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Design of a biosensor based on 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H* pyrrole

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ABSTRACT

Immobilization of polyphenol oxidase (tyrosinase, E.C. 1.14.18.1) was achieved on a copolymer of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole [SNS(NO_2)] with pyrrole ([SNS(NO_2)]/PPy) via electrochemical polymerization. Two different substrates; catechol and L-tyrosine were used for the characterization of biosensor. The kinetic parameters of the biosensor, maximum reaction rate of the enzyme (V_{max}) and Michaelis–Menten constant (K_m) were determined for two different substrates. V_{max} was found as 0.02 μmol/min electrode for both substrates. K_m values were determined as 250 and 2 mM for catechol and L-tyrosine respectively. Calibration curves for enzyme activity versus substrate concentration were plotted between 0.05 and 0.5 M catechol and between 0.8 and 2.5 mM L-tyrosine. Optimum temperature and pH, operational and storage stabilities of immobilized enzyme were examined.

1. Introduction

Conducting polymers (CPs) are intelligent materials containing π-electron backbone responsible for their electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity. This extended π-conjugated system of the conducting polymers have single and double bonds alternating along the polymer chain. Those polymers, called as 'synthetic metals', possess the electrical, electronic, magnetic and optical properties of a metal while retaining the ease of processing associated with a common polymer [1,2]. Many applications of conducting polymers including biosensing devices have been reviewed [3–5]. CPs have been studied extensively in the recent decades for various applications as light emitting diodes [6], batteries [7], solar cells [8], optical displays [9], enzyme immobilization matrices [10], electrochromic devices [11], chemical sensors and arrays [12].

Since the first demonstration of an enzyme as an electrode in a biosensor, the development of such electrochemical devices has made considerable progress [13,14]. Enzymes have been widely applied on biosensor construction as bioreceptor which is the main part of a biosensor. Since enzymes are very expensive biological catalysts, immobilization procedure is effective because immobilized enzyme can be recovered from the solution and can be repetitively used. The immobilization of biomolecules in electropolymerized films is gaining importance since the electrochemical formation of polymer layers of controlled thickness constitutes a reproducible procedure for biosensor fabrication [15]. The electrochemical method involves the entrapment of biomolecules in organic polymers during their electrogeneration on an electrode surface. The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers and biomolecules [16].

Tyrosinase is a tetrameric enzyme with a molecular size of 128 kD. The enzyme has two distinct substrate binding sites one with a high affinity for aromatic compounds including phenolic substrates, the other for metal-binding agents and oxygen. The latter site presumably involves enzyme copper. It is a copper containing oxidoreductase type enzyme with an EC number of 1.14.18.1. It catalyzes two different reactions: (Schemes 1 and 2) the orthohydroxylation of monophenols (cresolase activity), and the oxidoreduction of orthodiphenols to orthoquinones (catecholase activity) [17,18]. Tyrosinase is commonly found in yeast, mushroom, apples, and potatoes and responsible for enzymatic browning of some fruits and vegetables during handling and storage hence, affecting the taste and nutrition value of them [19]. In plants, sponges and many invertebrates, they are important components of wound healing and the primary immune [20,21]. In previous works tyrosinase was immobilized on different conducting polymer matrices [22–24].

We here report a novel biosensor based on immobilization of tyrosinase on a copolymer of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole [SNS(NO_2)] with pyrrole. Two different substrates; catechol and L-tyrosine were used for the characterization of biosensor.

2. Experimental

2.1. Reagents

Tyrosinase (o-diphenol; oxidoreductase; EC 1.14.18.1) obtained from mushroom as lyophilized powder, L-tyrosine, L-ascorbic acid,

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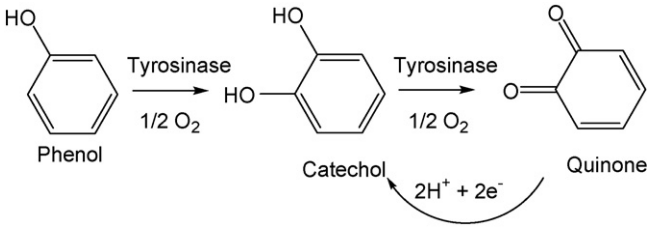


Scheme 1. Reaction mechanism of PPO when the substrate is catechol.

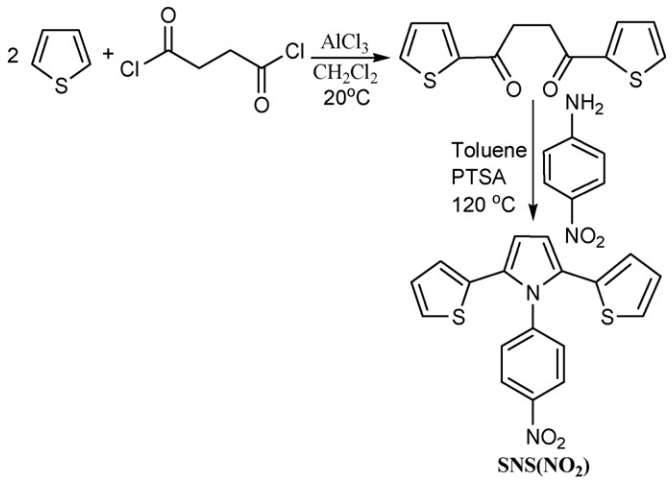
hydrochloric acid, sodium hydroxide, sodium molybdate, and sodium nitrite were purchased from Sigma and used as received without further purification. Sodium dodecylsulfate (SDS) was supplied from Merck. Pyrrole (Py) was purchased from Aldrich and used without further purification. $NaH_2PO_4{\cdot}H_2O$ (sodium phosphate monobasic) and $NaHPO_4{\cdot}7H_2O$ (sodium phosphate dibasic) were purchased from Fisher Scientific Company. Sodium dodecylsulfate (SDS) was supplied from Merck. For spectrometric activity determination, 3-methyl-2-benzothiazolinone hydrazone (MBTH), acetone and sulfuric acid were purchased from Sigma. Catechol was obtained from Sigma. For the preparation of citrate buffer, citric acid and sodium hydroxide were used as received.

2.2. Instrumentation

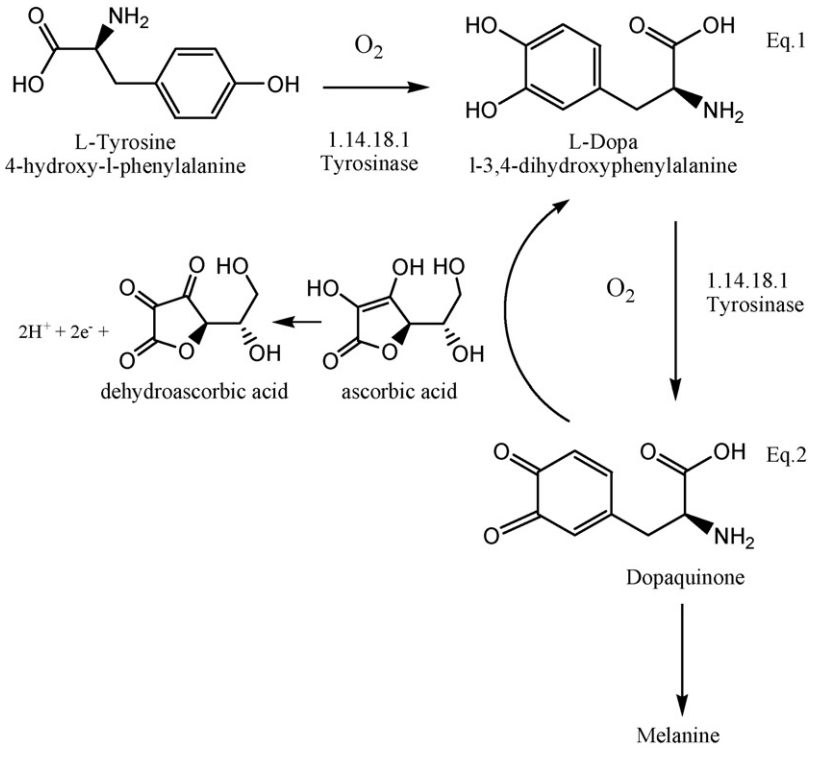
Potentioscan Wenking POS-73 potentiostat, Shimadzu UV-1601 spectrophotometer, Memmert D-91126 model water bath and JEOL JSM-6400 model scanning electron microscope (SEM) were used.

2.3. Synthesis of monomer

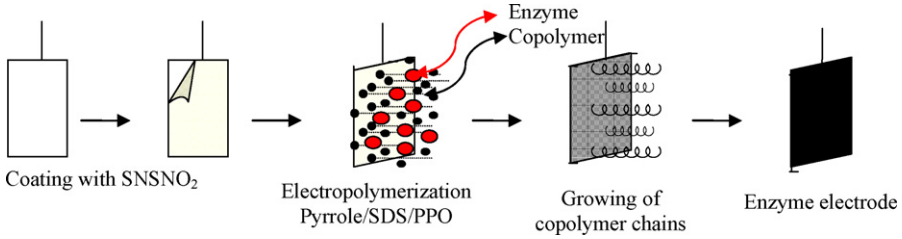
The structures of both the monomer and the soluble polymer were elucidated by Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) and Fourier Transform Infrared (FTIR). The average molecular weight has been determined by gel permeation chromatography (GPC) and the monomer was characterized in a previous study [25]. The monomer [SNS(NO_2)] was synthesized from 1,4-di(2-thienyl)-1,4-butanedione and 4-nitroaniline in the presence of *p*-toluene-sulphonic acid (PTSA). 1,4-Di(2-thienyl)-1,4-butanedione was obtained through the following pathway: To a suspension of $AlCl_3$ in CH_2Cl_2, a solution of 3-methylthiophene succinyl chloride in CH_2Cl_2 was added drop wise. The mixture was stirred at room temperature for 4 h, then poured into ice and concentrated HCl mixture. The dark green organic phase was washed with concentrated $NaHCO_3$ and brine, and then dried over $MgSO_4$. After evaporation of the solvent a blue green solid remained. Filtration and washing with ethanol yielded 1,4-bis(2-thienyl)butane-1,4-dione. A round-bottomed flask equipped with an argon inlet and magnetic stirrer was charged with 1,4-di(2-thienyl)-1,4-butanedione, 4-nitroaniline, PTSA and toluene. The



Scheme 3. Synthesis of [SNS(NO_2)].



Scheme 2. Reaction mechanism of PPO when the substrate is L-tyrosine.



Scheme 4. Schematic representation of enzyme immobilization procedure.

mixture was stirred and refluxed (110 °C) for 24 h under argon. [SNS(NO_2)] was obtained as pale brown powder after evaporation of the toluene, followed by flash column chromatography [12,26]. Schematic representation of the synthesis was shown in Scheme 3.

2.4. Immobilization of tyrosinase

Electrochemical polymerizations were achieved by constant potential electrolysis carried out in a three electrode cell consisted of a Pt working electrode coated with [SNS(NO_2)], a counter electrode, and a Ag wire as the pseudo reference electrode. For immobilization of tyrosinase in [SNS(NO_2)]/PPy biosensor, a solution of 0.1 mg/mL PPO, 2 mg/mL supporting electrolyte (SDS), 0.005 M pyrrole and 10 mL buffer were put in a typical three electrode cell. Immobilization was carried out at constant potential of 1.0 V for 20 min at room temperature. Tyrosinase was immobilized by entrapment during electropolymerization. Simple representation for immobilization is shown in Scheme 4.

2.5. Enzymatic assay

For catechol, immobilized enzyme activity determination was performed by Besthorn's hydrazone method [27]. In this method 3-methyl-2-benzothiozolinone (MBTH) interacts with the quinines produced by the enzyme to yield red products instead of brown colored pigments in the absence of color reagent [28]. Different concentrations of catechol solutions were prepared in citrate buffer (pH 6.5) and were put in water bath at 25 °C. 1.0 mL MBTH (0.3%) in ethanol was added to produce a red complex with the product. Enzyme electrode was immersed into the solution. After a specific reaction time 1 mL sample was drawn to achieve enzymatic assay.

For the determination of the enzymatic activity of [SNS(NO_2)]/PPy biosensor towards catechol, 1.0 mL sulfuric acid solution (5%, v/v) was added to stop the enzymatic reaction and 1.0 mL acetone was added to dissolve the colored complex. After mixing, absorbances were determined at 495 nm. For L-tyrosine, different concentrations of L-tyrosine and L-ascorbic acid solutions were prepared in phosphate buffer (pH 7). Electrodes were put into test tubes containing substrate solutions. After a specific reaction time 1 mL sample was drawn to achieve enzymatic assay. 1 mL HCl (2 M) was added to stop the reaction. 1 mL NaOH (2 M) was added to prevent bubbling and 1 mL 15% $NaMoO_4$ and $NaNO_2$ were added to have the yellow complex in specific reaction time intervals (5, 10 and 15 min). Since the formation of L-dopa complex is time dependent, L-dopa concentrations were determined by spectrochemical analysis at 460 nm exactly after 1 h. Enzyme electrodes were kept in phosphate buffer at 4 °C when not in use and daily prepared electrodes were used in all experimental steps. All experiments were done in constant temperature water bath while shaking.

Table 1
Properties of the soluble PPO and immobilized PPO on [SNS(NO_2)]/PPy.

	Substrate	V_{max} (μmol/min electrode)	K_m (mM)
Soluble PPO	Catechol	0.073	4
$SNSNO_2$/PPy/PPO	Catechol	0.02	250
Soluble PPO	L-Tyrosine	0.1	4
$SNSNO_2$/PPy/PPO	L-Tyrosine	0.02	2

2.6. Optimum pH and temperature experiments

The effect of pH was determined by changing reaction medium pH between 6.0 and 7.5 at a constant substrate concentration for both matrices. The effect of temperature was determined by changing the reaction medium temperature between 10 and 80 °C at a constant substrate concentration for both catechol and L-tyrosine. For all experiments the enzyme activity determination was performed as described above, and the relative enzyme activity was calculated by assigning the maximum value of activity as 100%.

2.7. Storage and operational stability experiments

The activity of immobilized enzyme on [SNS(NO_2)]/PPy biosensor after storage in citrate buffer at 4 °C were measured for 60 days with the experimental conditions given above.

Operational stability of the immobilized enzyme was studied by repetitive use of the same [SNS(NO_2)]/PPy electrode for 50 successive measurements in the same day. For all experiments, relative enzyme activity was calculated by assigning the maximum value of activity as 100%.

3. Results and discussions

3.1. Kinetic parameters for soluble and immobilized enzyme

When a biocatalyst is immobilized, maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) can undergo variations with respect to corresponding parameters of the soluble enzyme. These changes are results of several factors; such as protein conformational changes induced by the support, steric hindrances and diffusional effects. It is very important to know these variations since they reveal the relation between the substrate and the enzyme.

To determine kinetic parameters for [SNS(NO_2)]/PPy, enzymatic assay was applied for different concentrations of catechol and L-tyrosine solutions as mentioned in Section 2.5. V_{max} and K_m values were determined from Lineweaver–Burk plot at 25 °C pH 6.5 for catechol and pH 7 for L-tyrosine. In previous works kinetic parameters of soluble enzyme for both substrates were calculated [29,30]. The kinetic parameters of the free and immobilized enzymes are presented in Table 1. V_{max} of the immobilized enzyme was decreased fourfold upon immobilization for catechol whereas there was a fivefold decrease for L-tyrosine. K_m value for the catechol was higher than the soluble enzyme resulted from whenever the enzyme substrate comes together they gives products.

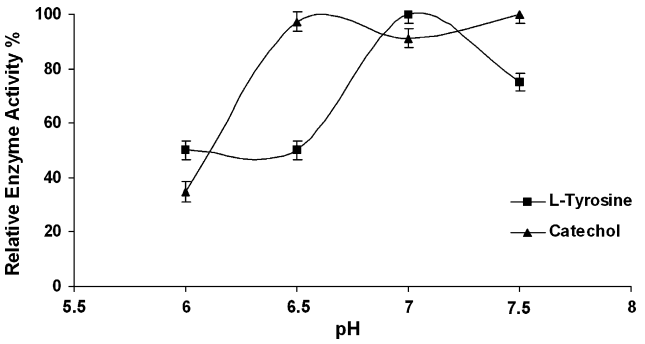


Fig. 1. Effect of pH on [SNS(NO_2)]/PPy biosensor.

3.2. Optimum pH

As shown in Fig. 1 the maximum activity was found at pH 6.5 for catechol. In previous works, the maximum activity was found at pH 5 for the soluble enzyme [29]. For L-tyrosine, both soluble (free) enzyme [30] and [SNS(NO_2)]/PPy biosensor have maximum activity at pH 7. For catechol at pH 6.5–7.5 [SNS(NO_2)]/PPy biosensor shows 90% activity, whereas for L-tyrosine maximum activity is achieved at pH 7. [SNS(NO_2)]/PPy biosensor can be used in wide pH range for catechol.

3.3. Optimum temperature

The effect of temperature on relative enzyme activity was examined between 10 and 80 °C as illustrated in Fig. 2. For both substrates, [SNS(NO_2)]/PPy biosensor has maximum activity at 60 °C. Biosensor shows higher activity towards catechol compare to L-tyrosine at lower temperatures. For the soluble enzyme, the maximum enzyme activities were seen at 30 and 40 °C for L-tyrosine and catechol respectively [29,30]. Enzyme stability toward temperature was achieved upon immobilization. Immobilized enzyme possesses a better heat resistance than free enzyme since immobilization procedure protects the enzyme active conformation from damage by heat exchange.

3.4. Storage and operational stabilities

For the storage stability measurements of the [SNS(NO_2)]/PPy biosensor, the activity of the enzyme electrode was checked for catechol and L-tyrosine every 5 days for 60 days (Fig. 3). At the end of the 35th day the biosensor activity was lost for catechol and the same happened at the end of the 60th day for L-tyrosine. The half-lives were calculated as 25 and 13 days for L-tyrosine and catechol respectively. Operational stability of the [SNS(NO_2)]/PPy biosensor for both substrates were also determined with 20 repetitive experiments. At the end of the 20th experiment for catechol, biosensor has retained 85% of its original activity. This is found to be 40% for L-tyrosine. The half-life value was determined as 14 assays for L-tyrosine. The operational stabilities of the enzyme electrodes were given in Fig. 4.

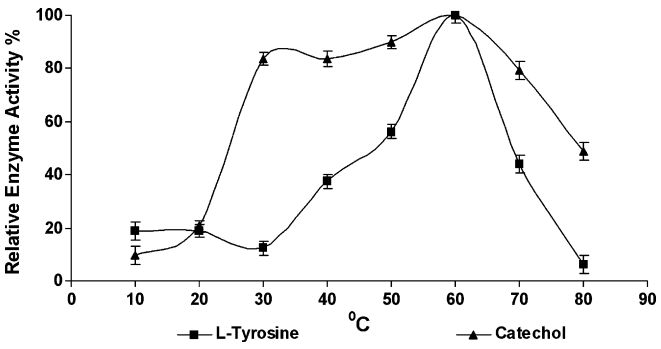


Fig. 2. Effect of temperature on [SNS(NO_2)]/PPy biosensor.

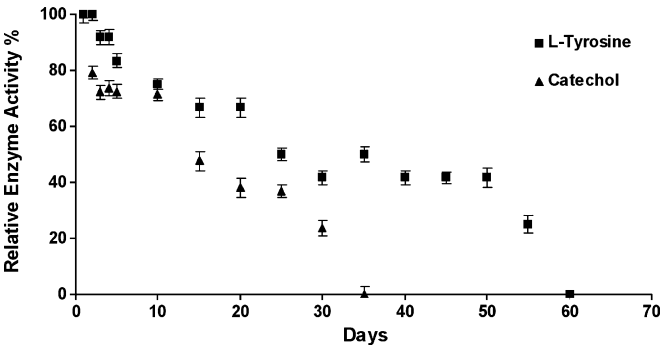


Fig. 3. Storage stabilities of [SNS(NO_2)]/PPy biosensor.

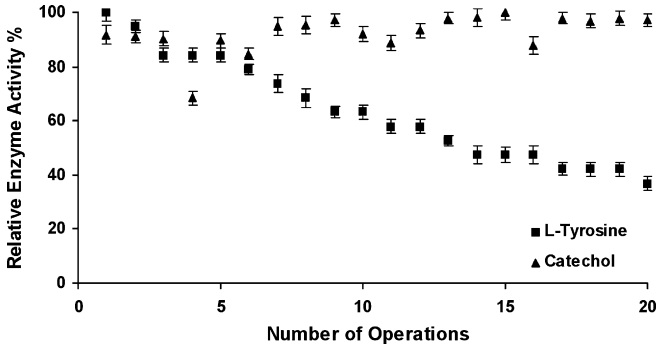


Fig. 4. Operational stabilities of [SNS(NO_2)]/PPy biosensor.

3.5. Analytical characteristics

The analytical characteristics of the [SNS(NO_2)]/PPy biosensor was examined using catechol in citrate buffer pH 6.5 and L-tyrosine in potassium phosphate buffer (pH 7) at 25 °C. Calibration curves were plotted for enzyme activities versus substrate concentrations. A linear calibration graph was obtained for enzyme activity versus substrate concentration between 0.05 and 0.5 M. A linear relation was defined by the equation of $y = 0.0388x + 0.001$ ($R^2 = 0.9387$) for catechol. For L-tyrosine a calibration graph was obtained for enzyme activity versus substrate concentration between 0.08 and 2.5 mM. A linear relation was defined by the equation of $y = 2.4904x + 0.0039$ ($R^2 = 0.9944$) for L-tyrosine. At higher concentrations enzyme activity remained constant reaching to saturation for both systems.

4. Conclusion

In this study, immobilization of polyphenol oxidase was carried out successfully on a copolymer of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole with pyrrole via electrochemical polymerization. The [SNS(NO_2)]/PPy biosensor was tested and characterized for two substrates: catechol and L-tyrosine. The kinetic parameters, of the biosensor were determined for the substrates. Analytical characterization was achieved by calibration curves. Optimization of the [SNS(NO_2)]/PPy biosensor was achieved by determining the optimum pH, temperature values. Storage and operational stabilities were also examined.

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References

[1] L.B. Groenendaal, G. Zotti, P.H. Aubert, S.M. Waybright, J.R. Reynolds, Adv. Mater. 11 (2003) 855–864.
[2] M. Gerard, A. Chaubey, B.D. Malhotra, Biosens. Bioelectron. 17 (2002) 345–359.
[3] M. Trojanowicz, T.K. vel Krawczyk, Mikrochim. Acta 121 (1995) 167–181.
[4] M. Situmorang, J.J. Gooding, D.B. Hibbert, D. Barnett, Biosens. Bioelectron. 13 (1998) 953–962.
[5] W. Schuhmann, C. Lehn, H.L. Schmidt, B. Grundig, Sens. Actuators B 7 (1992) 393–398.
[6] G. Gustafsson, Y. Cao, G.M. Treacy, F. Klavetter, N. Colaneri, A.J. Heeger, Nature 357 (1992) 477.
[7] K. Gurunathan, D.P. Amalnerkar, D.C. Trivedi, Mater. Lett. 57 (2003) 1642–1648.
[8] A. Watt, D. Blake, J.H. Warner, E.A. Thomsen, E.L. Tavenner, H. Rubinsztein-Dunlop, J. Paul Meredith, Appl. Phys. 38 (2005) 2006.
[9] J.C. Gustafsson, O. Inganas, A.M. Anderson, Synth. Met. 62 (1994) 17.
[10] S. Tuncagil, S. Kıralp, S. Varıs, L. Toppare, React. Funct. Polym. 68 (2008) 710–717.
[11] Y. Coskun, A. Cirpan, L. Toppare, Polymer 45 (2004) 4989–4995.
[12] U. Lange, N.V. Roznyatovskaya, V.M. Mirsky, Anal. Chim. Acta 614 (2008) 1–26.
[13] A.P.F. Turner, I. Karube, G.S. Wilson, Biosensors: Fundamentals and Applications, Oxford University Press, New York, 1987.
[14] J.M. Kauffmann, G.G. Guilbault, Bioanalytical Applications of Enzymes, vol. 36, Wiley, 1992.
[15] P.N. Bartlett, J.M. Cooper, J. Electroanal. Chem. 363 (1993) 1–12.
[16] S. Cosnier, Biosens. Bioelectron. 14 (1999) 443–456.
[17] K. Lerch, ACS Symp. Ser. 600 (1995) 64–80.
[18] A.M. Mayer, E. Harel, Phytochemistry 18 (1978) 193–215.
[19] H. Decker, R. Dillinger, F. Tuczek, Angew. Chem. Int. Ed. 39 (2000) 1587–1591.
[20] L. Cerenius, K. Soderhall, Immunol. Rev. 198 (2004) 116–126.
[21] H. Claus, H. Decker, Syst. Appl. Microbiol. 29 (2006) 3–14.
[22] A. Arslan, S. Kiralp, L. Toppare, Y. Yagci, Int. J. Biol. Macromol. 35 (2005) 163.
[23] H.B. Yildiz, L. Toppare, Y.H. Gursel, Y. Yagci, Enzyme Microb. Technol. 39 (2006) 945–948.
[24] I. Narlı, S. Kiralp, L. Toppare, Anal. Chim. Acta 572 (2006) 25–31.
[25] S. Varis, M. Ak, I.M. Akhmedov, C. Tanyeli, L. Toppare, J. Electroanal. Chem. 603 (2007) 8–14.
[26] S. Varis, M. Ak, C. Tanyeli, I.M. Akhemedov, L. Toppare, Solid State Sci. 8 (2006) 1477–1483.
[27] F. Mazzocco, P.G. Pifferi, Anal. Biochem. 72 (1996) 643–647.
[28] F. Ortega, E. Dominguez, G. Jonsson-Pettersson, L. Gorton, J. Biotechnol. 31 (1993) 289–300.
[29] A.E. Böyükbayram, S. Kıralp, L. Toppare, Y. Yağcı, Bioelectrochemistry 69 (2006) 164–171.
[30] S. Tuncagil, S.K. Kayahan, G. Bayramoglu, M.Y. Arica, L. Toppare, J Mol. Catal. B: Enzymatic 58 (2009) 187–193.