J Solid State Electrochem (2009) 13:657–663
DOI 10.1007/s10008-008-0590-7

ORIGINAL PAPER

Poly(pyrrole) versus poly(3,4-ethylenedioxythiophene): amperometric cholesterol biosensor matrices

Özlem Türkarslan · Senem Kıralp Kayahan · Levent Toppare

Received: 22 February 2008 / Accepted: 14 May 2008 / Published online: 10 June 2008

Abstract Two conducting polymers, poly(pyrrole) (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) were used as immobilization matrices for cholesterol oxidase (ChOx). The amperometric responses of the enzyme electrodes were measured by monitoring oxidation current of H_2O_2 at +0.7 V in the absence of a mediator. Kinetic parameters, such as K_m and I_{max}, operational and storage stabilities, effects of pH and temperature were determined for both entrapment supports. K_m values are found as 7.9 and 1.3 mM for PPy and PEDOT enzyme electrodes, respectively; it can be interpreted that ChOx immobilized in PEDOT shows higher affinity towards the substrate.

Keywords Amperometric biosensor · Enzyme electrodes · Conducting polymers · Cholesterol · Cholesterol oxidase

Abbreviations

Py	pyrrole
PPy	poly(pyrrole)
EDOT	3,4-ethylenedioxy thiophene
PEDOT	poly(3,4-ethylenedioxythiophene)
ChOx	cholesterol oxidase

Introduction

Steroids are lipids with ring structures. Although they have similar backbones, their functions in the body vary with different attached groups. Cholesterol, found in animal plasma membranes, is the precursor of several other steroids, including the vertebrate and sex hormones. Accumulation of cholesterol under the inner linings of arteries may lead to atherosclerosis [1]. Cholesterol determination in blood is clinically important in the diagnosis and prevention of cardiovascular diseases.

The first enzymatic determination of total serum cholesterol was done by Allain et. al. [2]; in their work, cholesterol esters were first hydrolyzed to free cholesterol by cholesterol ester hydrolase, and then this free cholesterol was oxidized to cholest-4-en-3-one by cholesterol oxidase accompanied with the production of hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of a peroxidase, yielding a chromogen which absorbs at 500 nm.

A biosensor is a device consisting of two main parts: (1) a recognition part in which a biorecognition element is immobilized on a solid surface and (2) a transducer part. The signal may be transduced by optical, thermal, electrical, or electronic elements [3]. Several cholesterol biosensors have been designed based on different analytical methods. The starting point is the immobilization of the enzymes, such as cholesterol oxidase and cholesterol esterase, and then enzyme–substrate interactions are detected by several techniques including spectrophotometry [4–6], surface plasmon resonance [7–10], quartz crystal microbalance [11], and electrochemistry [12–14]. As electrochemical biosensors offer fast, simple, and low-cost detection capabilities for biological binding events, they fulfill an important position among the biosensors [15].

Chronoamperometry is the preferred technique in electrochemical detection systems for cholesterol, as it allows monitoring either the oxygen consumption or the hydrogen peroxide production. In recent years, various enzyme

Ö. Türkarslan · S. K. Kayahan · L. Toppare (✉)
Department of Chemistry, Middle East Technical University, 06531 Ankara, Turkey
e-mail: toppare@metu.edu.tr

immobilization matrices were used in the construction of amperometric cholesterol biosensors, such as self assembled monolayers [16], lipid bilayer membranes [13], sol–gel/chitosan hybrid composite films on multi-walled carbon nanotubes [17], poly(pyrrole) [18], poly(pyrrole) (PPy)/Prussian blue layers [19], PPy-hydrogel membrane [20], diaminonaphthalene isomers [21], and polyaniline Langmuir–Blodgett films [22].

Immobilization of a biomolecule in electropolymerized films is a simple one-step method. In this physical entrapment, an appropriate potential is applied to the working electrode immersed in aqueous solution containing the biomolecule and the electropolymerizable monomer. This method enables reproducible and precise formation of polymeric films with controlled thickness and morphology. These coatings are equally stable in organic and aqueous solvents [23]. PPy [18], polyaniline (PAni) [24], poly(3,4-ethylenedioxy thiophene) (PEDOT) [25], and their derivatives are ideal candidates for enzyme immobilization matrices, thanks to the solubility of their monomers in water.

PPy is a famous enzyme immobilization matrix owing to its simple synthesis in aqueous medium. The alkoxy substituted polythiophene (PTh) derivative, PEDOT, can easily be electropolymerized, and it is highly stable in the oxidatively doped state [26]. As stated in the literature, PEDOT has enhanced electrochemical stability compared to PPy [27] and has a more ordered structure, as it is polymerized through α–α' positions only. All the biosensors consisted of PEDOT immobilization layer to date use either glucose oxidase [28–30] or polyphenol oxidase [31] as the redox enzymes. However, there is no study offering PEDOT as the ChOx immobilization matrix. The present work describes cholesterol sensors made up of cholesterol oxidase entrapped in PEDOT and PPy films. The amperometric responses were measured via monitoring oxidation current of H_2O_2 at +0.7 V (Scheme 1) without using a mediator. Although mediators enhance the sensitivity and selectivity [32–34], the cholesterol sensors produced has enough sensitivity and selectivity towards the substrate; hence, a mediator was not used in this study.

Materials and methods

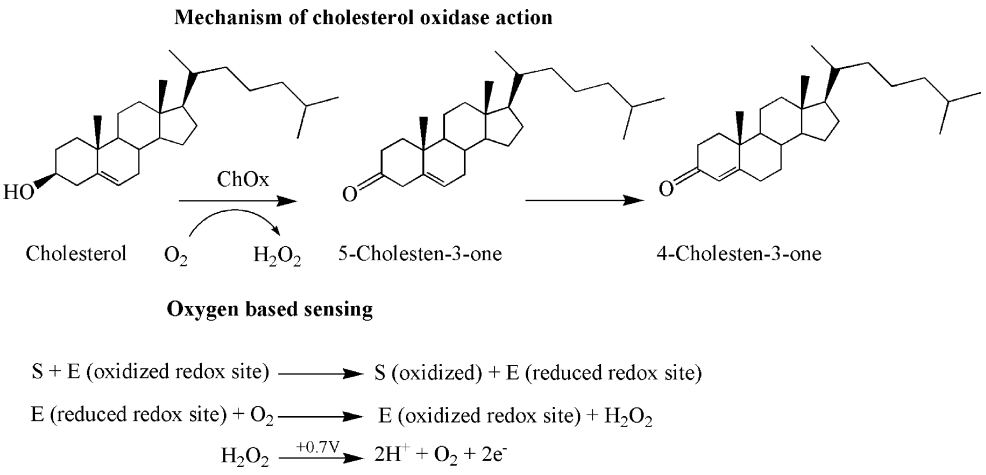
Chemicals and reagents

Pyrrole (Py), 3,4-ethylenedioxythiophene (EDOT), cholesterol oxidase (ChOx) [E.C.1.1.3.6] (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Sigma-Aldrich and used with no further purification. Ethanol (Merck) was of analytical grade and used as received for preparing the cholesterol stock solution (0.05 M). Phosphate buffer (pH=7) for the electrosynthesis was prepared by dissolving 0.025 mol of Na_2HPO_4 (Fisher Scientific Company) and NaH_2PO_4 (Fischer Scientific Company) in 1 l distilled water. On the other hand, the phosphate buffer (pH=7) utilized in the amperometric measurements consisted of 0.04 M Na_2HPO_4, 0.04 M KH_2PO_4 (Merck), and 0.1 M KCl (Fisher Scientific Company) to provide ionic conductivity.

Instruments

Electrochemical measurements were carried out in a cell equipped with Ag/AgCl reference electrode (silver wire dipped in 4 M KCl saturated with silver chloride, Fischer Scientific Company), platinum (Pt) plate working and counter electrodes with 0.12-cm^2 area each. All the electrosynthesis and amperometric experiments were carried out with Ivium CompactStat (The Netherlands) potentiostat via chronoamperometry.

Scheme 1 Mechanisms of cholesterol oxidase action and oxygen based sensing; enzyme and substrate are denoted with the capital letters '*E*' and '*S*', respectively



Preparation of the enzyme electrodes

Cholesterol oxidase was immobilized in two different conducting polymer matrices via constant potential application. PPy enzyme electrodes were prepared by applying +1.0 V vs reference electrode in the electrolysis cell described previously containing 0.042 M Py, 0.1 M SDS, and 0.72 mg of protein dissolved in 10 ml of phosphate buffer. In the case of PEDOT matrix, 0.028 M EDOT, 0.05 M SDS, 0.36 mg of protein, and 10 ml of phosphate buffer were used and +0.8 V was applied. SDS is not only the supporting electrolyte for the electrosynthesis but it also enhances solubility of Py and EDOT in aqueous solutions, thanks to its ionic surfactant property.

Preparation of cholesterol solution

Cholesterol stock solution (0.05 M) was prepared dissolving 0.387 g of cholesterol in 20 ml of ethanol at room temperature via gently mixing with constant speed to obtain a clear solution. The stock solution was stored at +4 °C in dark and consumed in 10 days. Triton X-100, the nonionic surfactant providing solubility of cholesterol in aqueous solutions, was added to the cholesterol solutions just before the experiments. High Triton X-100 concentration can inhibit the activity of ChOx. The concentration range of 0.8–1.2% (*v*/*v*) was found to be suitable by Tan et al. [35]. For all the experiments, this surfactant was added in the ratio of 1% (*v*/*v*) to the analyte solutions.

Amperometric biosensor measurements

Amperometric response studies were carried out for both enzyme electrodes in the phosphate buffer containing 0.1 M of KCl in open atmosphere. Operational, storage, pH, and temperature stabilities were determined via application of +0.7 V with respect to Ag/AgCl electrode to detect oxidation current of H_2O_2. Initially, the baseline current became constant, and then the analyte was added to the medium; the current immediately increased (response times were 2–3 s) and reached a steady state almost at the end of 150–200 s. Finally, the differences between these current values were recorded. During the experiments, the system was gently stirred. In the stability experiments, substrate solutions of 16 and 13 mM were used for PPy and PEDOT matrices.

Results and discussion

Optimization studies

The effect of film thickness on the oxidation current of hydrogen peroxide was initially studied. Deciding on the optimal thickness is important, as very thin polymeric films may be unable to protect the enzyme from the environmental effects. On the other hand, very thick films may complicate the diffusion process between solution and entrapment support, and as a result, the substrate may not associate with the recognition element. The thickness was controlled by fixing the charge at which the maximum amperometric response was obtained. First, Pt electrodes were coated with PPy depositing 1Q (0.0428 C-charge deposited in a minute), 2Q (0.0856 C), and 3Q (0.128 C), and then the experiments were carried out by adding 5 ml H_2O_2 (0.222 mM in a total volume of 15 ml) to medium. The current responses were found as 24, 49, and 40 μA/cm^2 for 1Q, 2Q, and 3Q PPy electrodes, respectively. In the case of PEDOT, only 1Q (0.0428 C) deposited electrode was utilized, as the polymeric films with higher thicknesses were instantly peeled off from the surfaces of the Pt electrodes. For 1Q PEDOT electrode, the amplitude of the response was calculated as 21 μA/cm^2. In summary, in all of the subsequent experiments, 2Q PPy (nearly 50 μm thick) and 1Q PEDOT (nearly 20 μm thick) electrodes were utilized. The thickness of the polymers was estimated using the charge required for the film coating on the electrode

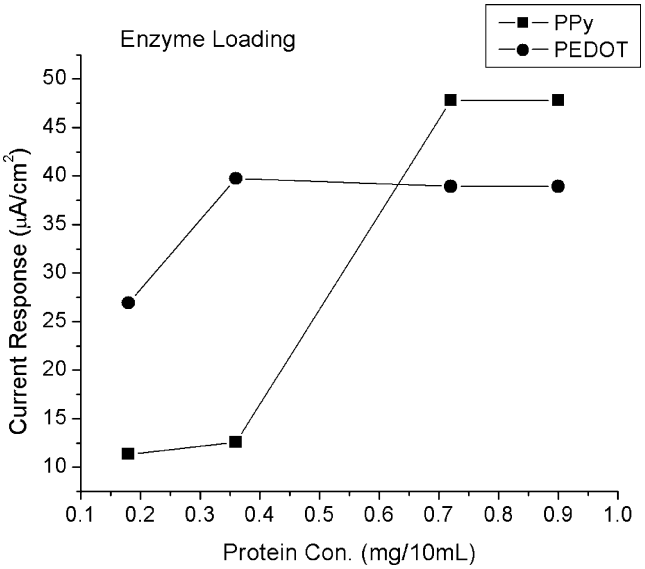


Fig. 1 Current responses of the enzyme electrodes containing different amounts of ChOx in the presence of 10 mM cholesterol solution

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Scheme 2 The classical equation showing enzyme–substrate–product interaction

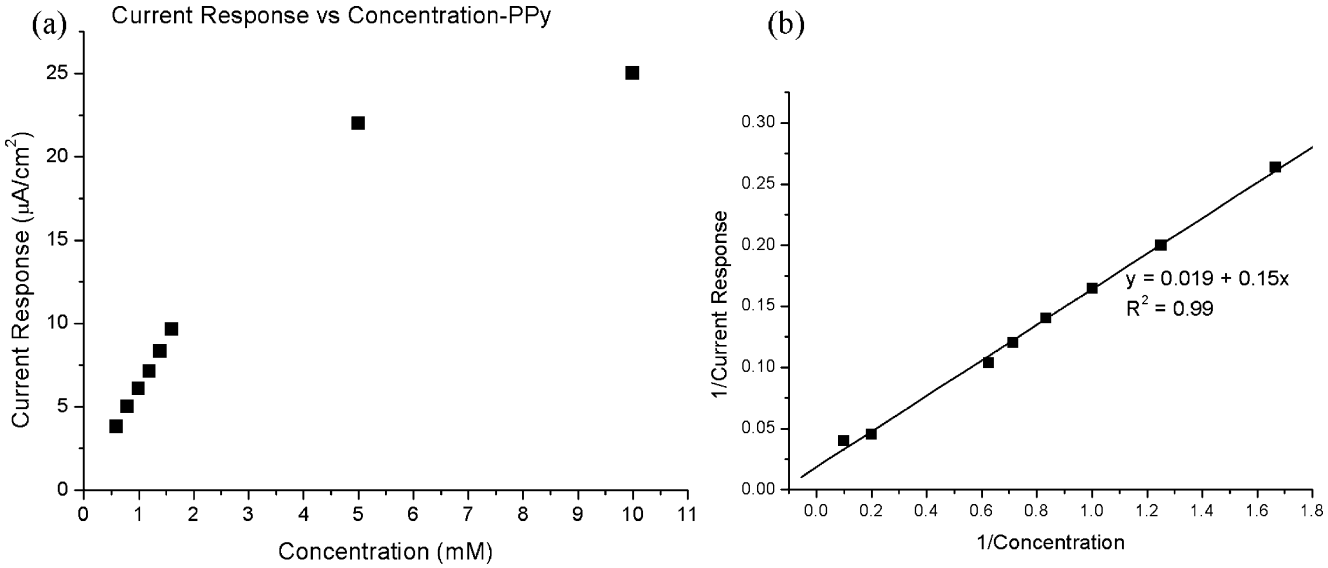


Fig. 2 **a** Current response vs concentration. **b** 1/Current response vs 1/concentration for the PPy enzyme electrode

surfaces. These values were checked with a micrometer after peeling off the polymer film from the electrode surface.

The responses of the enzyme electrodes containing different amounts of ChOx were also studied in the presence of 10 mM cholesterol to determine the optimal amount of enzyme loading, and the results for both sensors are shown in Fig. 1. The current response rose with increasing enzyme loading and then reached a saturation point. The maximum responses were obtained with 0.72 mg protein/10 ml and 0.36 mg protein/10 ml for PPy and PEDOT enzyme electrodes, respectively. In conclusion, in the preparation of 2Q PPy enzyme electrodes, 0.72 mg protein/10 ml was added to the polymerization medium, whereas 1Q PEDOT was loaded 0.36 mg protein/10 ml to obtain the highest sensitivity.

Enzyme kinetics

In the equilibrium model of Michaelis–Menten, the substrate binding step is assumed to be fast relative to the rate of breakdown of the enzyme–substrate complex (Scheme 2).

Therefore, this reaction is assumed to be at equilibrium, and the equilibrium dissociation constant for the complex, K_m, is a measure of the affinity of enzyme for its substrate

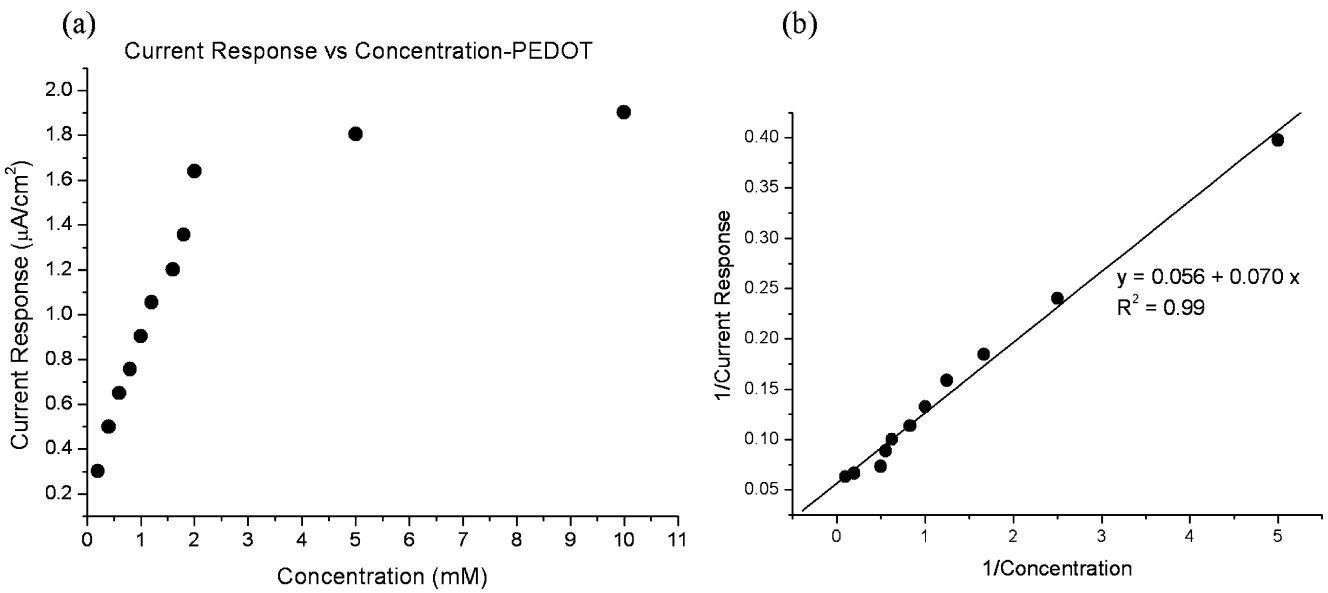


Fig. 3 **a** Current response vs concentration. **b** 1/Current response vs 1/concentration for the PEDOT enzyme electrode

and corresponds to substrate concentration at 1/2 V_{max} [36]. In the case of amperometric sensors, K_m and I_{max} (instead of V_{max}) were determined from the Lineweaver–Burk plot by plotting 1/current response vs 1/substrate concentration. After linear regression, an equation of type $y = A + Bx$ was obtained. Inverse of 'A' gives I_{max}, as at that point, x is zero, whereas K_m was calculated as $B(A)^{-1}$, as at that point, y is zero. Figures 2a and 3a reveal an increasing current response with the increasing substrate concentration. Lineweaver–Burk plots were given in Figs. 2b and 3b. Figure 4 aims to compare the current response with respect to concentration for the two polymers in concern. As regards to the minimum detectable concentrations, the current responses are 3.8 and 2.5 μA/cm^2 for PPy and PEDOT, respectively. All the experiments were carried out at constant temperature (24 °C) and pH (pH=7). The important parameters obtained for the enzyme electrodes were summarized in Table 1. As K_m is inversely proportional to the affinity of the enzyme for its substrate, in PEDOT matrix, more ChOx was associated with cholesterol; however, the intensity of the response was lower compared to PPy matrix, which could be interpreted as less product formation. Sensitivities [37, 38] were calculated as 6.7 and 14.3 μAmM^{-1} cm^{-2} for PPy and PEDOT enzyme electrodes, respectively, by dividing I_{max} to K_m. It can be concluded that with small K_m value and comparatively high sensitivity, the PEDOT enzyme electrode revealed better biosensor characteristics.

Operational and storage stabilities

The response time and reproducibility of a biosensor are the important parameters when testing the analytical performance. On the other hand, biomolecules have limited stabilities, especially when removed from their native microdomains; in addition, their stabilities and performances decrease upon immobilization. Thus, their storage times should be determined [39]. Operational and storage stabilities were shown in Figs. 5 and 6, respectively. The operational stability of enzyme electrodes in terms of repetitive use was obtained running several measurements on the same day. For ChOx entrapped in PPy matrix, 70% activity loss was observed at the 13th use (60% activity loss at the ninth measurement), and the PEDOT enzyme electrode lost 35% of its activity at the ninth use. Although PPy matrix was likely to protect the enzyme better, as it is thicker than PEDOT matrix, it is more vulnerable to environmental effects, such as oxygen and hydrogen peroxide, due to structural irregularities (α–α as well as α–β linkages and open positions susceptible to possible attacks of water) [40]. To determine the storage stability or shelf-life of the enzyme electrodes, the same electrode was used for consecutive 28 days. On the 28th day, 60% of the activity was lost for the PEDOT enzyme electrode, whereas the PPy enzyme electrode lost 90% of its activity. PEDOT was a better support for ChOx, as it could be used for 20 days with only 20% loss. The fluctuations were related to conformational changes and diffusional effects [41].

Table 1 Kinetic parameters

	PPy enzyme electrode	PEDOT enzyme electrode
K_m	7.9±0.2 mM	1.3±0.1 mM
I_{max}	52.6±0.6 μA/cm^2	17.9±0.7 μA/cm^2
Sensitivity	6.7 μA mM^{-1} cm^{-2}	14.3 μA mM^{-1} cm^{-2}

K_m and I_{max} values shown in the text and table are the averages of four experiments.

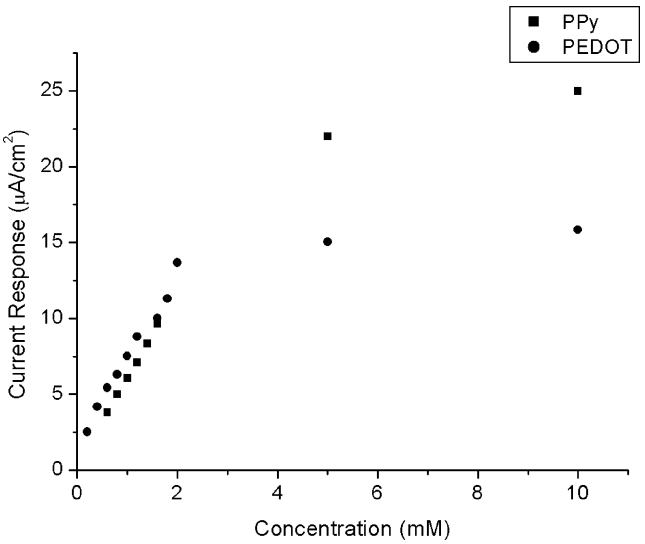


Fig. 4 Current response vs concentration graphs

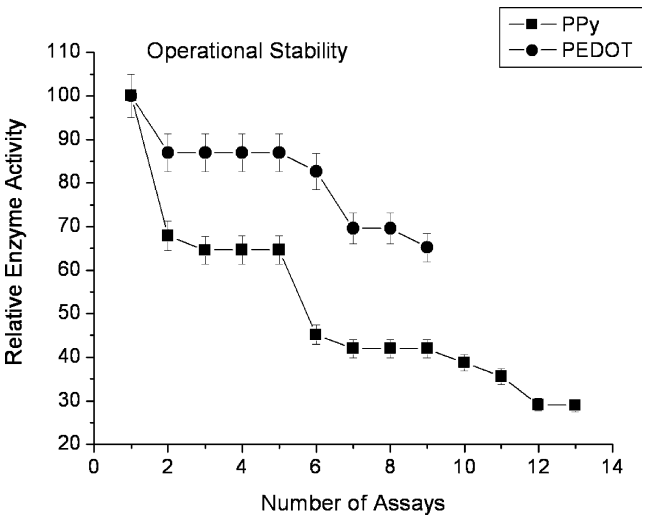


Fig. 5 Operational stabilities of the PPy and PEDOT enzyme electrodes. Each data point represents the average of data collected by four electrodes

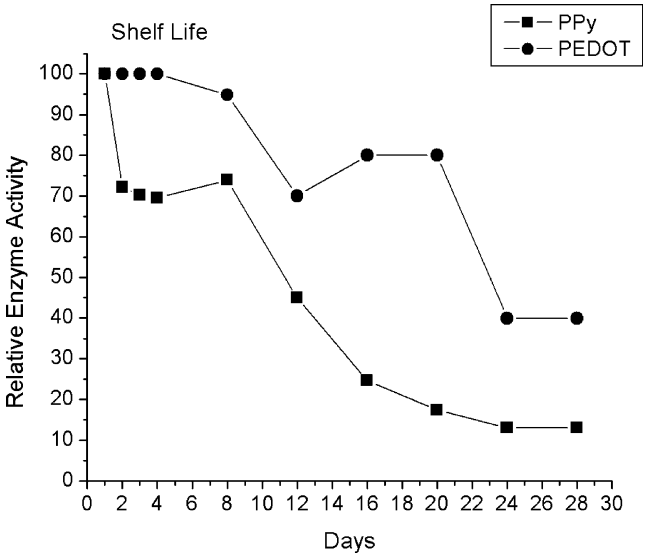


Fig. 6 Storage stabilities of the PPy and PEDOT enzyme electrodes; the amperometric responses of these enzyme electrodes were regularly checked during 28 days

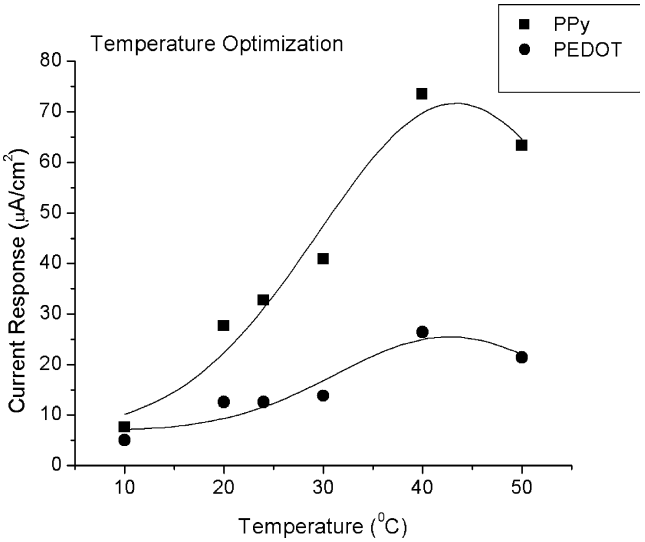


Fig. 8 Effects of temperature on PPy and PEDOT enzyme electrodes. Each data was collected with freshly prepared enzyme electrodes

Effect of pH

Optimum pH values should be determined in enzyme assays, as pH changes in the medium (especially high and low pH) cause denaturation. The experiments were carried out with freshly prepared enzyme electrodes. In addition, the same electrodes were used two or three times in either alkaline or acidic medium. As shown in Fig. 7, amplitudes of current responses for the PPy enzyme electrode were higher, as the I_{max} was greater. However, PEDOT matrix demonstrated a better protection for ChOx from pH changes; for this reason, a broader curve was obtained. In both cases, the maximum activities were observed at pH 7, but high acidity or basicity resulted in the loss of activity.

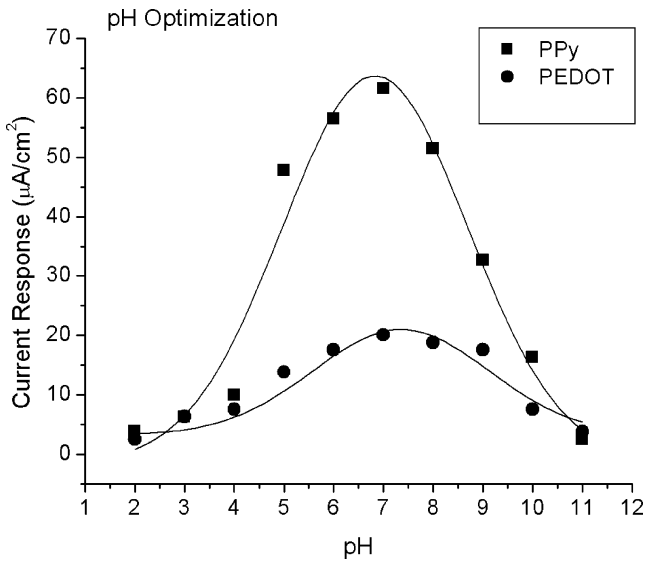


Fig. 7 Effects of pH on PPy and PEDOT enzyme electrodes. Data collected with freshly prepared electrodes refer to the averages of three experiments

Effect of temperature

Cholesterol oxidase is optimally active at 37 °C and pH 7.0 in aqueous medium [42]. The effect of temperature between 10 and 50 °C were investigated via checking the current responses of the freshly prepared electrodes and illustrated in Fig. 8. As ethanol was used to dissolve cholesterol, activities were not checked at higher temperatures to prevent vaporization. Current response gradually increased with increasing temperature and reached a maximum at 40 °C for both matrices. As seen in Fig. 8, the PPy enzyme

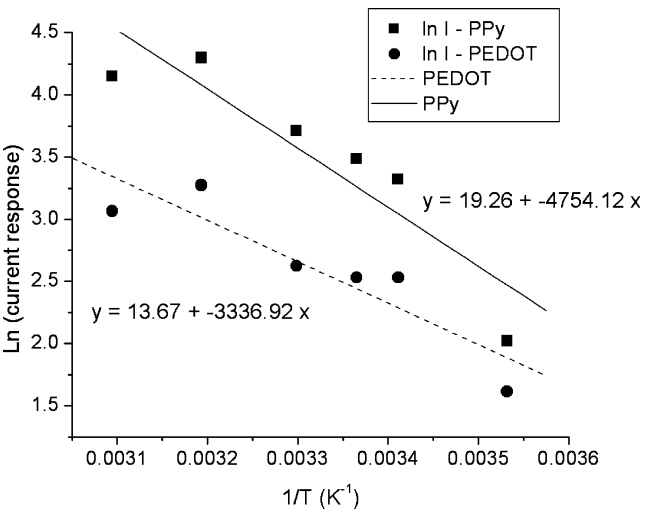


Fig. 9 Determination of the activation energy

electrode showed high intensity responses as predicted due to its high I_{max}. These results demonstrated that both enzyme electrodes showed the same trend.

Activation energies were also calculated from the Arrhenius equation:

$$I(T) = I_0 \exp(-E_a/RT)$$

$$\mathrm{Ln}\, I = \mathrm{Ln}\, I_0 - E_a/R(1/T).$$

Ln I (current response) vs $1/T$ graphs (Fig. 9) were plotted, and after linear regressions were done, equations of type '$y = A + Bx$' were obtained, and then activation energies were calculated from '$E_a = B\,R$'. The activation energies for the enzymatic reactions in PPy and PEDOT matrices were found as 39.5 and 27.7 kJ/mol, respectively. The smaller E_a means that the entrapped enzyme in PEDOT matrix possesses higher enzyme activity and the sensor exhibits higher affinity towards its substrate, which is in agreement with lower K_m value [17].

Conclusions

The redox enzyme, cholesterol oxidase, was immobilized in two different polymeric matrices. The polymers were grown on Pt working electrodes in the presence of buffer, enzyme, water soluble monomers, pyrrole and 3,4-ethylenedioxythiophene, and sodium dodecyl sulfate. The enzyme immobilized in PEDOT matrix had high sensitivity, small K_m and E_a, which could be interpreted in terms of high affinity towards the substrate, cholesterol. Moreover, thanks to its structural order, PEDOT matrix revealed a better stability and protected the enzyme from environmental changes, such as pH and temperature. Although the enzyme in the PPy support had higher I_{max}, which is the measure of product formation, the sensitivity was lower compared to the PEDOT support.

Acknowledgments The authors gratefully thank to DPT 2005K 120580, TUBITAK 105T385 and BAP-2007-01-03-03 and TUBA grants.

References

1. Madder S (1996) Biology, 5th edn. Times Mirror Higher Education Group, Inc., USA, pp 51, 86, 87, 638, 639
2. Allain CC, Poon LS, Chan CSG, Richmond W, Fu PC (1974) Clin Chem 20:470
3. Sadana A (2001) Engineering biosensors: kinetics and design applications, 1st edn. Academic, USA, pp 1
4. Çırpan A, Alkan S, Toppare L, Cianga I, Yağcı Y (2003) Des Monomers Polym 6:237
5. Kumar A, Pandey RR, Brantley B (2006) Talanta 69:700
6. Singh S, Chaubey A, Malhotra BD (2003) J Appl Polym Sci 91:3796
7. Arya SK, Solanki PR, Singh SP, Kaneto K, Pandey MK, Datta M, Malhotra BD (2007) Biosens Bioelectron 22:2516
8. Arya SK, Solanki PR, Singh RP, Pandey MK, Datta M, Malhotra BD (2006) Talanta 69:918
9. Arya SK, Prusty AK, Singh SP, Solanki PR, Pandey MK, Datta M, Malhotra BD (2007) Anal Biochem 363:210
10. Solanki PR, Arya SK, Nishimura Y, Iwamoto M, Malhotra BD (2007) Langmuir 23:7398
11. Chong KT, Su X, Lee EJD, O'Shea SJ (2002) Langmuir 18:9932
12. Özer BC, Özyörük H, Çelebi SS, Yıldız A (2007) Enzyme Microb Technol 40:262
13. Devadoss A, Palencsar MS, Jiang D, Honkonen ML, Burgess JD (2005) Anal Chem 77:7393
14. Vidal JC, Espuelas J, Castillo JR (2004) Anal Biochem 333:88
15. Bakker E, Qin Y (2006) Anal Chem 78:3965
16. Zhou N, Wang J, Chen T, Yu Z, Li G (2006) Anal Chem 78:5227
17. Tan X, Li M, Cai P, Luo L, Zou X (2005) Anal Biochem 337:111
18. Singh S, Chaubey A, Malhotra BD (2004) Anal Chim Acta 502:229
19. Vidal JC, Espuelas J, Garcia-Ruiz E, Castillo JR (2004) Talanta 64:655
20. Brahim S, Narinesingh D, Guiseppi-Elie A (2001) Anal Chim Acta 448:27
21. Garcia-Ruiz E, Vidal JC, Aramendia MT, Castillo JR (2004) Electroanal 16:497
22. Matharu Z, Sumana G, Arya SK, Singh SP, Gupta V, Malhotra BD (2007) Langmuir 23:13188
23. Cosnier S (2005) Electroanal 17:1701
24. Singh S, Solanki PR, Pandey MK, Malhotra BD (2006) Anal Chim Acta 568:126
25. Kros A, Sommerdijk NAJM, Nolte RJM (2005) Sensor Actuator B 106:289
26. Argun AA, Aubert PH, Thopson BC, Schwendeman I, Gaupp CL, Hwang J, Pinto NJ, Tanner DB, MacDiarmid AG, Reynolds JR (2004) Chem Mater 16:4401
27. Sotzing GA, Reddinger JL, Reynolds JR (1997) Synth Met 84:199
28. Kros A, Van Hövell SWFM, Sommerdijk NAJM, Nolte RJM (2001) Adv Mater 13:1555
29. Fabiano S, Tran-Minh C, Piro B, Dang LA, Pham MC, Vittori O (2002) Mater Sci Eng 21:61
30. Nien PC, Tung TS, Ho KC (2001) Electroanal 18:1408
31. Vedrine C, Fabiano S, Tran-Mink C (2003) Talanta 59:535
32. Vidal JC, Garcia E, Castillo JR (2002) Anal Sci 18:537
33. Jia F, Yu C, Gong J, Zhang L (2008) J Solid State Electrochem DOI 10.1007/s10008-008-0521-7
34. Pauluikaite R, Ghica ME, Barsan M, Brett CMA (2007) J Solid State Electrochem 11:899
35. Tan X, Li M, Luo L, Zou X (2005) Anal Biochem 337:111
36. Marangoni AG (2003) Enzyme kinetics: a modern approach. Wiley, USA, pp 44–60
37. Mignani A, Luciano G, Lanteri S, Leardi R, Scavetta E, Tonelli D (2007) Anal Chim Acta 599:36
38. Niculescu M, Erichsen T, Sukharev V, Kerenyi Z, Csöregi E, Schuhmann W (2002) Anal Chim Acta 463:39
39. Lundgren JS, Bright FV (1996) Anal Chem 12:3377
40. Thomas CA, Zong K, Schottland P, Reynolds JR (2000) Adv Mater 12:222
41. Arslan A, Kıralp S, Toppare L, Bozkurt A (2006) Langmuir 22:2912
42. MacLachlan J, Wotherspoon ATL, Ansell RO, Brooks CJW (2000) J Steroid Biochem 72:169