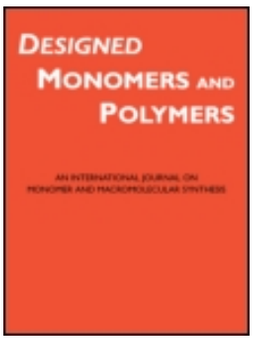

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Immobilization of cholesterol oxidase in a conducting copolymer of thiophene-3-yl acetic acid cholesteryl ester with pyrrole

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Abstract—Cholesterol oxidase has been immobilized in conducting copolymers of thiophene-3-yl acetic acid cholesteryl ester with pyrrole (CM/PPy) and polypyrrole (PPy) via electropolymerization. *p*-Toluene sulphonic acid was used as the supporting electrolyte. Kinetic parameters (V_{max} and K_m) and operational stability of enzyme electrodes were investigated. Surface morphology of the films was examined by scanning electron microscope.

Keywords: Cholesterol oxidase; immobilization; conducting polymer; polypyrrole; electrochemical polymerization.

1. INTRODUCTION

Enzymes are biological catalysts that have the property of catalyzing specific chemical reactions in living organisms. Since they do not change during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products, it is difficult to separate them. Immobilized enzymes offer advantages over free enzymes for their repeated use, easy separation of enzyme and products, enhanced stability and significant reduction in the operation cost.

Cholesterol oxidase is one of the industrially important enzymes. It catalyzes the oxidation of cholesterol and forms equimolar amounts of cholest-4-en-3-one and hydrogen peroxide. Cholesterol oxidase was immobilized in a sol–gel film [1],

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polyaniline [2], polypyrrole [3–6], octyl-agarose gel [7], nylon membrane [8] and in copolymers of methyl and glycidal methacrylates [9].

In this study, CM/PPy and PPy were used as the supporting materials to immobilize cholesterol oxidase. Immobilization was achieved by electrochemical polymerization. The presence of the cholesteryl group on the monomer (CM) was investigated in terms of enzyme activity. Synthesis and characterization of the conducting copolymer of thiophene-3-yl acetic acid cholesteryl ester with pyrrole (CM/PPy) has been reported in an earlier study [10].

2. EXPERIMENTAL

2.1. Materials

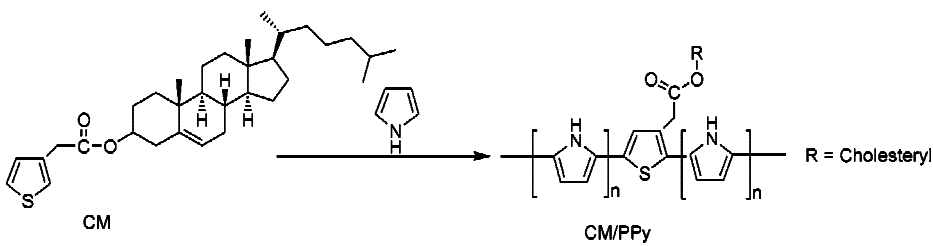
Cholesterol oxidase (COD) (E.C. 1.1.3.6) with a specific activity of 4.2 U/mg solid and horseradish peroxidase (HRP) (E.C. 1.11.1.7) with a specific activity of 157 U/mg solid were obtained from Sigma. Cholesterol, Triton X-100 and *p*-toluene sulphonic acid (PTSA) were purchased from Sigma and used as received. Pyrrole (Sigma) was distilled before use and stored at 4°C. All the reagents used were of analytical grade.

2.2. Synthesis of copolymer

The electropolymerization of thiophene-3-yl acetic acid cholesteryl ester (CM) with pyrrole is given in Scheme 1.

2.3. Preparation of enzyme electrodes (PPy/COD, CM/PPy/COD)

The electropolymerization process was carried out using a potentioscan Wenking POS-73 model potentiostat with a three-electrode configuration consisting of platinum foil as the working and counter electrode and Ag wire as the reference electrode. A 10 ml solution of 0.1 M phosphate buffer (pH 7.0) containing 0.2 M pyrrole, 6 mg/ml PTSA as the supporting electrolyte and 1 mg/ml COD were used for immobilization via electropolymerization. For the preparation of the CM/PPy/COD electrode, the Pt electrode was coated with CM and used as the



Scheme 1. Electrochemical synthesis route for copolymerization.

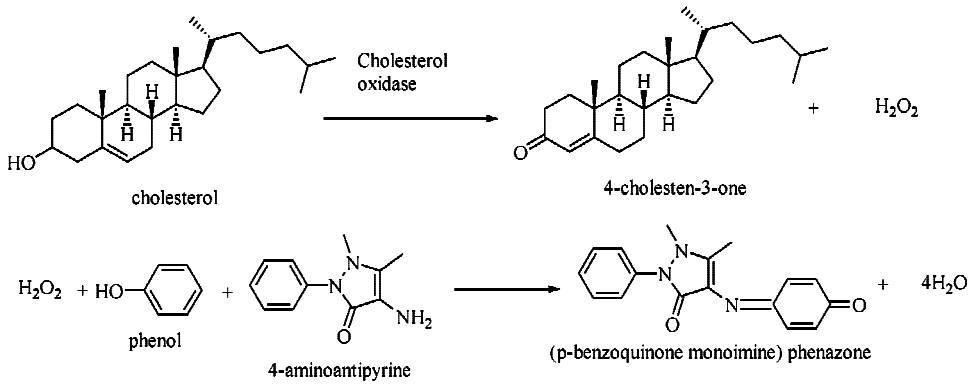
anode for the electropolymerization. Electrolyses were performed at room temperature under nitrogen atmosphere at the oxidation potential of pyrrole (+1.0 V). After immobilization was achieved, electrodes were removed and washed several times with distilled water to remove the supporting electrolyte. Next, the electrode was placed in phosphate buffer for 10 min and solution was examined for the enzyme activity due to unbound enzyme. This procedure was repeated for several times with phosphate buffer until no activity was observed. Electrodes were stored in phosphate buffer at 4°C when not in use.

2.4. Preparation of cholesterol solution

Cholesterol is insoluble in water. However, it is soluble in alcohols and also in the presence of surfactants [1, 8, 11, 12]. The cholesterol was dissolved in phosphate buffer, isopropanol and Triton X-100 (*t*-octylphenoxypolyethoxyethanol) in a weight ratio of 86 : 10 : 4. The surfactant and isopropanol were used due to the poor solubility of cholesterol in pure water. The cholesterol solutions were prepared daily by dissolving the cholesterol in isopropanol, then adding Triton X-100 and finally the phosphate buffer (pH 7.0).

2.5. Enzyme activity measurements

The activity of cholesterol oxidase was determined spectrophotometrically using the method of Kumar *et al.* [1]. One unit will convert 1.0 μmol of cholesterol to 4-cholesten-3-one per min at pH 7.0 at 25°C. 4 ml cholesterol solution was incubated at 37°C for 5 min. A 1-ml aliquot of cholesterol oxidase solution (2 mg in 50 ml phosphate buffer, pH 7.0) was added to a cholesterol solution. After the reaction, 3 ml of a solution containing 4-aminoantipyrine (158 mg/ml), phenol (146 mg/ml) and peroxidase (HRP; 10 mg/dl) was added. The appearance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol and peroxide was measured at 500 nm with a double beam spectrophotometer (Shimadzu Model, UV-1601). One unit of activity was defined as the formation of 1 μmol hydrogen



Scheme 2.

peroxide per min at 37°C and pH 7.0. The V_{max} and K_m values were estimated from Lineweaver–Burk plots [13]. Reaction mixtures composed of 0–5 mM substrate solution were used to calculate kinetic parameters. The complete reaction sequence is shown in Scheme 2.

3. RESULTS AND DISCUSSION

3.1. Kinetic studies of free and immobilized COD

Kinetic studies of the immobilized COD were performed at various concentrations of cholesterol. Maximum velocity, V_{max}, and the apparent Michaelis–Menten constant, K_m, were estimated from the Lineweaver–Burk plot. The kinetic constants for the oxidation of cholesterol by free and immobilized cholesterol oxidase are given in Table 1. Usually, there is a decrease in activity of an enzyme upon insolubilization, which can be attributed to denaturation of the enzymic protein caused by the coupling process. Once an enzyme has been insolubilized, however, it finds itself in a microenvironment that may be drastically different from that existing in free solution. The new microenvironment might be a result of the physical and chemical character of the support matrix alone, or it may result from interactions of the matrix with substrates or products involved in the enzymatic reaction. The Michaelis–Menten constant has been found to decrease by more than one order of magnitude when substrate of opposite charge to the carrier matrix was used. Again, this only happened at low ionic strengths and when neutral substrates were used. The diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme can limit the rate of the enzyme reaction. The rate at which substrate passes over the insoluble particle affects the thickness of the diffusion film, which in turn determines the concentration of substrate in the vicinity of the enzyme and hence the rate of reaction. The molecular weight of the substrate has also significant effect. Diffusion of large molecules will obviously be limited by steric interactions with the matrix, and this is reflected in the fact that the relative activity of bound enymes towards high molecular weight substrates has been generally found to be lower than towards low molecular weight substrates. Although the different matrices were used, the K_m and V_{max} values were in the same order. This

Table 1.
Kinetic parameters of free and immobilized enzyme

	V_{max}	K_m (mol/l)
Free COD	3.0×10^{-1} [a]	2.6×10^{-4}
PPy/PTSA/COD	4.0×10^{-3} [b]	8.2×10^{-5}
CM/PPy/PTSA/COD	7.0×10^{-3} [b]	7.8×10^{-5}

[a] In μmol/ml per min.
[b] In μmol/min per electrode.

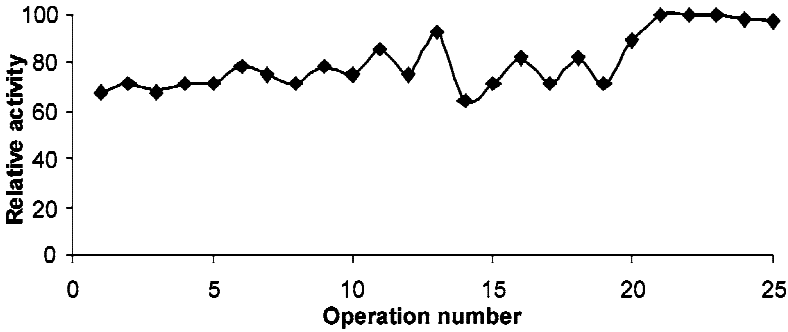


Figure 1. Operational stability of the PPy/PTSA/COD electrode.

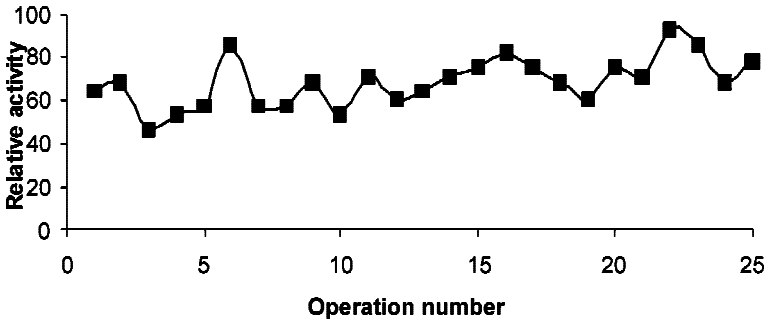


Figure 2. Operational stability of the CM/PPy/PTSA/COD electrode.

means that cholesteryl group on monomer does not affect the kinetic parameters of the immobilized COD enzyme.

3.2. Operational stability of immobilized COD

Operational stability is an important consideration for an immobilized enzyme. To determine this parameter, activities of the enzyme electrodes were checked for 25 successive measurements. Figures 1 and 2 show the operational stability of the PPy/PTSA/COD and CM/PPy/PTSA/COD electrodes respectively. The response of the electrodes did not change significantly, the slight increase in the response of PPy/PTSA/COD enzyme electrode is related to the swelling of the polymer structure and reorganization of the enzyme molecules in the PPy matrix (Fig. 1). Slight decrease in the response is due to desorption of the enzyme molecules initially adsorbed on the CM/PPy layer (Fig. 2). Hence, the enzyme retains its catalytic activity for 25 measurements.

3.3. Surface morphologies of enzyme-entrapped film

Scanning electron microscopy (SEM; JEOL JSM-6400) was used to observe the surface changes of the films when the enzyme was anchored. The films were washed before analysis in order to remove unbound enzymes. The surface morphologies

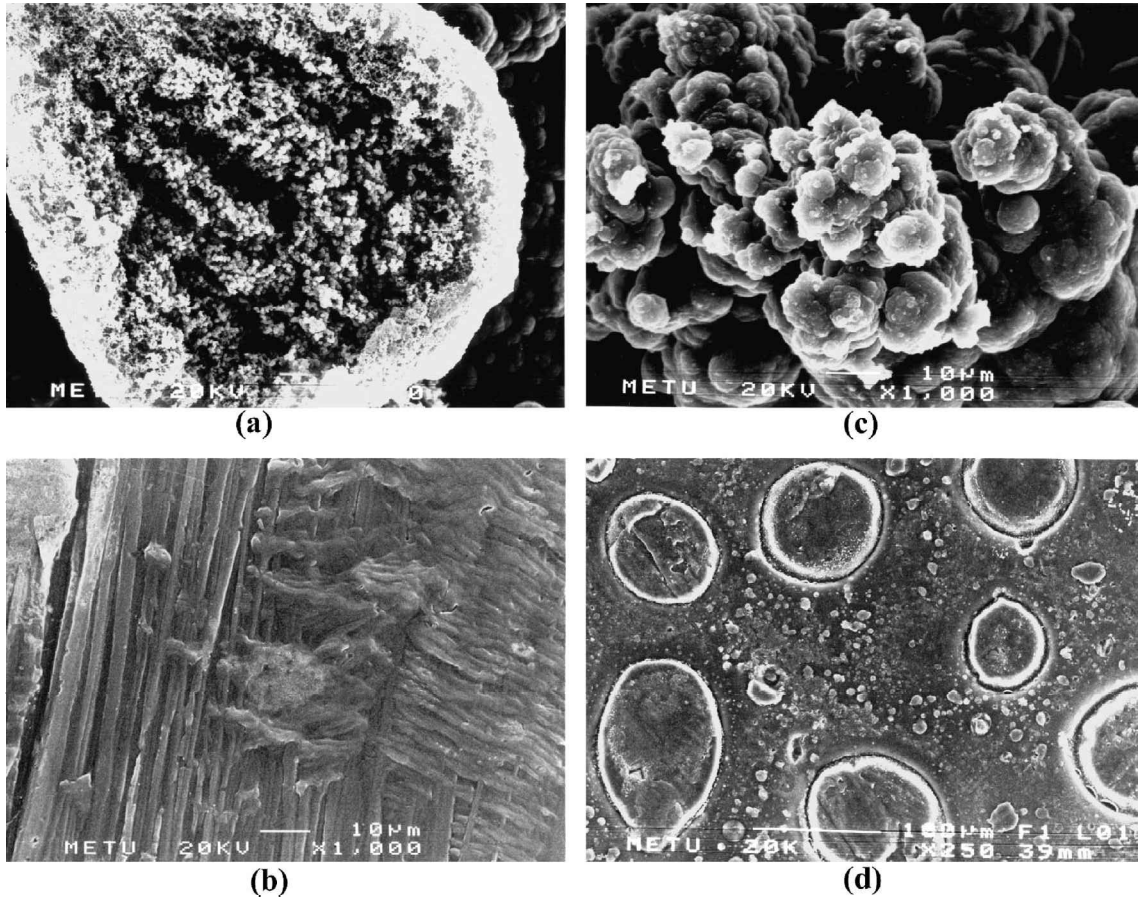


Figure 3. Scanning electron micrographs of (a) the solution side of PPy/PTSA/COD electrode, (b) the electrode side of PPy/PTSA/COD electrode, (c) the solution side of CM/PPy/PTSA/COD electrode and (d) the electrode side of CM/PPy/PTSA/COD electrode.

of these films were completely different compared to the films produced in the absence of COD. On the solution sides of the films, the cauliflower-like structure was significantly damaged when COD was anchored in each polymer matrix (Fig. 3a and c). Enzyme clusters, however, could not be observed on the electrode side (Fig. 3b and d). Small islands were observed for the electrode side of CM/PPy/PTSA film in the presence of cholesterol oxidase enzyme. In the absence of COD enzyme, electrode side of CM/PPy/PTSA film exhibited different morphology [10].

4. CONCLUSIONS

In this study, immobilization of cholesterol oxidase has been achieved in conducting PPy and CM/PPy matrices. Kinetic parameters, operational stability and surface morphology of the enzyme electrodes were examined. This study showed that the presence of the cholesteryl group on the monomer (CM) does not affect the enzyme activity.

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