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Preparation and optimization of a bienzymic biosensor based on self-assembled monolayer modified gold electrode for alcohol and glucose detection

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ABSTRACT

The aim of this project was to develop a bienzymic biosensor, which was based on co-immobilization of alcohol oxidase and glucose oxidase on the same electrode by formation of self-assembled monolayer (SAM) for selective determination of ethanol and glucose.

In the biosensor construction the enzymes and the mediator, tetrathiafulvalene (TTF), were immobilized with cross-linking agents glutaraldehyde and cysteamine by forming a self-assembled monolayer (SAM) on a gold disc electrode. Amounts of ethanol and glucose were amperometrically detected by monitoring current values at reduction potential of TTF^+, 0.1 V. Decreases in biosensor responses were linearly related to glucose concentrations between 0.1 and 1.0 mM and ethanol concentrations between 1.0 and 10 mM. Limits of detection of the biosensor for ethanol and glucose were calculated to be 0.75 and 0.03 mM, respectively. In the optimization studies of the biosensor some parameters such as optimum pH, optimum temperature, enzyme amount, effect of TTF concentration and duration of SAM formation were investigated.

1. Introduction

The quantitative measurement of alcohol and glucose is very important for clinical and forensic purposes in the analyses of human breath and blood. The food and beverage industries need their analysis especially to control the fermentation process and product quality (Patel et al., 2001).

Some analytical methods have been developed during the years for the determination of ethanol and glucose. Enzymatic methods (Gonchar et al., 2001; Atwater et al., 1997), colorimetry (Zanon et al., 2007), as well as advanced techniques such as high performance liquid chromatography (Tagliaro et al., 1991; Lefebvre et al., 2002) and gas chromatography (Buckee and Mundy, 1993; McLachlan et al., 1999) are widely used for determination of ethanol.

For quantitative determination of glucose some methods such as spectrophotometry (Edmiston and Williams, 2000; Vasilarou and Georgiou, 2000; Harms et al., 1999), chemiluminescent (Panoutsou and Economou, 2005; Economou et al., 2006), and HPLC (Coquet et al., 1994) have been reported.

Self-assembled monolayers (SAMs) are thin organic films that are formed by the strong chemisorption between electrode surface and head group of selected organic molecule. (Dubois and Nuzzo, 1992; Bain and Whitesides, 1989). This simple way of getting a well-defined and organized monolayer on surface is carried out by immersion of electrode in a dilute solution (ca 10–100 mM) of the adsorbate at room temperature (Chaki and Vijayamohanan, 2002). Then the well-ordered monolayers formed by alkanethiols on metal surfaces, particularly gold, can be used to immobilize enzymes, proteins, nucleic acids, etc. close to an electrode surface (Gooding and Hibbert, 1999). SAMs have been particularly used in fabrication of immunosensors, enzyme biosensors and DNA-hybridization biosensors, because they have considerable advantages such as lower background current, reproducibility and robustness (Campuzano et al., 2002).

Alcohol oxidase (AOX; Alcohol:O_2 oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme consisting of eight identical sub-units arranged in a quasi-cubic arrangement, each containing a strongly bound cofactor, flavin adenine dinucleotide (FAD) molecule (Vonck and van Bruggen, 1990). Thus AOX is responsible for the oxidation of low molecular weight alcohols, such as ethanol, to the corresponding aldehyde, using molecular oxygen (O_2) as the electron acceptor. A variety of biosensors based on NAD^+-dependent alcohol dehydrogenase (Leca and Marty, 1997; Ivanova et al., 2003; Gautier et al., 1990), alcohol dehydrogenase (Niculescu et al., 2002; Luo et al., 2008; Niculescu et al., 2003), alcohol oxidases (Patel et al., 2001; Wen et al., 2007; Barsan and Brett, 2008), and microbial (Akyilmaz and Dinckaya, 2005; Rotariu et al., 2004; Tkac et al., 2003) were proposed and applied for the determination of ethanol in complex samples.

Glucose oxidase (GOX; beta-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) is a well-characterised enzyme, which catalyses the oxidation of beta-D-glucose to D-gluconolactone and hydrogen

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peroxide (Kleppet, 1966; Wilson and Turner, 1992). Glucose oxidase is commonly used to construct amperometric biosensors for medical (Wilkins and Atanasov, 1996; Newman and Turner, 2005) and food industry (Mello and Kubota, 2002).

Tetrathiafulvalene (TTF) has been used as an ideal material for the modification of electrode surfaces due to its reasonable conductivity at room temperature, low solubility in water, and simplicity of application (Cao et al., 2008). Furthermore tetrathiafulvalene provides a promising system in the development of electroactive SAMs, due to the possibility to change its redox potential by means of either electron donor or acceptor substituents (Campuzano et al., 2006).

The aim of this project is to develop a bienzymic second generation biosensor, which is based on co-immobilization of alcohol oxidase and glucose oxidase on the same electrode surface for selective detection of ethanol and glucose.

In the biosensor construction, two enzymes and the mediator, TTF, were immobilized by cross-linking agent glutaraldehyde and cysteamine by forming self-assembled monolayer (SAM) on a gold disc electrode.

2. Experimental

2.1. Chemicals

Alcohol oxidase (AOX) (12 U/mg protein) from *Hansenulla* sp., glucose oxidase (GOX) (190.000 U/g solid) from *Aspergillus niger*, tetrathiafulvalene, cysteamine, glutaraldehyde (25%), acetone, L-ascorbic acid, ethanol, D(+)-glucose and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions used in the experiments were prepared just before their use.

2.2. Apparatus

In the experiments PalmSens potentiostat (Netherlands), a three-electrodes system from CH Instruments (USA) that contains a CHI 101 model Au working electrode (2 mm diameter), a CHI 111 model Ag/AgCl reference electrode and a CHI 115 model platinum wire counter electrode, Gilson P100 and P1000 automatic pipettes (France), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used.

2.3. Preparation of the biosensor

Prior to coating, Au electrode (AuE) surface was polished with alumina slurries on microfiber cloth to obtain a mirror surface. After that, it was thoroughly rinsed with double distilled water and sonicated first in absolute ethanol and then in double distilled water for 10 min to remove undesired adsorbed particles. At the next step, the electrode was cleaned by five successive cyclic voltammetric sweeps between −1.0 and +1.0 V in the 0.1 M HNO_3.

The clean gold electrode was soaked in 100 mM cysteamine solution for 15 h at room temperature to form an Au-Cys SAM electrode. At the end of this period the modified electrode (AuE-Cys) was rinsed with double distilled water to remove unbounded molecules on the electrode surface. Co-immobilization of two enzymes and the mediator was carried out as follows: 3 μL of the 0.4 M TTF solution (prepared in acetone) was deposited on the cysteamine-modified AuE and let to dry. Then, 5 μL of 0.5 mg/mL GOX-AOX enzyme mixture were also deposited on modified AuE and let to dry again. Finally, the electrode was immersed in the 25% glutaraldehyde solution for 1 h at 4 °C. This modified immobilization procedure was described previously by Campuzano et al. (2002).

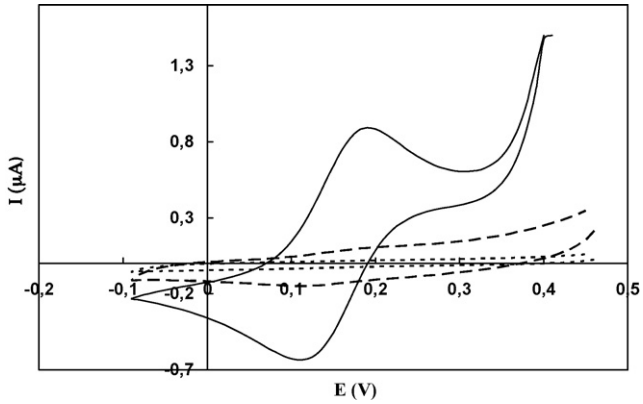

Fig. 1. Cyclic voltammograms of the biosensor at different stages obtained from the experiments. (. . .) Bare Au electrode; (- - -) Cysteamine-modified Au electrode; (—) Au-Cys-TTF-AOX-GOX (phosphate buffer; pH 7.0, 50 mM; *T*: 30 °C). Scan rate is 25 mV s^{-1}.

2.4. Principle of measurements

Principle of measurements was based on monitoring decrease in current on reduction potential of TTF^+ (at 0.1 V vs. Ag/AgCl) by using cyclic voltammetry method. Cyclic voltammograms were taken at a potential range between −0.1 and 0.4 V vs. Ag/AgCl. Correlations between decreases in biosensor responses and glucose oxidase or alcohol oxidase activity were monitored.

3. Results and discussion

3.1. Immobilization

Cyclic voltammograms were carried out at a potential range between −0.1 and 0.4 V by using bare electrode, cysteamine-modified electrode (AuE-Cys) and AuE-Cys-TTF-AOX-GOX for establishing the formation of SAM and immobilization of enzymes and mediator. When cysteamine monolayer formed on the electrode an indistinct cathodic (at 0.1 V) and anodic (at 0.2 V) peak current appeared. After formation of TTF-AOX-GOX certain cathodic and anodic peak currents were observed which are attributed to the good conductive properties of the AuE-Cys-TTF-AOX-GOX modified surface. The cyclic voltammograms shown in Fig. 1 were obtained with the bare electrode, cysteamine-modified Au electrode and AuE-Cys-TTF-AOX-GOX. Cyclic voltammograms showed that immobilization of TTF and enzymes brought about prominent oxidation and reduction peaks which facilitated the monitoring of enzyme activity.

Some chronoamperometric measurements were taken to confirm responses of the biosensor for substrates. Fig. 2(A and B) presents the results on the steady-state current at the Cys-TTF-AOX-GOX coated electrode in solutions containing different concentrations of ethanol and glucose. The data in the experiments were acquired at a static potential (+0.10 V for glucose and +0.15 V for ethanol vs. Ag/AgCl). From the figure it can be said that the biosensor gave effective results for both ethanol and glucose at different applied potentials. So, this result showed that the biosensor can be used for quantitative determination of these substrates selectively.

3.2. Optimization of bioactive layer

To determine effect of enzyme amount, TTF concentration and SAM duration on the biosensor responses, different amounts of enzymes, TTF concentrations and different duration of SAM were carried out.

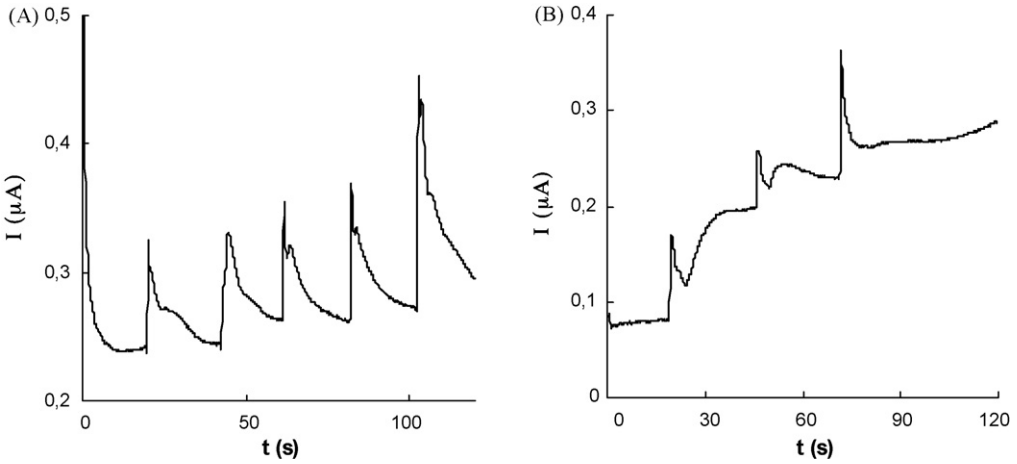

Fig. 2. Chronoamperometric responses of the biosensor: (A) at 20, 40, 60, 80 and 100th second 5.0 mM ethanol was added at 0.15 V potential; (B) at 25, 50 and 75th second 0.5 mM glucose was added at 0.1 V potential. (Phosphate buffer; pH 7.0, 50 mM; *T*: 30 °C). All the potentials were referred to saturated Ag/AgCl.

3.2.1. Detection of the effect of the amount of enzymes on biosensor responses

To determine the effect of glucose oxidase and alcohol oxidase amount on the biosensor response, different enzyme amounts were used.

From the results, higher biosensor responses and more acceptable calibration curves were achieved when biosensor was used, that contained 0.5 mg/mL both glucose oxidase and alcohol oxidase. On the other hand, when enzyme concentrations higher than 0.5 mg/mL were used in biosensor construction, some deformations and diffusion barrier occurred in SAM. As a result, biosensor responses decreased dramatically. In case of 0.25 mg/mL enzyme, biosensor responses as well as linearity of calibration curve were decreased explicitly.

3.2.2. Investigation of TTF concentration on biosensor responses

Different TTF concentrations were prepared and used, for determination of the effect of TTF concentration on the biosensor response. For this purpose three biosensors that contained 0.2, 0.4 and 0.8 M TTF were constructed.

According to results, in case of using 0.8 M TTF the biosensor responses were lower than a biosensor that contained 0.4 M TTF. The lowest biosensor responses were obtained by using 0.2 M TTF. Thus, due to not only better biosensor responses but also accuracy 0.4 M TTF was chosen to be the most suitable amount in the construction of the biosensor.

3.2.3. Duration of SAM formation

The density of hydrocarbon chains was assigned by the duration of SAM deposition, therefore SAM duration had a great importance. For the investigation of the duration of SAM formation the electrodes were immersed in 100 mM cysteamine solution for 7.5, 15, and 22.5 h.

Biosensor that immersed for 15 h had highest biosensor responses for both glucose and ethanol. When electrode had been immersed for 22.5 h, response of the biosensor decreased dramatically as a result of deformations on SAM related to duration time. When the electrode had been immersed for 7.5 h, linearity of calibration curve was deteriorated.

From the results of all experiments in the optimization of bioactive layer it can be concluded that the most suitable biosensor responses and also calibration curves were obtained by using 0.5 mg/mL solutions of enzymes, 0.4 M TFF and with 15 h duration of SAM.

3.3. Optimization of working conditions

3.3.1. Working temperature and pH effect on the biosensor responses

Experiments were carried out at 15, 20, 25, 30 and 35 °C for determination of temperature effect on biosensor responses.

According to the results, optimum temperature was found to be 30 °C for both glucose oxidase and alcohol oxidase. As a result of increase in temperature, some defects and deformations occurred on SAM surface as well as on the activities of the enzymes, thus 30 °C was chosen to be the working temperature for the biosensor.

In order to investigate the pH effect on biosensor responses different buffer solutions were prepared. For this purpose 50 mM phosphate buffers at different pH values (pH 6.0, 6.5, 7.0, 7.5 and 8.0) were prepared and used.

From the results, optimum pH values were found 7.0 for both glucose oxidase and alcohol oxidase. If we consider the optimum pH values of the enzymes in free form it can be said that the immobilization procedure used in the biosensor construction almost did not affect the optimum pH values.

3.4. Analytical characteristics of biosensor

3.4.1. Linear range for glucose and ethanol

Responses of biosensor depend linearly on glucose and ethanol concentration between 0.1–1.0 and 1.0–10.0 mM, respectively. Voltammograms obtained from the experiments are shown in Fig. 3.

The cyclic voltammograms indicated that when concentrations of ethanol and glucose were increased, the biosensor responses decreased correlately at reduction potential of TTF^+ (0.1 V vs. Ag/AgCl). This result was observed for both ethanol and glucose and from the cyclic voltammograms it can be said that both enzymes use TTF^+ to be an effective mediator in the regeneration of the enzymes. Calibration curves for glucose and ethanol are shown in Fig. 4.

Limits of detection of the biosensor for ethanol and glucose were calculated to be 0.75 and 0.03 mM, respectively. Quantification limits of the biosensor were also determined and these values were 2.5 and 0.1 mM for ethanol and glucose, respectively. Comparisons of performances of biosensors for ethanol and glucose are shown in Table 1.

According to the table, although linear ranges of biosensors for glucose were similar to each other, our biosensor had a lower detec-

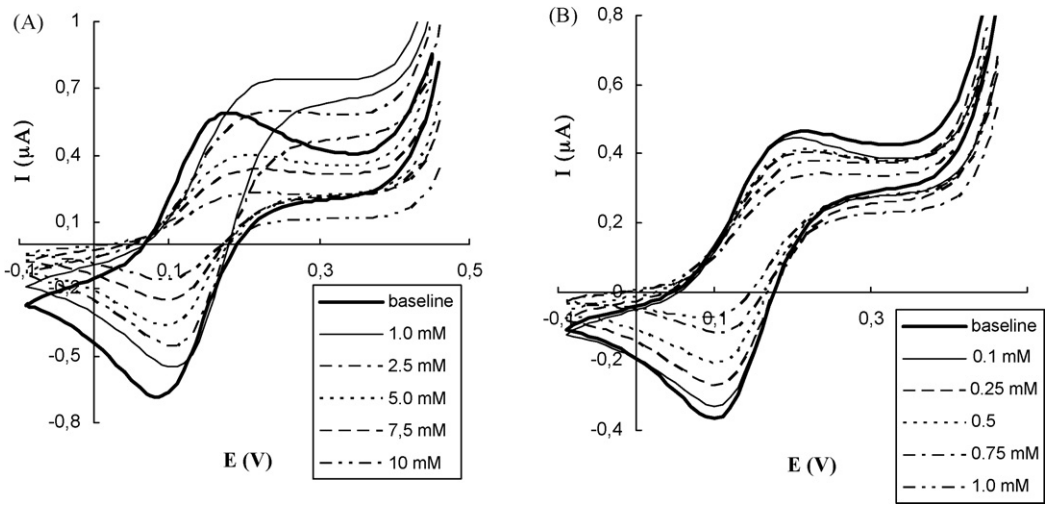

Fig. 3. Cyclic voltammograms obtained from the experiments during linear range studies. (A) Results for ethanol; (B) glucose. (Phosphate buffer; pH 7.0, 50 mM; *T*: 30 °C). The amount of glucose oxidase and alcohol oxidase, concentration of TTF and cysteamine, duration of SAM and the percentage of glutaraldehyde were kept constant at 0.5 mg/ml, 0.4 M, 100 mM, 15 h and 25%, respectively. Scan rate is 25 mV s^{-1} and potentials were referred to Ag/AgCl reference electrode.

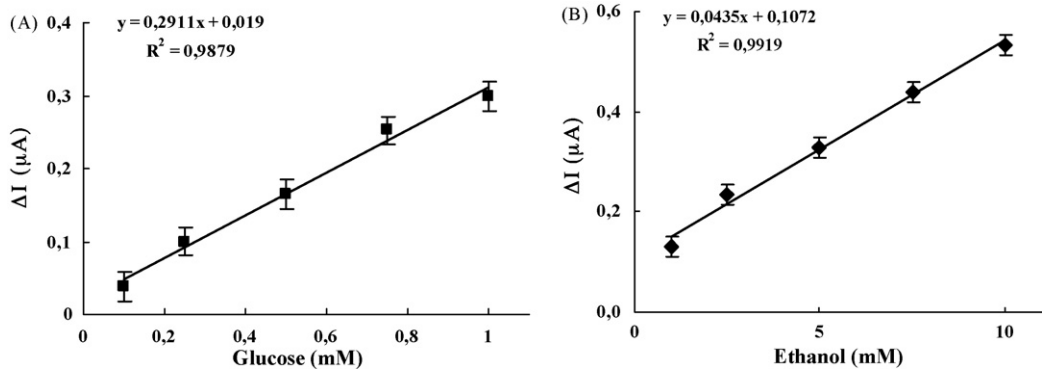

Fig. 4. Calibration curves of the biosensors: (A) for ethanol and (B) for glucose (phosphate buffer; pH 7.0, 50 mM; *T*: 30 °C). The amount of glucose oxidase and alcohol oxidase, concentration of TTF and cysteamine, duration of SAM and the percentage of glutaraldehyde were kept constant at 0.5 mg/ml, 0.4 M, 100 mM, 15 h and 25%, respectively.

tion limit value than the others. For ethanol, other biosensors had wider linear range and lower detection limit. However, the construction of our biosensor was more cost effective and easier than the other biosensors. Moreover, our biosensor allowed simultaneous determination of ethanol and glucose.

For testing simultaneous detection of ethanol and glucose with the biosensor developed both ethanol (5.0 mM) and glucose (0.5 mM) were added into the reaction cell and measurements were performed. Responses obtained by the biosensor in the presence of both substrates were nearly equal to total ethanol and glucose responses that were achieved one by one. Therefore, it can be said that these results enabled to detect ethanol and glucose simultaneously.

3.4.2. Reproducibility

The reproducibility of the biosensor was determined for 5.0 mM ethanol ($n = 6$). From the experiments, average value, standard deviation (SD) and coefficients of variation (CV%) were calculated to be 5.08 mM, ±0.25, and 4.98%, respectively.

The reproducibility of the biosensor was also determined for 0.5 mM glucose ($n = 7$). Average value, standard deviation (SD) and coefficients of variation (CV%) were calculated to be 0.51 mM, ±0.01, and 1.82%, respectively.

3.4.3. Substrate selectivity

For determination of substrate selectivity, 5.0 mM of standards of various compounds such as methanol, propanol, butanol,

Table 1
Comparison of performances of ethanol and glucose biosensors.

Method	Linear range (mol/L)	Detection limit (mol/L)	Reference
(A) ADH immobilized within PVA-MWCNT on GC electrode	$(0.1–1.5) \times 10^{-3}$	1.3×10^{-5}	Tsai et al. (2007)
Graphite–Teflon–AOD–GOD-HRP–ferrocene composite electrode	$(0.2–20) \times 10^{-4}$	4.0×10^{-5}	Prada et al. (2004)
PPY films modified Au electrode	$(0.01–0.75) \times 10^{-3}$	1.0×10^{-5}	Carelli et al. (2006)
AOD-GOD-TTF immobilized onto SAM modified Au electrode	$(1.0–10) \times 10^{-3}$	7.5×10^{-4}	Present work
(B) GOD immobilized on eggshell membrane with glutaraldehyde	$(0.1–13) \times 10^{-4}$	no data	Wu et al. (2004)
GOD/Au_{nano}/Pt_{nano}/CNT modified Au electrode	$(0.5–17.5) \times 10^{-3}$	4.0×10^{-4}	Chu et al. (2007)
Graphite-Teflon-AOD- GOD-HRP-ferrocene composite electrode	$(0.1–9) \times 10^{-4}$	5.0×10^{-5}	Prada et al. (2004)
AOD-GOD-TTF immobilized onto SAM modified Au electrode	$(1.0–10) \times 10^{-4}$	3.0×10^{-5}	Present work

(A) For glucose, (B) for ethanol.

Table 2
Substrate selectivity and interference effects of some compounds.

Substrate[a] (0.5 mM)	Response (%)	Substrate[b] (5.0 mM)	Response (%)
Glucose	100	Ethanol	100
Fructose	40	Methanol	94
Galactose	69	Propanol	38
Maltose	36	Butanol	25
Citric acid	7	Ascorbic acid	20
Uric acid	8	Uric acid	16
(Glucose + fructose)	135	(Ethanol + methanol)	158
(Glucose + galactose)	112	(Ethanol + propanol)	125
(Glucose + maltose)	101	(Ethanol + butanol)	108
(Glucose + citrate)	107	(Ethanol + ascorbic acid)	106
(Glucose + uric acid)	91	(Ethanol + uric acid)	87

[a] For glucose oxidase.
[b] For alcohol oxidase.

ascorbic acid and uric acid, and 0.5 mM of standards of various compounds such as galactose, fructose, maltose, ascorbic acid and uric acid were examined by using the biosensor. Responses of the biosensor obtained for the other substrates were compared to the responses of ethanol and glucose. Results obtained from the experiments are shown in Table 2.

Considering the results of the experiments it can be concluded that the biosensor gave responses for primer aliphatic alcohols and sugars. As primary alcohols are natural substrates of alcohol oxidase, their biosensor responses were reasonable. Galactose showed higher responses than other sugars, it may be possible that galactose can be converted by glucose oxidase (Semashko et al., 2003), because galactose is an epimer of glucose.

3.4.4. Interference effects of some compounds on biosensor responses

Interference effects of some sugars (fructose, galactose and maltose), alcohols (methanol, propanol and butanol) and other potential interfering (citrate, ascorbic acid and uric acid) compounds were investigated. Concentrations of compounds tested for detection of interfering on ethanol responses were 5.0 mM and concentrations of compounds tested for detection of interfering on glucose responses were 0.5 mM. Results obtained from the experiments are shown in Table 2.

The experiments were carried out at optimum working conditions (T: 30 °C and phosphate buffer: pH 7.0, 50 mM). The amount of glucose oxidase and alcohol oxidase, concentration of TTF and cysteamine, duration of SAM and the percentage of glutaraldehyde were kept constant at 0.5 mg/mL, 0.4 M, 100 mM, 15 h and 25%, respectively.

3.4.5. Storage stability of the biosensor

The storage stability of the biosensor has been studied over a period of 10 days. The sensor was stored in phosphate buffer (pH 7.0, 50 mM) at 4 °C when not in use. The catalytic current response could maintain about 50% of the initial signal. The first reason of decrease in biosensor responses was oxidation of TTF by oxygen and light. The other reason was defects and deformations occurring on SAM day by day.

4. Conclusions

In the paper, a new bienzymic biosensor based on alcohol oxidase and glucose oxidase has been described. The biosensor was prepared by using a tetrathiafulvalene and cysteamine-modified Au electrode. In optimization studies of the biosensor, some parameters such as optimum pH, optimum temperature and effect of TTF concentration, enzyme amount and duration of SAM formation were investigated. We found out that the best conditions were detected to be 30 °C, phosphate buffer (pH 7.0, 50 mM), 0.4 M TTF and 15 h SAM duration. Furthermore, the biosensor has a decent reproducibility for glucose and ethanol detection and also interferences on biosensor responses were within acceptable limits.

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