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ETHANOL BIOSENSOR BASED ON IMMOBILIZATION OF ALCOHOL OXIDASE IN A CONDUCTING POLYMER MATRIX *VIA* CROSSLINKING WITH GLUTARALDEHYDE

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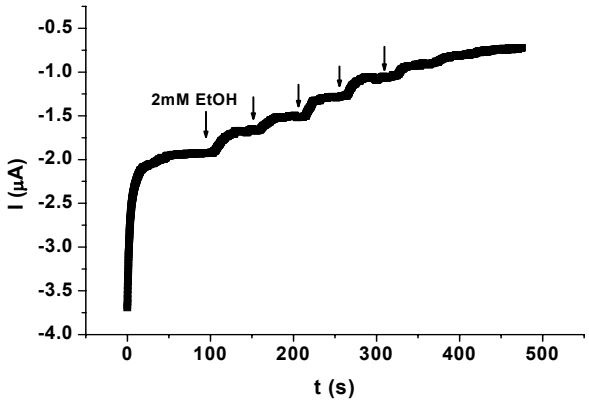
An amperometric biosensor was designed using immobilizing alcohol oxidase in conducting polymer of poly 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine matrix via crosslinking with glutaraldehyde on platinum electrode. An electrochemical technique was used for the polymerization of conducting polymer onto the electrode. Carbon nanotubes and gold nanoparticles were also added to the biosensing system to observe the effects of nanoparticles. The proposed biosensor was characterized and optimized in terms of conducting polymer thickness, enzyme loading, pH, nanoparticle effects, linear range, repeatability and operational stability. The proposed biosensor showed good linear range, and repeatability as well as high operational stability. The proposed biosensor optimized with the conditions of potassium phosphate buffer (50 mM, pH 7), 20 cycles of $SNSNH_2$, 1.5 µL AOx (1.56U), 1% glutaraldehyde and –0.7 V applied potential. Linear analytical range was obtained between 0.1-5 mM ethanol and after 6 hours with 25 measurements $pSNSNH_2$-AOx biosensor lost only 7% of its activity. The biosensing system was successfully applied to real samples vodka and whisky with good recovery.



INTRODUCTION

Analytical devices for analyzing, detection, quantification or monitoring of specific chemical species have gained a great deal of importance which resulted in a very hot topic named biosensors.[1-3] Enzymes, DNA, tissues, bacteria, yeast, antibodies, antigens, liposomes, and organelles are generated in the biorecognition part of the biosensors.[4] Most of the biological molecules have very short life times in solution phase; therefore, they have to be fixed in a suitable matrix.[5] The success of the biosensor, the activity of immobilized molecules depends on surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and especially on the immobilization method.[5-7]

The analytical power of electrochemical techniques and the specificity, selectivity of

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biological recognition part collaborate in electrochemical biosensors. Therefore, they provide fast, simple, and low-cost detection for biological events. Amperometric detection has proven to be very useful for quantification due to their good selectivity, sensitivity, rapid response, miniature size, and reproducible results.[8-11]

Enzyme electrodes have been shown to be extremely useful for monitoring a wide variety of substrates of analytical importance in clinical, environmental, and food samples.[4, 12, 13] Hence, enzymes combine the recognition and amplification steps, as needed, for many sensing applications.[13, 14]

Alcohol oxidase (AOx; Alcohol:O_2 oxidoreductase, EC 1.1.3.13) is a homooctameric flavoprotein and oligomeric enzyme consisting of eight identical sub-units, each containing a strongly bound cofactor, flavin adenine dinucleotide molecule.[15-17] Alcohol oxidase catalyzes the oxidation of low molecular weight alcohols to their corresponding aldehydes, using molecular oxygen as the electron acceptor.[18-20] Biosensors based on alcohol oxidase are easy prepared since alcohol oxidase uses only molecular oxygen (O_2) as the cofactor. O_2 is involved in the reaction of oxidation of ethanol to acetaldehyde and hydrogen peroxide. Therefore, the catalytic reaction can be easily followed amperometrically.[21, 22]

$$RCH_2OH + O_2 + AOx \rightarrow RCHO + H_2O_2$$

Recently, studies on conducting polymers (CPs) appeared as a new field of research and development in designing biosensors.[23,24] The advantage of using conducting polymers is that a soluble polymer enables a nondestructive analysis of the sample and has the ability to transfer electric charge produced by the biochemical reaction hence, they serve as the immobilizing matrices for biomolecules and provide a suitable environment for immobilization.[25]

Due to their large specific surface area and high surface free energy, nanoparticles can adsorb biomolecules strongly. Importantly, the adsorption of such biomolecules onto the surfaces of nanoparticles can retain their bioactivity due to the biocompatibility of nanoparticles.[26,27] Nanoparticles are generally used to promote the electron transfer in redox proteins due to their electronic and structure properties. Gold nanoparticles (AuNPs) have the advantage in preparing biosensors since they provide a stable immobilization platform where biomolecules retain their bioactivity. AuNPs allow direct electron transfer between electrode and redox proteins thus; there is no need for electron transfer mediators in designing biosensors.[26-28] Carbon nanotubes (CNTs) have the simple chemical composition and atomic bonding pattern, and have diversity in structure and properties. These unique properties make CNTs extremely attractive for electrochemical biosensors.[29-32]

In this work, alcohol oxidase was immobilized by crosslinking method in conducting polymer of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine ($pSNSNH_2$) matrix on platinum electrode ($pSNSNH_2$-AOx). AuNPs effect on CP modified biosensor ($pSNSNH_2$-AuNPs-AOx) and CNTs effect on CP modified biosensor ($pSNSNH_2$-CNTs-AOx) were also used to realize nanoparticle effect on ethanol biosensing. Schematic representation of biosensor preparation was shown in Scheme 1.

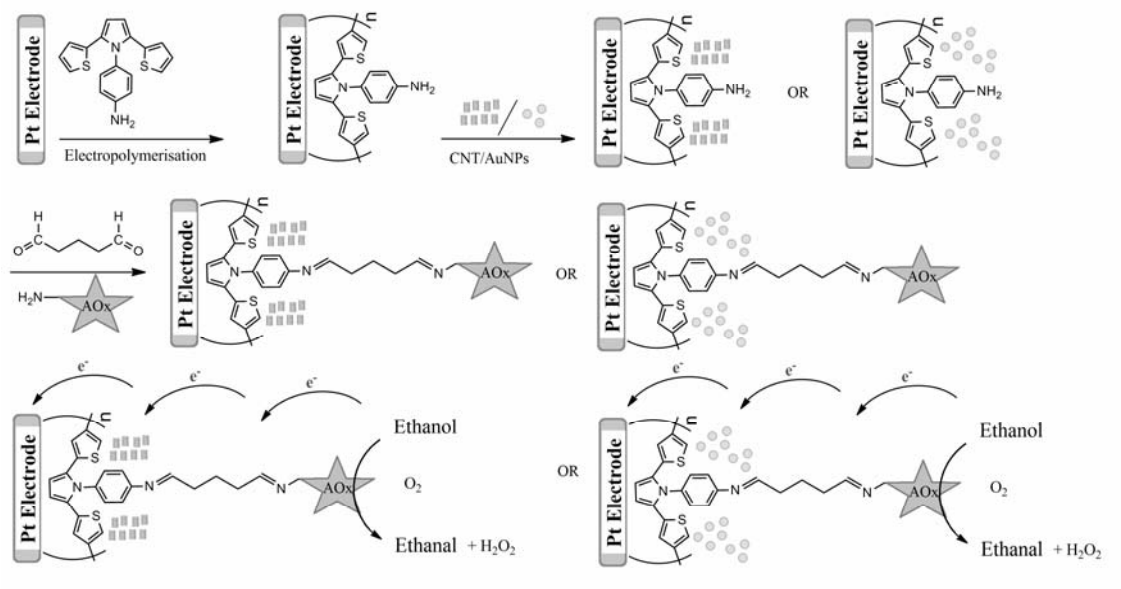
RESULTS AND DISCUSSION

pH optimization

pH of the environment is important for the enzymes due their fragile nature; they can easily be affected from the pH changes. For the $pSNSNH_2$-AOx biosensor the maximum current response towards ethanol was obtained at pH 7 phosphate buffer as shown in Fig. 1. For further experiments, pH 7 phosphate buffer was used as the optimum pH.

Effect of polymer thickness on p($SNSNH_2$)-AOx biosensor

Since conducting polymers offer different chemical structures and functional groups, they can be modified according to the need for immobilizing biological material. For that matter, cross-linking method can be utilized using some functional groups like -NH_2, -COOH in order to bind protein molecules directly to the conducting polymers. This method can improve rapidness, sensitivity, and adaptability of biosensors in analytical sense. The number of voltammetric cycles can control the film thickness, and films formed with a very high number of cycles are very thick and have a passive electrode. After 15, 20, and 50 scan of $SNSNH_2$ electropolymerization, the biosensor response was checked for 2mM ethanol. Scan numbers and the corresponding biosensor responses were stated in Fig. 2. When the platinum electrode surface was coated with 20 and 50 scan $SNSNH_2$, nearly same responses were obtained ($\sim$ 0.50 μA). In order to design easily prepared biosensor 20 scan was chosen as optimum. Therefore, this number of scan was used in further experiments.



Scheme 1 – Schematic representation of pSNSNH$_2$-AOx, pSNSNH$_2$-CNTs-AOx and pSNSNH$_2$-AuNPs-AOx biosensors.

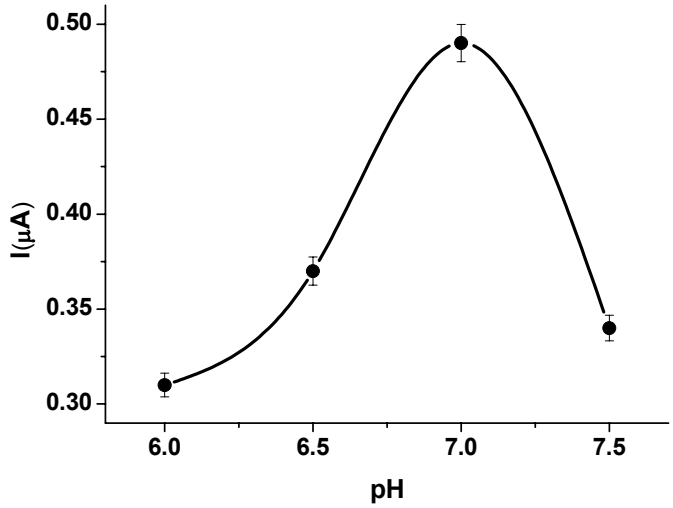


Fig. 1 – Effect of pH on pSNSNH$_2$-AOx biosensor.

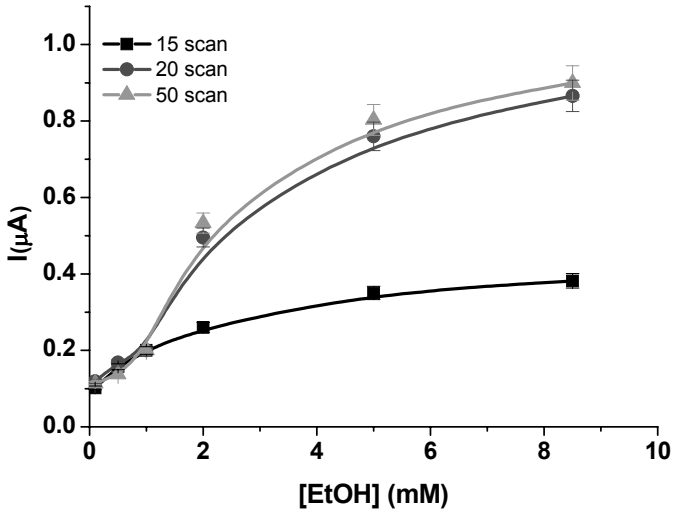


Fig. 2 – Effect of polymer thickness on pSNSNH$_2$-AOX biosensor.

Effect of enzyme loading on biosensing

Different amount of alcohol oxidase (0.5-2μL) were mixed with 1% glutaraldehyde, spread over the $pSNSNH_2$ coated platinum electrode and allowed to dry 1 hour. Each enzyme electrodes were tested for 1mM ethanol. 1.5 μL (1.56 U, 0.055 mg protein) was found as optimum enzyme amount for ethanol biosensing. For further experiments this amount was used for immobilizing alcohol oxidase (Fig. 3).

Analytical characterization of the biosensor

Analytical characterization of the biosensor was performed under optimized conditions; pH 7 phosphate buffer, 20 scans $SNSNH_2$, 1.5 μL alcohol oxidase (1.56 U, 0.055 mg protein). Dynamic ranges and the equations were obtained to characterize the proposed $pSNSNH_2$-AOx biosensor analytically at the optimized electrode configuration and following conditions; potassium phosphate buffer (50 mM, pH 7), 20 cycle $SNSNH_2$, 1.5 μL AOx (1.56U), %1 glutaraldehyde, –0.7 V applied potential. Linear analytical range was obtained between 0.1-5 mM ethanol with an equation; y=0.1415x+0.1353 (r^2= 0.999). Repeatability of the $pSNSNH_2$-AOx biosensor was tested for 2 mM alcohol (n= 4). The standard deviation and coefficient of variation were calculated as 0.015 mM and 3.1 % respectively. Stability of $pSNSNH_2$-AOx biosensor was tested for 6 hours and 25 measurements were carried out in the presence of 2 mM ethanol at operational conditions. $pSNSNH_2$-AOx biosensor lost only 7 % of its activity. Moreover at optimized conditions 2mM ethanol was added to system continuously. Upon addition of ethanol, increase in current can be clearly seen from Fig. 4.

Modification of the $pSNSNH_2$-AOx biosensor with AuNPs and CNTs

After deposition of 20 scan $SNSNH_2$ by cyclic voltammetry on platinum electrode with 0.5-2μL, gold nanoparticles were added onto the electrode. Biosensing response was checked against 1 mM and 2mM ethanol. Maximum biosensing response was obtained when 1μL (10nm, 0.01%Au) was added to the electrode (Fig. 5A). Ethanol biosensing responses were checked for 1 and 2mM ethanol for CNT modified electrodes with different amounts of CNT (0.5 μL, 1 μL, 1.5 μL, and 2μL). Maximum biosensing response was obtained when 1.5 CNT was added to biosensor (Fig. 5B).

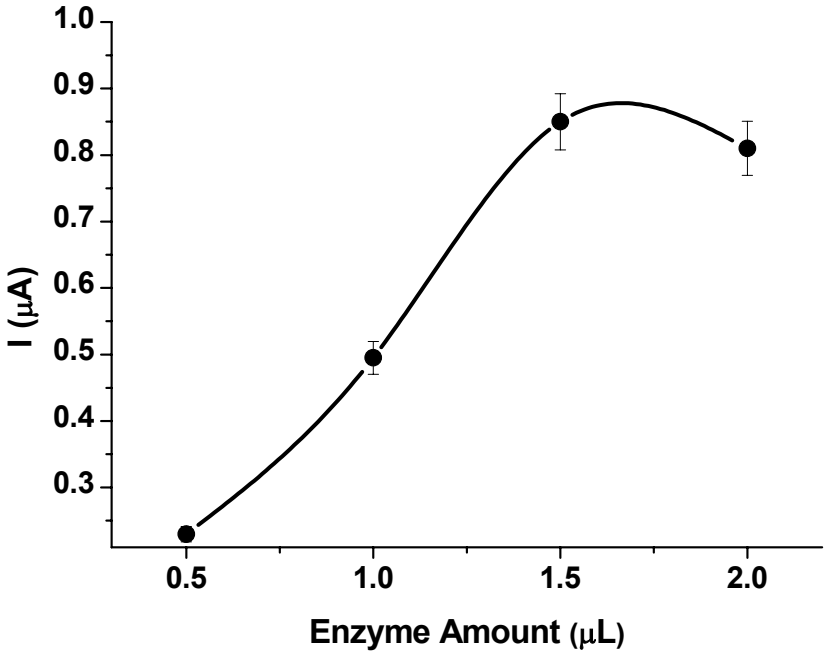


Fig. 3 – Effect of enzyme amount on $pSNSNH_2$-AOX biosensor.

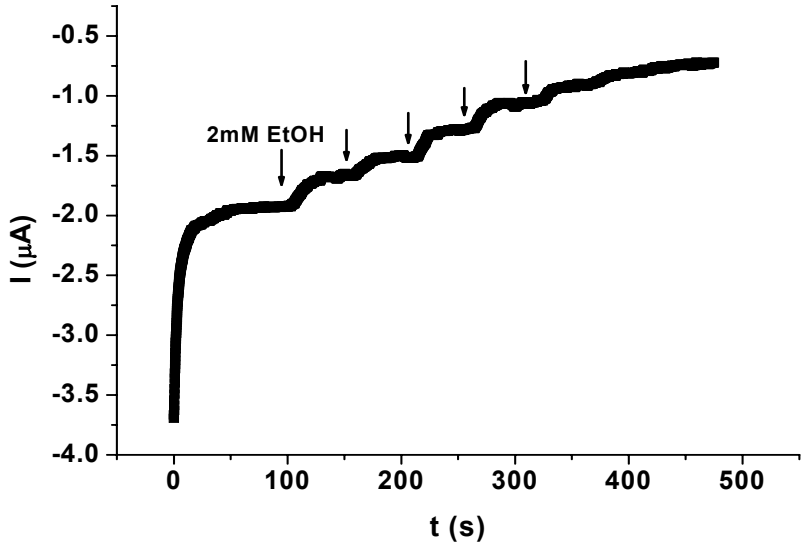


Fig. 4 – Continuous addition of 2mM Ethanol to pSNSNH$_2$-AOx biosensing system.

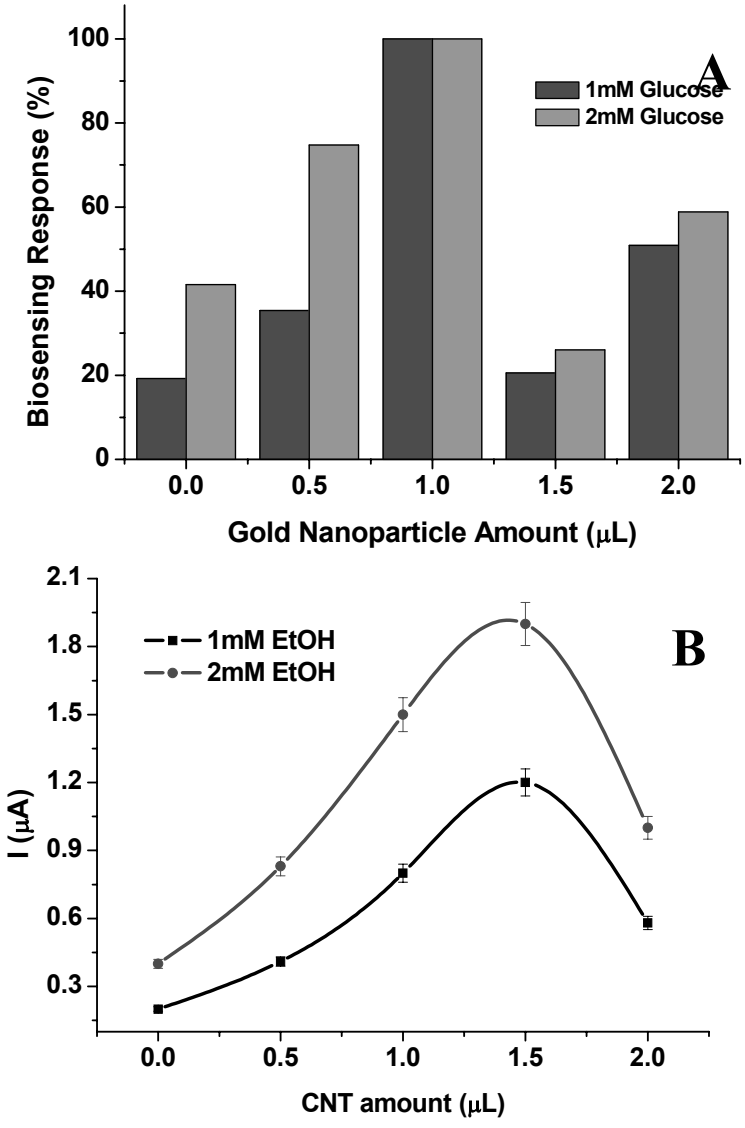


Fig. 5 – A) Gold Nanoparticle Effect on pSNSNH$_2$-AOx 1mM ve 2mM ethanol biosensing,
B) Carbon nanotube effect on pSNSNH$_2$-AOx ethanol biosensor for 1mM ve 2mM ethanol biosensing.

Ethanol analysis in commercial samples

The pSNSNH2-AOx biosensor was used for ethanol analysis in vodka and whisky. The data were compared with label values. The comparison of the results obtained from both systems was summarized in Table 1. Clearly, with the proposed biosensor very similar results obtained with label values.

Table 1

Analysis of ethanol in real samples *via* $pSNSNH_2$-AOx biosensor

	$pSNSNH_2$-AOx	**Label Value**
Vodka (%)	39.4 ± 2.69	40
Viski (%)	41.3 ± 1.80	40

EXPERIMENTAL

Reagents

Alcohol oxidase, (AOx, Pichia pastoris, 28 Unit/ mg protein, 37mg protein/ mL) was purchased from Sigma. $LiClO_4$, $NaClO_4$, $AlCl_3$, succinyl chloride, benzene-1,4-diamine propionic acid, nitromethane, iron(III) chloride, propylene carbonate, poly(methylmethacrylate), dichloromethane, toluene, ethanol were purchased from Sigma. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany, www.merck.com). All other chemicals were of analytical grade and purchased either from Merck or from Sigma. Cetyltrimethylammonium bromide (CTAB), Multiwall carbon nanotubes (diameter; 110 –170 nm, length; 5-9 μm) and gold nanoparticles (0.01% Au, 10 nm) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without any pre-treatment.

Instrumentation

Chronoamperometry measurements were carried out with a PalmSens Instruments (PalmSens, Houten, The Netherlands). A platinum electrode (0.16 cm^2, Metrohm, Switzerland) was used as a working electrode, an auxiliary electrode and an Ag/AgCl (3 M KCl saturated with AgCl as an internal solution, Radiometer Analytical) as the reference electrode.

Synthesis of $SNSNH_2$ and $p(SNSNH_2)$

For the synthesis of the monomer; 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine ($SNSNH_2$), firstly 1,4-di(2-thienyl)-1,4-butanedione was obtained via double Friedel–Crafts reaction in the presence of $AlCl_3$ and CH_2Cl_2 at 15 ºC. In the presence of catalytic amount of propionic acid, toluene 1,4-di(2-thienyl)-1,4-butanedione and benzene-1,4-diamine was refluxed for 4 h (yield 78%) in a round-bottomed flask with an argon inlet and magnetic stirrer. Resultant mixture was stirred and refluxed for 24 h under argon. After evaporation of the toluene, the desired compound as a pale yellow powder was obtained by flash column chromatography (SiO_2 column, elution with dichloromethane).[33]

$p(SNSNH_2)$ was obtained on the platinum electrode ($0.16mm^2$) surface through running 20 cycles by cyclic voltammetry. The polymerization was achieved in acetonitrile solution containing 5 mg/mL $SNSNH_2$ monomer, 0.1 M $NaClO_4$ and 0.1 M $LiClO_4$ at a scan rate of $0.5Vs^{-1}$.

Preparation of immobilized alcohol oxidase biosensor ($pSNSNH_2$-AOx)

For the immobilization of alcohol oxidase, proper amounts of AOx and 1% glutaraldehyde in 10 μL in potassium phosphate buffer solution (50mM, pH 7.0) were spread over the surface of platinum electrode which was covered with $pSNSNH_2$ and allowed to dry at ambient conditions for nearly 1 hour. Daily prepared electrodes were used in all experimental steps.

Effect of Gold Nanoparticle and Carbon Nanotubes on ethanol biosensing

For the preparation of gold nanoparticle modified ethanol biosensors, 10nm gold nanoparticle colloidal solution (0.01% Au) was used. After electropolymerization of $SNSNH_2$, different electrodes including 0.5-2μL AuNP with 1.5μL AOx and 1μL glutaraldehyde were prepared and ethanol biosensing responses were checked for 1 and 2mM ethanol.

To realize carbon nanotubes effect on ethanol biosensing, 1.0 mg CNT was dissolved in 1.0 mL CTAB. The use of CTAB offers a quick and effective method to disperse carbon nanotubes.[34] After electropolymerization of $SNSNH_2$, CNT was spread over the electrode with different amounts of 0.5-2μL. After adding glutaraldehyde and alcohol oxidase on the surface of electrodes, electrodes allowed drying for 1 hour. Ethanol biosensing responses were checked for 1 and 2mM ethanol.

Amperometric response measurements

All experiments were conducted at optimized conditions in an electrochemical cell with three electrode configuration, containing 10 mL buffer using magnetic stirring with 210 rpm. After each measurement the electrode was washed with distilled water and kept in buffer. The electrode was initially equilibrated in buffer, and then the substrate was added to the electrochemical cell. The biosensor responses were registered as current (μA) by following the oxygen consumption at -0.7 V due to biological activity of the immobilized material. After every amperometric response measurements, the enzyme or microbial electrode was washed with distilled water and the buffer of the electrochemical cell was refreshed.

pH optimization of the biosensors

Biosensing responses were checked in the pH of the potassium phosphate (pH 6.0-7.5, 50 mM) to see the effect of pH on the biosensor response. The current density was adjusted as 100 % to the maximum response pH, and other values were calculated relative to this value.

Effect of AOx amount for biosensors

Biosensors containing different amount of AOx were prepared and their responses were checked to determine the

appropriate AOx amount. Different amount of alcohol oxidase (0.5- 2μL) were mixed with 1% glutaraldehyde, spread over the $pSNSNH_2$ coated platinum electrode and allowed to dry 1 hour. Each enzyme electrodes ($pSNSNH_2$-AOx) were tested for 1mM ethanol.

Effect of electropolymerization time

Effect of electropolymerization time of the polymer, which is directly correlated with the thickness of the polymer on the graphite electrode, was determined by preparing electrodes with 5, 10, 15, and 20 min of electropolymerization (referring to 50, 100, 150 and 200 scan numbers). Charges related with the scan number were also calculated.

Analytical characterization of biosensors

The analytical characteristics of the biosensors in terms of linear dynamic ranges and the equations were examined under optimized conditions. Calibration curves were plotted for current versus substrate concentration (where y is the sensor response in terms of current μA and x is the substrate concentration in mM). Repeatability of the biosensors was estimated by repetitive measurements with their substrates. Furthermore, the standard deviation and coefficient of the variation were calculated.

CONCLUSIONS

Conducting polymers have concerned much attention due to providing suitable matrices for biological materials. Numerous numbers of papers on the advantages of using CPs for novel catalytic surfaces have been published. The use of conducting polymers with particular properties with the immobilized biological systems enables to develop novel biomicroelectronic devices. In future, biosensors based on CPs would be gradually more miniaturized due to the flexibility of electrodeposition with in micro or nano order. Moreover, it can be possible to obtain microbial or enzymatic sensors in required scope with the appropriate immobilization method.

In this work, biosensor design was achieved with immobilizing alcohol oxidase on platinum electrode via crosslinking with glutaraldehyde. Covalent binding of the redox enzyme onto the CNT modified polymeric material yielded a nanobiocomposite structure. The proposed biosensor was characterized and optimized in terms of thickness, enzyme loading, pH, AuNPs, CNTs, linear range, repeatability and operational stability. The biosensor optimized analytically with the conditions of potassium phosphate buffer (50 mM, pH 7), 20 cycle $SNSNH_2$, 1.5 μL AOx (1.56U), %1 glutaraldehyde, –0.7 V applied potential. Linear analytical range was obtained between 0.1-5 mM ethanol and after 6 hours with 25 measurements $pSNSNH_2$-AOx biosensor lost only 7% of its activity. Addition of CNT dispersion and AuNP on the electropolymerized $SNSNH_2$ provided attractive matrix properties with unique and versatile properties for the biomolecule immobilization. Proposed system was used as the appropriate microenvironment to efficiently immobilize the biomolecule on the surface of CP containing functional NH_2 groups. It can be concluded that the combination of CP and AuNP cause the immobilized enzyme to have higher bioactivity which results fast, stable and sensitive responses to the substrate.

REFERENCES

1. M. Gerard, A. Chaubey and B.D. Malhotra, *Biosen. Bioelectron.*, **2002**, *17*, 345-359.
2. S. B. Adeloju and G. G. Wallace, *Analyst*, **1996**, *121*, 699-703.
3. D. R. Thevenot, K. Toth, R. A. Durst and G. S. Wilson, *Biosens. Bioelectron.*, **2001**, *16*, 121-131.
4. P. N. Bartlett and J. M. Cooper, *J. Electroanal. Chem.*, **1993**, *362*, 1-12.
5. S. Tuncagil, D. Odaci, E. Yildiz, S. Timur and L. Toppare, *Sensor. Actuat. B-Chem.*, **2009**, *137*, 42-47.
6. S. Tuncagil, S. Varis and L. Toppare, *J. Mol. Catal. B- Enzym.*, **2010**, *64*, 195-199.
7. J. F. Kennedy and J. M. Cabral, "Enzyme immobilization", H. J. Rehm, G. Reed and J. F. Kennedy (Eds.), Vol. in "Enzyme Technology, Biotechnology", VCH Verlagsgesellschaft Gmbtt, Weinheim, Germany, 7A, 1987, p. 347.
8. S. Tanrıverdi, S. Tuncagil and L. Toppare, *J. Macromol. Sci. A* **2012**, *49*, 185-190.
9. S. Cosnier, *Biosens. Bioelectron.*, **1999**, *14*, 443-456.
10. J. Wang, *J. Pharm. Biomed. Anal.*, **1999**, *19*, 47-53.
11. E. Bakker and M. Telting-Diaz, *Anal. Chem.*, **2002**, *74*, 2781-2800.
12. Alaa M. T. Al Laylaa, Ö. Türkarslan, S. Kurbanoglu, S. T. Sulaiman, K. A. Al-Flayeh and L. Toppare, *J. Macromol. Sci. A*, **2013**, *50*, 914-922.
13. M. Trojanowicz, *Microchim. Acta*, **2003**, *43*, 75-91.
14. H. B. Yildiz, M. Kamaci, H. Azak, O. Secgin and O. Suer, *J. Mol. Catal. B- Enzym.*, **2013**, *91*, 52-58.
15. J. Mohns and W. Kuennecke, *Deut. Lebensm-Rundsch*, **1996**, *92*, 1-4.
16. N. G. Patel, S. Meier, K. Cammann and G. C. Chemnitius, *Sensor. Actuat. B-Chem.*, **2001**, *75*, 101-110.
17. J. Vonck and E. F. van Bruggen, *Biochim. Biophys. Acta*, **1990**, *1038*, 74-79.
18. A. M. Azevedo, D. M. F. Prazeres, J. M. S. Cabral and L. P. Fonseca, *Biosens. Bioelectron.*, **2005**, *21*, 235-247.
19. M. M. Barsan and C. M. A. Brett, *Talanta*, **2008**, *74*, 1505-1510.
20. F. Tagliaro, D. De-Leo, M. Marigo, R. Dorizzi and G. Schiavon, *Biomed. Chromatogr.*, **1990**, *4*, 224-228.

21. H. B. Yildiz and L. Toppare, *Biosens. Bioelectron.*, **2006**, *21*, 2306-2310.
22. A. G. Prada, N. Peña, M.L. Mena, A.J. Reviejo and J.M. Pingarron, *Biosens. Bioelectron*., **2003**, *18*, 1279-1288.
23. P. N. Bartlett and P.R. Birkin, *Synthetic Met.*, **1993**, *61*, 15-21.
24. W. Schuhmann, *Mikrochim. Acta.*, **1995**, *121*, l-29.
25. G. G. Wallace, M Smyth and H Zhao, *Trend. Anal. Chem.*, **1999**, *18*, 245-251.
26. X. Luo, A. Morrin, A. J. Killard and M.R. Smyth, *Electroanal.*, **2006**, *18*, 319-326.
27. A. Gole, C. Dash, V. Ramakrishnan, S. R. Sainkar, A. B. Mandale, M. Rao and M. Sastry, *Langmuir*, **2001**, *17*, 1674-1679.
28. M. C. Daniel and D. Astruc, *Chem. Rev.*, **2004**, *104*, 293-346.
29. J. Wang, *Electroanal.*, **2005**, *17*, 7-14.
30. J. J. Gooding, *Electrochim. Acta*, **2005**, *50*, 3049-3060.
31. M. Trojanowicz, *Trend. Anal. Chem.*, **2006**, *25*, 480-489.
32. G. A. Rivas, M. D. Rubianes, M. C. Rodrıguez, N. F. Ferreyra, G. L. Luque, M. L. Pedano, S. A. Miscoria and C. Parrado, *Talanta*, **2007**, *74*, 291-307.
33. E. Yildiz, P. Camurlu, C. Tanyeli, I.M. Akhmedov and L. Toppare, *J. Electroanal. Chem.*, **2008**, *612*, 247-256.
34. S. Wang, F. Xie and G. Liu, *Talanta*, **2009**, *77*, 1343-1350.