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Immobilization of Tyrosinase in Poly(2-thiophen-3-yl-alkyl ester) Derivatives

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In this study, construction of novel biosensors for the determination of phenolic compound was performed via immobilization of tyrosinase during the electrochemical synthesis of conducting block copolymers of 2-thiophen-3-yl-alkyl ester derivatives with 3,4-ethylenedioxythiophene and synthesis of poly(3,4-ethylenedioxythiophene) (PEDOT). The resultant biosensors were characterized in terms of their maximum reaction rates, Michaelis–Menten constants (K_m), temperature and pH stabilities. All the copolymer matrices represented higher reaction rates and higher K_m values in comparison to both polypyrrole and PEDOT matrices and a relation between the morphology of the matrice and the kinetic parameters was observed. Biosensors maintained their activity even at temperatures as high as 80°C and could be used at pHs higher than 8 with high precision. The amount of phenolics in actual samples (red wines) was investigated using electrodes, and results were compared with those found from Folin–Ciocalteau method. Hence, the present study has proven the suitability of these copolymers to be used as polymer matrices for enzyme immobilization.

Keywords: Tyrosinase, immobilization, phenolic compounds, electrochemistry, conducting polymer, copolymer, biosensor

1. Introduction

Up to date extensive research has been devoted to investigate the possible use of enzymes in biotechnological applications. Use of enzyme in free form has significant drawbacks such as thermal instability, susceptibility to attack by proteases, activity inhibition, high sensitivity to several denaturing agents. Hence, immobilization of the enzyme in a matrice is preferred and is proven to be more advantageous. There are various enzyme immobilization techniques like, adsorption, crosslinking, covalent attachment and entrapment in a matrice. However, electrochemical polymerization offers a simple and facile approach where enzyme entrapment is achieved during the synthesis of conducting polymers (1).

In recent years there has been a growing interest in the development of biosensors based on conducting polymers due to their 1) direct and easy deposition on sensor electrode by electrochemical oxidation of monomer at any size and geometry, 2) control of film thickness, 3) their chemical structures achieved via structural modification of the parent monomer. Thanks to these properties, the use of polypyrrole and its amphiphilic derivatives has been thoroughly investigated and a review has been published (2). Although polypyrrole and it derivatives met some of the requirements like easy oxidation, low cost of monomer (3), these polymers are known to be less stable compared to thiophene derivatives. Monomers of simple polythiophenes have generally high oxidation potentials and are not water soluble (4). Poly(3,4-ethylenedioxythiophene (PEDOT) on the other hand shows remarkable stability, provides homogeneous films (5) and can be synthesized electrochemically in aqueous medium (6).

The quantification of phenolic compounds in environmental, industrial and food samples is of great interest. Tyrosinase (polyphenol oxidase, PPO) is a very well known enzyme which catalyzes the oxidation of phenols and diphenols to orto-quinones in the presence of oxygen (7). PPO has also proved to be very useful in the analytical determination of cyanide, azide, aromatic amines, phenols and catechols (8).

Current study reports the immobilization of polyphenol oxidase in poly(3,4-ethylenedioxythiophene) and block copolymers of poly(hexanedioic acid bis-(2-thiophen-3-yl-ethyl) ester) (PHABTE/PEDOT), poly(decanedionic acid bis-(2-thiophene-3-yl-ethyl)ester) (PDATE/PEDOT), poly(octanoic acid 2-thiophen-3-yl-ethyl ester) (POTE/PEDOT) with PEDOT. Immobilization of enzyme was

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achieved via entrapment method during the electrochemical polymerization of 3,4-ethylenedioxythiophene (EDOT). This article presents kinetic parameters (K_m and V_{max}) of the biosensors, the optimum operation conditions such as, pH and temperature. The total amount of phenolic compounds in Turkish red wines was also investigated to check the validity of the method.

2. Experimental

2.1. *Materials*

Tyrosinase (PPO) (1530 U/mg) [E.C 1.14.18.1] was purchased from Sigma. Acetonitrile (AN) (Merck) and tetrabutylammonium tetrafluoroborate (TBAFB) and borontrifluoride ethylether (BFEE) (Sigma), were used without further purification. Sodium dodecylsulfate (SDS), 3,4-ethylenedioxythiophene (EDOT), 3-methyl-2-benzothiozolinone (MBTH), acetone, catechol and sulfuric acid were obtained from Sigma. For the preparation of citrate buffer, tri-sodium citrate-2 hydrate and citric acid were used as received. All catechol solutions were prepared in citrate buffer. Hexanedioic acid bis-(2-thiophen-3-yl-ethyl) ester (HABTE) (9), decanedionic acid bis-(2-thiophene-3-yl-ethyl)ester (DATE) (10) and octanoic acid 2-thiophen-3-yl-ethyl ester (OTE) (11) monomer and their corresponding homopolymers namely, PHABTE, PDATE, POTE were synthesized according to previous studies.

2.2. *Preparation of enzyme electrodes*

Immobilization of PPO was achieved via electropolymerization of EDOT either on bare or previously PHABTE, POTE, PDATE coated platinum electrodes. Electropolymerization was performed in a typical three electrode cell, consisting of Pt (1.5 cm^2 each) foils as the working and counter electrodes and a Ag wire pseudo reference electrode. Enzyme electrodes were prepared in 6 mL citrate buffer (pH = 6.5) containing 5 mg/mL SDS (as the supporting electrolyte),15 μL EDOT and 1.5 mg/mL PPO. Immobilization was carried out at a constant potential of +1.0 V (Wenking POS 73 potentiostat) for 20 min at room temperature. During electrochemical deposition, PPO was entrapped within the matrice. Enzyme electrodes were kept at 4°C in citrate buffer solution when not in use.

2.3. *Determination of PPO activity*

Free and immobilized PPO activities were determined using Besthorn's hydrazone method. For determination of free PPO activity, different concentrations of catechol (0.01 M–0.8 M) were prepared. Solutions contain 1.0 mL citrate buffer, 0.5 mL MBTH (0.3% in ethanol) and 0.5 mL catechol. One minute of reaction time was given after the addition of 0.5 mL enzyme solution (0.1 mg/mL). Sulfuric acid (0.5 mL, 5% (v/v)) was added to stop the enzymatic reaction. Quinone reacts with MBTH to form a red color complex and this complex was dissolved by adding 3.0 mL acetone in the test tube. After mixing, absorbance was measured at 495 nm using a Shimadzu UV-Vis spectrophotometer. For immobilized PPO, different concentrations of catechol were prepared (3.0 mL) and put in a water bath at 25°C. 1 mL of MBTH solution was added. An enzyme electrode was immersed in the solution and shaken for 5 min 1 mL of sulfuric acid and 1mL acetone were added for a total volume of 6 mL. After mixing, absorbances were measured at 495 nm.

2.4. *Determination of optimum temperature and pH*

Optimum temperature and pH determinations were carried out by changing incubation temperature and pH between 10 and 90°C, and 2.5 and 12, respectively. The rest of the procedure was the same as the determination of PPO activity.

2.5. *Determination of total phenolic compounds in red wines*

The procedure of measuring the amount of total phenolic compounds was the same as the activity measurement procedure given in section 2.3. The only difference is the use of actual sample, red wine, instead of catechol solution. Two brands of red wines were investigated.

3. Results and discussion

Immobilization of PPO in pure PEDOT, PHABTE/PEDOT, PDATE/PEDOT, POTE/PEDOT matrices has been successfully achieved after optimizing the immobilization conditions. Experiments showed that the thickness of the polymeric films, especially in the case of PEDOT, was critical since thick (>30 μm) films were easily stripped off the Pt electrode surface. Control of the film thickness (ca. 20 μm) was achieved by monitoring the charge passing during polymerization reaction.

Investigation of kinetic parameters of an enzyme electrode is of utmost importance due to interest in understanding the effectiveness of catalysts and it is among the standard protocol. Hence, in this study, the maximum reaction rate, V_{max}, and Michaelis–Menten constant (the analyte concentration yielding a response equal to half of its maximum value), K_m, of the free enzyme and enzyme electrodes were obtained according to Lineweaver–Burk plots (12) (Figure 1). The V_{max} values obtained have unit of μmol/min mL. The activity of enzyme electrodes was measured in 4 mL.

As reflected in Table 1, reaction rates (V_{max}) declined upon immobilization of the enzyme in any of the matrice. However, this took place profoundly in the case of PEDOT matrice. Michaelis–Menten constatnt, $K_{m,}$ is (roughly) an

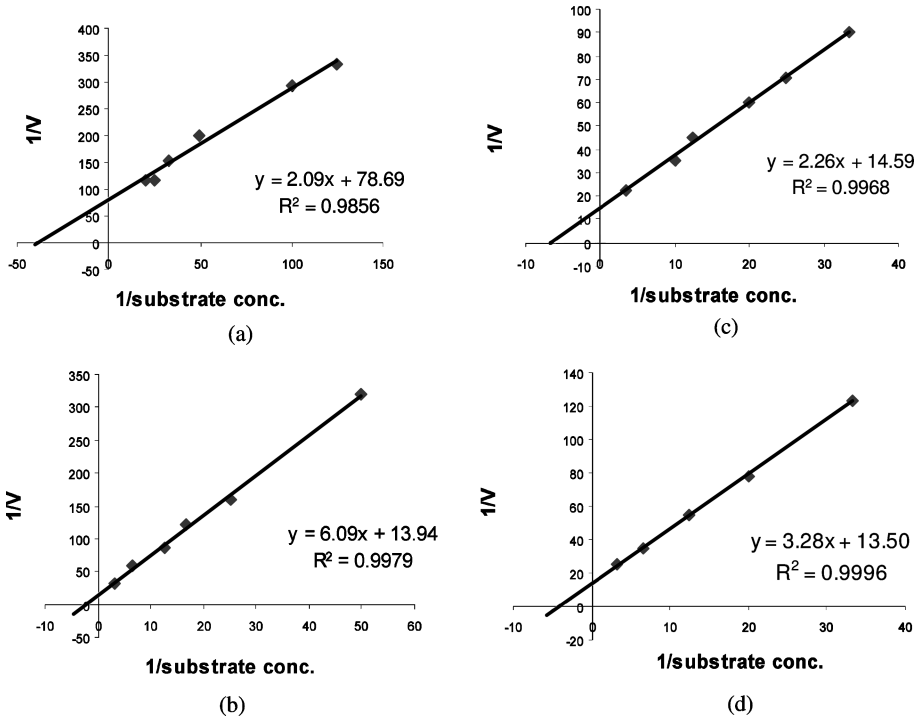

Fig. 1. Lineweaver–Burk plots for immobilized enzyme in (a) PEDOT, (b) PHABTE/PEDOT, (c) POTE/PEDOT, (d) PDATE/PEDOT.

inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the K_m, the greater the affinity which indicates that the ES complex is held together very tightly and rarely dissociates to form product. A large K_m indicates that the substrate and enzyme do not prefer to be together for a long time. As a general trend, all enzyme electrodes exhibited larger K_m values compared to free PPO. Cases where the measured K_m is much larger than that of the free enzyme, mean that a significant substrate diffusion barrier is present between the sample and the reaction layer which is by the nature of the entrapment itself. Hence, K_m is a parameter that is directly related with morphology of the matrice along with the effect the microenviroment of the enzyme within the matrice. Figure 2 represents the scanning electron micrographs of the enzyme electrodes. PEDOT reveals a much more compact morphology than PPy which might prohibit both the substrate and product diffusion, leading to a rather low reaction rate and K_m. However, this adverse effect has been overcome by the synthesis of the copolymers which represented high reaction rates owing to their morphology. Differences among the K_m values of the copolymers could be due to both the morphological differences and flexibility of the matice arising from the starting materials' structure. As a matter of fact, both POTE/PEDOT and PDATE/PEDOT copolymers revealed K_m values of the same magnitude which is in accordance with the similarities in their morphologies (Figure 2 (c,e)). Structures of DATE and OTE with alkyl chains might provide flexibility. PHABTE/PEDOT enzyme electrode however, revealed a high K_m which might again be related to the compactness of its morphology and the difference in its monomeric structure.

Table 1. Kinetic parameters for free and immobilized PPO

Matrice	V^b_{max}	K^b_m *(mM)*
Free PPO[a]	11.2 μmol/min mg protein	4
Polypyrrole[a]	0.110 μmol/min electrode 0.025 μmol/min mL	100
PEDOT	0.05 ± 0.005 μmol/min electrode 0.0125 μmol/min mL	26 ± 1
PHABTE/ PEDOT	0.28 ± 0.01 μmol/min electrode 0.07 μmol/min mL	440 ± 5
POTE/ PEDOT	0.28 ± 0.01 μmol/min electrode 0.07 μmol/min mL	150 ± 3
PDATE/ PEDOT	0.30 ± 0.01 μmol/min electrode 0.075 μmol/min mL	240 ± 3

[a](13)
[b]Average of four experiments

The influence of the reaction temperature (between 10 and 80°C) on the activity of immobilized PPO is illustrated in Figure 3. According to a previous study (13), the temperature at which the maximum activity of the free enzyme and the enzyme entrapped in PPy matrice was found to be 40°C.

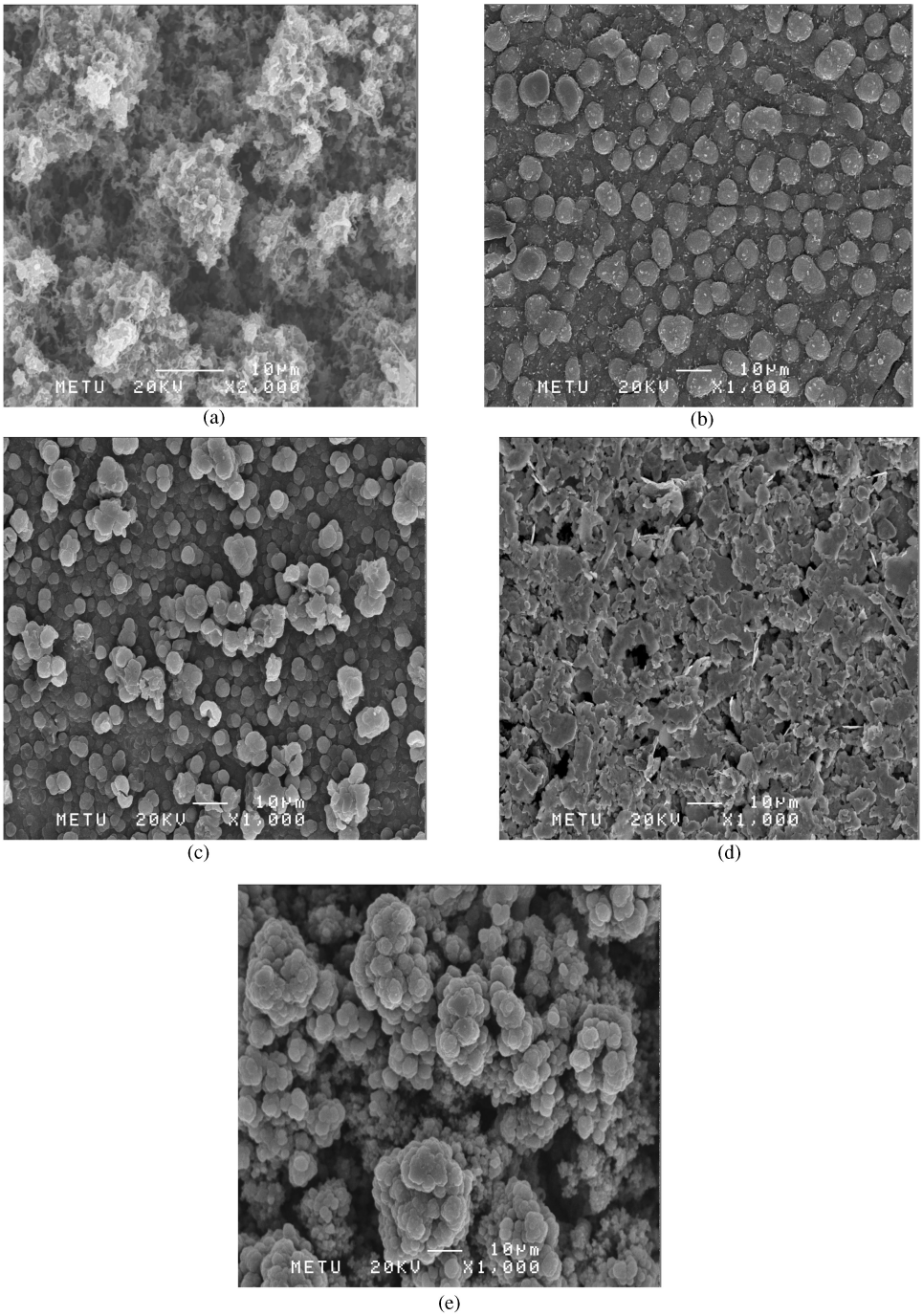

Fig. 2. SEM micrographs of (a) PPy/PPO, (b) PEDOT/PPO, (c) PDATE//PEDOT/PPO, (d) PHABTE//PEDOT/PPO, (e) POTE//PEDOT/PPO.

At 50°C, free PPO was shown to lose its activity completely, whereas PPy/PPO electrode lost only 40% of its activity. As a general trend, POTE/PEDOT and PDATE/PEDOT revealed the highest activity and a similar temperature dependency where the PEDOT based matices revealed a maximum enzyme activity at 50°C. The electrodes maintained their activities even at temperatures as high as 80°C.

Enzymes can easily lose their catalytic activity and be denatured, especially high or low pHs could have a detrimental effect. However, for the sake of simplicity the ability to use them in a wide range of pH should be rather practical. Hence, this part of the study is devoted to investigate the relative enzymatic activity of the electrodes in a broad extensive pH range. Previous studies declared the optimum

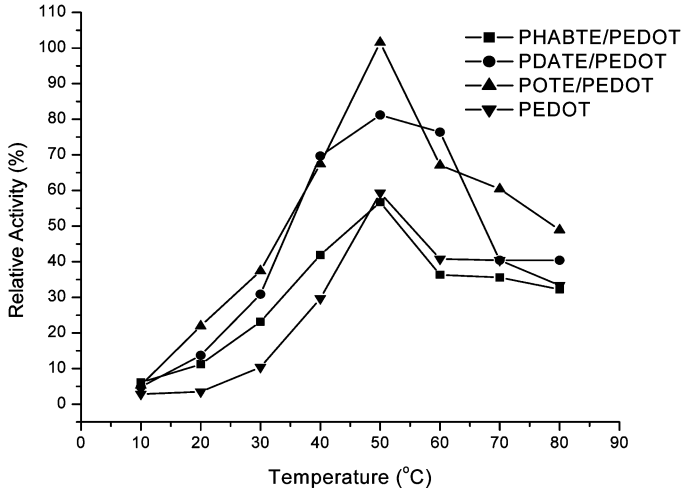

Fig. 3. Effect of incubation temperature on enzyme entrapped in PEDOT, PDATE/PEDOT, PHABTE/PEDOT, POTE/PEDOT.

pH as 5 for free PPO (13). The PPO immobilized in PPy revealed a maximum activity at pH 7. However, other 4 matrices, namely PEDOT, PDATE/PEDOT, POTE/PEDOT, PHABTE/PEDOT enzyme electrodes reveal maximum activity through a pH range of 7 to 11. Results imply the drastic enhancement in the pH stability of the enzyme upon immobilization. The underlying reason could be the effect of microenviroment of the enzyme within the matrice where the partitioning of protons might occur. The negative groups of the dopant anions around the polymer chains cause protons to be concentrated around the enzymes and protect them against the high concentration of hydroxide ions. This tendency might result in an apparent pH around enzyme which might be lower than the bulk. The enzyme electrodes could be used at pH values higher than 8 with a high precision, since more than 80% of the relative activity is preserved (Figure 4). As a result, the copolymer

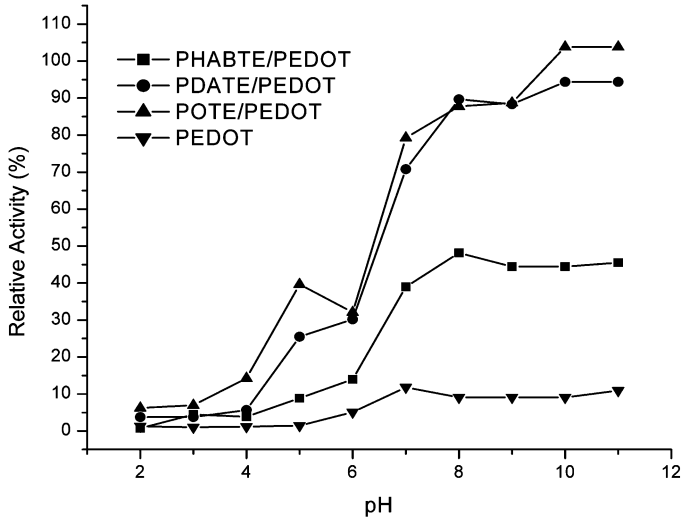

Fig. 4. Effect of pH on enzyme immobilized in PEDOT, PDATE/PEDOT, PHABTE/PEDOT, POTE/PEDOT.

Table 2. Total phenolic amounts of wines

	Brand 1	*Brand 2*
Folin–Ciocalteau method[a]	4100 mg/L	2300 mg/L
Polypyrrole[a]	4000 mg/L	2200 mg/L
PEDOT[b]	2300 mg/L	1100 mg/L
PHABTE/PEDOT[b]	3900 mg/L	2100 mg/L
POTE/PEDOT[b]	4300 mg/L	2700 mg/L
PDATE/PEDOT[b]	3800 mg/L	1900 mg/L

[a](13)
[b]values ±50 mg/L

electrodes are rather superior than the PPy/PPO electrode which reveals maximum activity at pH values between 6 and 8.

The concentration of phenols, estimated by analyzing total phenols as gallic acid equivalents (GAE) (13), were presented in Table 2. Similar results were obtained when wines were analyzed with the Folin–Ciocalteau method (14), a common procedure where its validity is accepted by wine producers.

4. Conclusions

In this study, construction of novel biosensors for phenolic compound determination was achieved via immobilization of tyrosinase during the electrochemical synthesis of conducting block copolymers of 2-thiophen-3-yl-alkyl ester derivatives with EDOT. The produced biosensors were characterized in terms of their maximum reaction rates, Michaelis–Menten constants (K_m), temperature and pH stabilities. Copolymer based biosensors revealed higher V_{max} and K_m values compared to pristine polymers namely PPy and PEDOT. The underlying reason was attributed to the morphological differences and the flexibility of the matrice. As regards to temperature and pH stabilities, copolymer based enzyme electrodes yielded superior results where the electrodes maintained their activity at high temperatures. They could also be used at pHs higher than 8 with a high precision. PEDOT, on the other hand, revealed rather low reaction rate and K_m along with its inferior behavior in terms of thermal stability. As a conclusion the current study has shown the possible use of new copolymers as polymer matrices for enzyme immobilization owing to their high thermal and pH stabilities.

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References

1. Besombes J.L., Cosnier S. and Labbe P. (1997) *Talanta*, 44, 2209–2215.
2. Ramanavicius A., Ramanaviciene A. and Malinauskas A. (2006) *Electrochim. Acta*, 51, 6025–6037.
3. Diaz, A.F., Castillo, J.I., Longon, J.A. and Lee, W.Y.J. (1981) *J. Electroanal. Chem*., 129, 115–132.
4. Védrine C., Fabiano S. and Tran-Minh, C. (2003) *Talanta*, 59, 535–544.
5. Yamato H., Ohwa M. and Wernet W. (1995) *J. Electroanal. Chem*., 397, 163–170.
6. Garreau, S., Louarn, G., Buisson, J.P., Froyer, G. and Lefrant, S. (1999) *Macromolecules*, 32, 6807–6812.
7. Duran, N., Rosa, M.A., D'Annibale, A. and Gianfreda, L. (2002) *Enzyme Micro. Technol*., 31, 907–931.
8. Khan, A.A., Akhtar, S. and Husain, Q.D. (2006) *J. Mol. Cat. B: Enzymatic*, 40, 58–63.
9. Camurlu, P., Cirpan, A. and Toppare, L. (2004) *Synth. Met*., 146, 91–97.
10. Aslan, E., Camurlu, P. and Toppare, L. (2005) *J. Macromol. Sci., Part A: Pure and Appl. Chem*., 42, 451–462.
11. Camurlu, P., Cirpan, A. and Toppare, L. (2005) *Mat. Chem. Phys*., 92, 413–418.
12. Palmer, T. Understanding Enzymes, Prentice Hall: London, 113–120, 1995.
13. Kiralp, S., Toppare, L. and Yagci, Y. (2003) *Int. J. Biol. Macromol*., 33, 37–41.
14. Kiralp, S. and Toppare, L. (2006) Proc. Biochem., 41, 236–239.