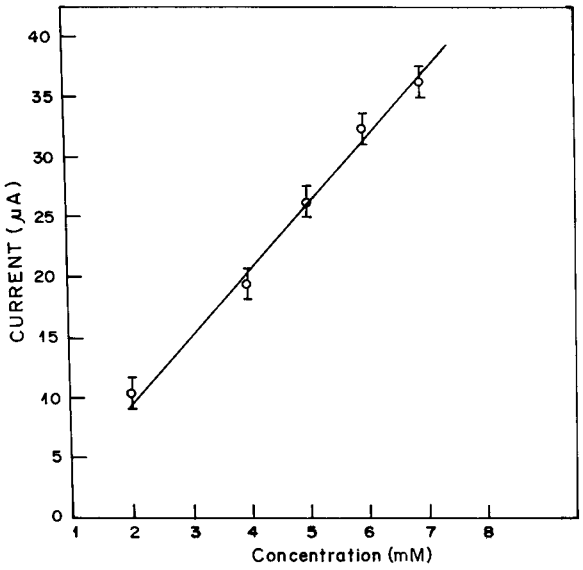

Figure 3 Amperometric response obtained with ChOx/DBS–PPY electrode in phosphate buffer (pH 7.0) as a function of cholesterol concentration. (2–8 mM).

CONCLUSIONS

The enzyme ChOx can be physically adsorbed on unmediated and mediated (potassium ferricyanide) electrochemically prepared DBS ion-doped PPY films prepared on ITO glass plates. The short response time (30 s) and the observed linearity (2–8 mM) of the mediated ChOx/Fe^{3+}/DBS–PPY/ITO electrode indicates that these electrode scan be utilized for clinical application. Experiments are in progress to improve the shelf life beyond 8 weeks at room temperature.

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