# Preparation and Characterization of an Enzyme Electrode Based on Cholesterol Esterase and Cholesterol Oxidase Immobilized onto Conducting Polypyrrole Films

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**ABSTRACT:** Cholesterol esterase (ChEt) and cholesterol oxidase (ChOx) enzymes were entrapped within polypyrrole (PPy) films on a platinum disc electrode during electrochemical polymerization. The characteristics of the PPy/ChEt/ChOx enzyme electrode thus prepared were investigated as a function of the time, pH, temperature, and concentration of cholesteryl palmitate by a spectrophotometric method. PPy/ChEt/ChOx electrodes can be used for the estimation of cholesteryl palmitate concentrations from 1 to 8 m*M*, can be used least 10 times, and have a shelf life of about 1 month at 4–10°C. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 91: 3769–3773, 2004

**Key words:** polypyrroles

## INTRODUCTION

There has been increased interest in the development of devices for various applications, including clinical analysis, pharmaceuticals, and food and dairy. In this context, the immobilization of enzymes and biomolecules has significantly contributed toward the development and fabrication of biosensing devices[1–5] for cost-effective, stable, selective, and highly sensitive estimations for clinical diagnosis. It is well known that enzymes are highly unstable in the solution phase. There is, therefore, a need to immobilize them on a solid support. Several techniques, such as physical adsorption, covalent linkage, crosslinking, and entrapment,[6–9] have been reported for the immobilization of enzymes on a suitable matrix. Among these, the entrapment method has been considered to be advantageous because it involves the entrapment of enzymes and biomolecules within a solid matrix during the course of polymerization, thus avoiding the additional step required for the immobilization of enzymes after the matrix has been prepared. Moreover, no chemical treatment resulting in a reduced loss of enzyme activity is required in this method of immobilization.

Conducting polymers have extensively been used as immobilization matrices for enzymes as well as physicochemical transducers.[10–12] Polypyrrole (PPy) and its derivatives have been considered interesting materials as immobilization matrices because of their high conductivity, mechanical stability, and tendency to form freestanding films.[13–15]

Cholesterol estimation has been found to be important for clinical diagnosis because the accumulation of cholesterol in blood leads to a number of fatal diseases. Its increased level is associated with arteriosclerosis, myxoderma, and diabetes mellitus, whereas a decreased level is associated with certain anemia, malabsorption, and wasting syndrome. Biosensors are playing leading roles in the estimation of various biologically important analytes.[16] It is, therefore, highly desirable to develop a biosensor that allows the conventional and rapid determination of cholesterol. Most cholesterol biosensors reported in the literature are based on cholesterol oxidase (ChOx), which catalyzes the oxidation of cholesterol to 4-cholestenone.[17–20] Reports are available on the immobilization of ChOx onto PPy films.[19–22] Kajiya et al.[21] immobilized ChOx and ferrocene carboxylate in PPy electrochemically and observed increased sensitivity. Tretnakk and Wolfbeis[22] immobilized ChOx (ChOx) onto PPy and performed cholesterol measurements in a batch flow-through system. Kumar et al.[23] used dodecyl benzene sulfonate doped PPy films for the immobilization of ChOx by physical adsorption.

Attempts have been made to coimmobilize cholesterol esterase (ChEt) and ChOx for total cholesterol estimation.[24–26] Krug et al.[27] developed an enzyme-based fiber optic device for the determination of total and free cholesterol. Motonaka and Faulkner[28] developed an enzyme microsensor with ChOx and ChEt with a redox mediator $[Os(bpy)_3](PF_6)_2]$. Krug et al.[27]

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immobilized ChOx and ChEt on a preactivated nylon membrane. They related the cholesterol concentration to the color that developed with a kinetic evaluation. However, this time-consuming and sensitive method of electrode modification involved hydroxymethyl ferrocene and horseradish peroxidase. Recently, Suman and Pundir[29] reported the coimmobilization of ChEt, ChOx, and peroxidase (POD) onto alkylamine glass beads for total cholesterol estimation in serum. The procedure involved coupling through glutaraldehyde, which could lead to a loss in the enzyme activity with a long response time (12 min). Although the immobilization of ChOx and ChEt by electropolymerization in a PPy matrix was reported,[30] the method involved the simultaneous incorporation of an inorganic additive such as laponite clay. The analytical characteristics of the enzyme electrode were improved via laponite clay additives for free and total cholesterol estimation.

In this article, we report the preparation of an enzyme electrode through the electrochemical entrapment of ChEt and ChOx within PPy films during the course of polymerization onto platinum disc electrodes. The activity of the enzyme electrode was studied by a spectrophotometric method. Attempts were also made to characterize the PPy/ChEt/ChOx electrodes as a function of the pH, temperature, cholesteryl palmitate concentration, and storage time, and this may result in an efficient method of cholesterol estimation.

## EXPERIMENTAL

### Reagents

Cholesterol, ChEt, ChOx, cholesteryl palmitate, 4-aminoantipyrine (4-AAP), POD, and polyoxyethylene-9-lauryl ether, all from Sigma Chemical Co., were used without further purification. Pyrrole (Aldrich) was distilled before being used. Potassium phosphate, potassium orthophosphate, potassium hydroxide, sodium chloride, and phenol were analytical-grade. A cholesteryl palmitate solution was prepared with polyoxyethylene-9-lauryl ether as a surfactant (1 mL for 10-mL solution), and this was followed by the addition of a hot sodium chloride solution with constant stirring. The solution was stored at 4°C. A ChEt solution containing 0.2–0.4 units/mL and a ChOx solution containing 4 units/mL were prepared in a cold phosphate buffer (400 m*M*) of pH 7.0, and a solution of POD (40 units/mL) was prepared in deionized water.

### Preparation of the enzyme electrode

The electrochemical polymerization solution consisted of predistilled pyrrole (0.1*M*), *p*-toluene sulfonate (1*M*), and the enzymes ChOx (4.0 U/mL) and ChEt (0.2–0.4 U/mL). The electrochemical synthesis was carried out in a two-electrode cell with a platinum disc (1 $mm^2$) as a working electrode and a platinum wire as a counter electrode at a constant potential of 0.8 V with a programmable electrometer (model 617, Keithley), after which the electrodes were thoroughly washed. The conductivity of the polymer films obtained by this method was found to be about $3 \times 10^{-3}$ S/cm.

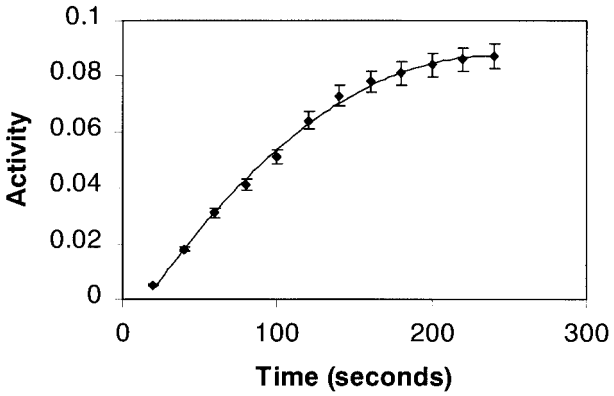


**Figure 1** Response of PPy/ChEt/ChOx electrodes in the presence of cholesteryl palmitate (4 m*M*), 4-AAP, phenol, and POD at pH 7.0.

### Activity measurements

The activity of ChEt and ChOx entrapped within PPy films was measured by a photometric assay with an ultraviolet–visible spectrophotometer (model 160A, Shimadzu). The reaction solution consisted of a potassium phosphate buffer (400 m*M*, pH 7.0), POD (40 units), a cholesteryl palmitate solution (4 m*M*), a phenol solution (6 w/v %), and 4-AAP (1.76 w/v %). The enzyme electrode was dipped in the reaction solution for 2 min, and then the increase in the concentration of the quinone imine dye enzymatically produced was monitored at 500 nm through the measurement of the increase in the absorbance. The activity measurements were performed as a function of the pH, temperature, different concentrations of cholesteryl palmitate, and storage stability to optimize the working conditions for the enzyme electrode.

## RESULTS AND DISCUSSION

### Activity measurements of the enzyme electrode

To determine the catalytic behavior of ChOx and ChEt entrapped within the PPy films, we determined the activity of ChOx and ChEt by measuring the optical density for the quinone imine dye at 500 nm (Fig. 1) with a Shimadzu 160A ultraviolet–visible spectrophotometer. The basic principle of the activity measurements involves the hydrolysis of cholesterol ester into

cholesterol and fatty acids. This reaction is catalyzed by ChEt entrapped within a PPy film:

$$\text{Cholesteryl palmitate} + H_2O \xrightarrow{\text{ChEt}} \text{Cholesterol} + \text{Fatty acids} \quad (1)$$

Cholesterol produced in eq. (1) undergoes oxidation in the presence of ChOx, which is present in the PPy/ChEt/ChOx electrode; therefore, cholestenone and $H_2O_2$ are produced, as shown in eq. (2):

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholestenone} + H_2O_2 \quad (2)$$

4-AAP acts on the $H_2O_2$ produced in eq. (2) in the presence of POD (present in the reaction solution) as shown:

$$H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\textit{Peroxidase(POD)}} H_2O + \text{Quinoneimine} \quad (3)$$

When the reaction was monitored at 500 nm for about 4 min, a regular increase in the absorbance at 500 nm was observed for 3 min because of the production of the quinone imine dye; thereafter, the absorbance at 500 nm did not change much. These results show that in about 3 min, the reaction equilibrium is reached, which leads to the steady-state value.

### Effect of the substrate concentration on the response of the PPy/ChEt/ChOx electrodes

Spectrophotometric studies were carried out for PPy/ChEt/ChOx electrodes at different concentrations of cholesteryl palmitate. The measurements were also carried out with different electrodes to determine the response at each concentration. Figure 2 shows the results of the responses obtained for the PPy/ChEt/ChOx electrodes to different cholesteryl palmitate concentrations (1–8 m*M*). Each point in the curve is the average of three measurements carried out under the same conditions of pH (7.0) at room temperature. A variation of about 5% in the response was observed, implying that the enzyme electrodes showed reproducible results. A linear relationship between the activity and cholesteryl palmitate concentration was obtained from 1 to 8 m*M*. However, when the response studies were carried out for higher cholesteryl palmitate concentrations, no significant change in the absorbance was observed. This may be due to the substrate saturation effects at very high substrate concentrations. These results indicated that the PPy/ChEt/ChOx electrodes could be used for the estimation of cholesteryl palmitate from 1 to 8 m*M*.

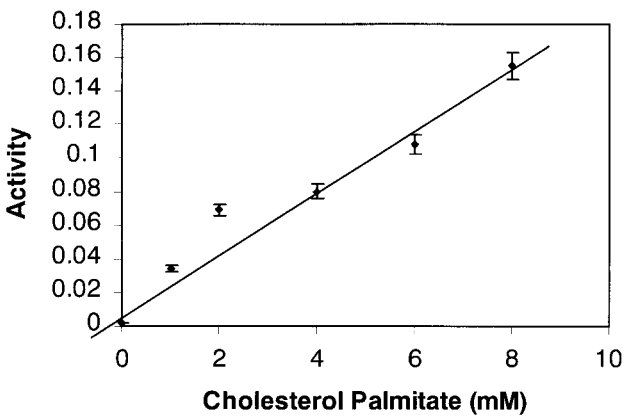


**Figure 2** Response of PPy/ChEt/ChOx electrodes to different cholesteryl palmitate concentrations.

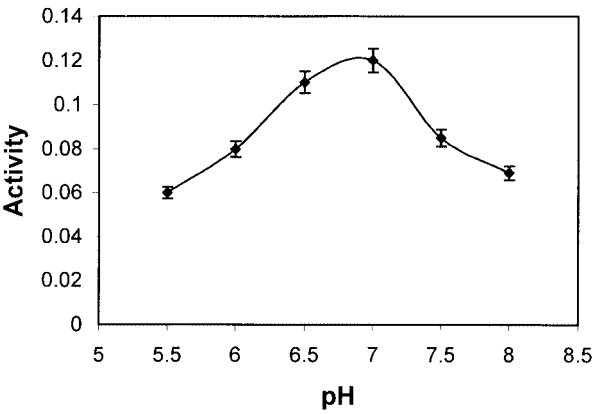


**Figure 3** Effect of the pH on the activity of PPy/ChEt/ChOx electrodes in the presence of 4 m*M* cholesteryl palmitate, 4-AAP, phenol, and POD.

### Effect of pH on the response of the PPy/ChEt/ChOx electrodes

It is known that the pH of a reaction solution affects the activity of enzyme electrodes. The effect of pH on the activity of the PPy/ChEt/ChOx electrodes was examined in the pH range of 5.5–8.0 (Fig. 3). A maximum response (pH optimum) was observed between pH 6.5 and 7.0 because of the increased production of the quinone imine dye (500 nm) in this range, which differs from those of cholesterol ester (7.0) and ChOx (7.5). This shift of the pH optimum for the enzyme electrode can be attributed to the presence of negatively charged *p*-toluene sulfonate ions in the PPy films. This results in the accumulation of positively charged ions ($H^+$) on the electrode surface immediately in contact with the surrounding solution. Consequently, pH at the carrier surface is lower than that of a bulk solution.[31] A similar pattern was observed by Motonaka and Faulkner.[28] About a 4.5% experimental variation was observed, which indicated good reproducibility. Thus, it can be concluded that PPy/ChEt/

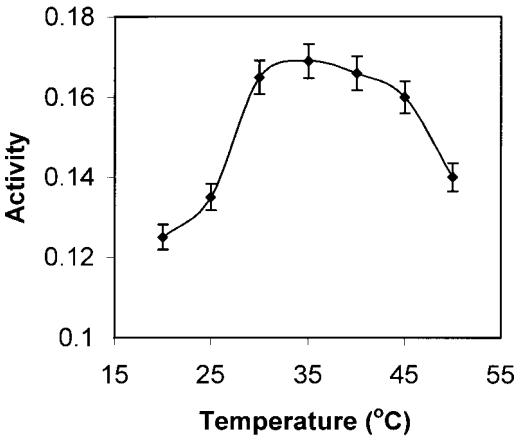


**Figure 4** Effect of the temperature on the response of PPy/ChEt/ChOx electrodes in the presence of 4 m*M* cholesteryl palmitate, 4-AAP, phenol, and POD at pH 7.0.

ChOx electrodes can work effectively in the pH range of 6.5–7.0.

### Effect of the temperature on the response of the PPy/ChEt/ChOx electrodes

To study the temperature tolerance of the PPy/ChEt/ChOx electrodes, we carried out spectrophotometric studies at different temperatures ranging from 20 to 50°C at pH 7.0 with 4 m*M* cholesteryl palmitate. The measurements were performed with three different electrodes at the same temperature. The activity of the PPy/ChEt/ChOx electrodes increased with an increase in the temperature up to 30°C, after which no significant variation was observed up to 40°C (Fig. 4). Thus, a bell-shaped curve was obtained at an optimum temperature of 35°C. However, the enzyme activity at 30 and 40°C was found to be similar. During the measurements, approximately a 3.5% error (with three different electrodes) was observed, this might be attributed to the fact that each electrode could undergo denaturation to a different degree, and this resulted in a slight change in the enzyme activity at different temperatures. After 45°C, a sharp fall in the activity of PPy/ChEt/ChOx was observed with an increase in the temperature. This may be attributed to the partial denaturation of the enzymes at higher temperatures. These results reveal that the PPy/ChEt/ChOx electrodes can work effectively and efficiently in the temperature range of 30–45°C. This increase in the thermal stability can be attributed to the PPy matrix acting as a protecting layer, resulting in reduced conformational changes that may prevent enzyme denaturation at higher temperatures.

### Storage stability of the PPy/ChEt/ChOx electrodes

Figure 5 shows the results of the spectrophotometric studies carried out for the activity measurements of the PPy/ChEt/ChOx electrodes at intervals of 3 days up to about 4 weeks with 4 m*M* cholesteryl palmitate. When the electrodes were not in use, they were stored at 4–10°C. A partial fall in the activity of the PPy/ChEt/ChOx electrodes was observed during the storage. It can be seen from Figure 5 that about 84% enzyme activity was retained for about 18 days, after which a slight fall in activity occurred. After about 18 days, a sharp decrease in activity was observed, which was attributed to the fact that with the storage time, the enzymes underwent partial denaturation. The electrodes showed 70% of the initial activity. The results were found to be repeatable and reproducible during the activity measurements in this period of storage with approximately a 5% experimental error.

## CONCLUSIONS

It has been shown that ChEt and ChOx enzymes can be immobilized in PPy films with electrochemical entrapment. Spectrophotometric studies were carried out for PPy/ChEt/ChOx electrodes as a function of the cholesteryl palmitate concentration, pH, temperature, and shelf life. The optimum pH for the enzyme electrodes was found to be in the range of 6.5–7.0, which could also be used to confirm the presence of ChEt and ChOx in the PPy/ChEt/ChOx electrodes; they were stable in the temperature range of 30–45°C. The shelf life of these electrodes was found to be about 4 weeks. These electrodes can be used about 10 times for the estimation of cholesterol ester, for concentrations of 1–8 m*M*, by a spectrophotometric method. Studies on the application of PPy/ChEt/ChOx electrodes for total cholesterol estimation are presently under way, and the results will be reported later.

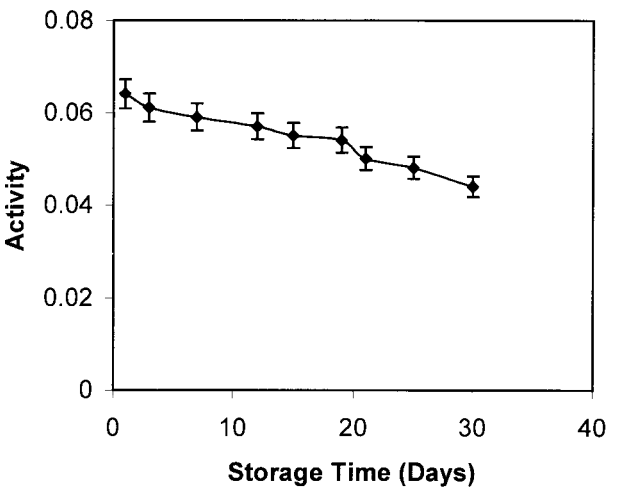


**Figure 5** Effect of the storage time on the response of PPy/ChEt/ChOx electrodes in the presence of 4 m*M* cholesteryl palmitate, 4-AAP, phenol, and POD at pH 7.0.

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## References


1. Guilbault, G. G. Analytical Uses of Immobilized Enzymes; Marcel Dekker: New York, 1984.
2. Turner, A. P. F.; Karube, I.; Wilson, G. S. Biosensor: Fundamentals and Applications; Oxford University Press: Oxford, 1987; p 86.
3. Narang, U.; Dunbar, R. A.; Bright, F. V.; Prasad, P. N. Appl Spectrosc 1993, 47, 1700.
4. Bright, F. V. In Spectroscopic Characterization of Immobilized Proteins in Immobilized Biomolecules in Analysis: A Practical Approach; Cass, T.; Ligler, F. S., Eds.; Oxford University Press: Oxford, 1998; Chapter 10.
5. Wilson, G. S.; Hu, Y. Chem Rev 2000, 100, 2693.
6. Gambhir, A.; Gerard, M.; Mulchandani, A. K.; Malhotra, B. D. Appl Biochem Biotechnol 2001, 96, 249.
7. Ramanathan, K.; Pandey, S. S.; Kumar, R.; Gulati, A.; Murthy, A. S. N.; Malhotra, B. D. J Appl Polym Sci 2000, 78, 662.
8. Suad-Chagny, M. F.; Gonon, F. G. Anal Chem 1986, 58, 412.
9. Nakaminami, J.; Ito, S.; Kuwabata, S.; Yoneyama, H. Anal Chem 1999, 71, 4278.
10. Gerard, M.; Chaubey, A.; Malhotra, B. D. Biosens Bioelectron 2002, 17, 345.
11. Shinohara, H.; Chiba, T.; Aizawa, M. Sens Actuators 1988, 13, 79.
12. Trojanowicz, M.; Krawczyk, T. K. Mikrochim Acta 1995, 121, 167.
13. Kumar, A.; Rajesh; Chaubey, A.; Grover, S. K.; Malhotra, B. D. J Appl Polym Sci 2001, 82, 3486.
14. Vidal, C. J.; Garcia, E.; Castillo, J. R. Anal Sci 2002, 18, 537.
15. Chaubey, A.; Gerard, M.; Singhal, R.; Singh, V. S.; Malhotra, B. D. Electrochim Acta 2000, 46, 723.
16. Chaubey, A.; Gerard, M.; Malhotra, B. D. In Handbook of Polymers in Electronics; Malhotra, B. D., Ed.; Rapra Technology: United Kingdom, 2002; p 297.
17. Ram, M. K.; Bertoncello, P.; Ding, H.; Paddeu, S.; Nicolini, C. Biosens Bioelectron 2001, 16, 849.
18. Galban, J.; Sierra, J. F.; Lopez, J. M.; Marcos, S. D.; Castillo, J. R. Appl Spectrosc 2000, 54, 1157.
19. Kumar, A.; Rajesh; Malhotra, B. D.; Grover, S. K. Anal Chim Acta 2000, 414, 43.
20. Kumar, S. Development of Conducting Polymer Based Cholesterol Biosensor; Major Project Report for M.E. (Polymer Technology); Delhi College of Engineering: Delhi, India, 2002.
21. Kajiya, Y.; Tsuda, R.; Yoneyama, H. J Electroanal Chem 1991, 301, 155.
22. Tretnakk, W.; Wolfbeis, O. S. Anal Biochem 1990, 184, 124.
23. Kumar, A.; Rajesh; Chaubey, A.; Grover, S. K.; Malhotra, B. D. J Appl Polym Sci 2001, 82, 3486.
24. Karube, I.; Hara, K.; Mastsuoka, H.; Suzuki, S. Anal Chim Acta 1982, 139, 127.
25. Crumbliss, A. L.; Stonehuerner, J. G.; Whenkens, R. Biosens Bioelectron 1993, 8, 331.
26. Tabata, M.; Endo, J.; Murachi, T. J Appl Biochem 1981, 3, 84.
27. Krug, A.; Suleiman, A. A.; Guilbault, G. G. Anal Chim Acta 1992, 256, 263.
28. Motonaka, J.; Faulkner, L. R. Anal Chem 1995, 65, 3258.
29. Suman; Pundir, C. S. Curr Appl Phys 2003, 3, 129–133.
30. Besomes, J. L.; Cosnier, S.; Labbe, P.; Reverdy, G. Anal Chim Acta 1995, 317, 275.
31. Guilbault, G. G. Analytical Uses of Immobilised Enzymes; Marcel Dekker: New York, 1984; p 137.