

A promising enzyme anchoring probe for selective ethanol sensing in beverages

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ABSTRACT

A newly designed amperometric biosensor for the determination of ethanol through one-step electrochemical coating of (4,7-di(thiophen-2-yl)benzo[c][1,2,5]selenadiazole-co-1*H*-pyrrole-3-carboxylic acid) (TBeSe-co-P3CA) on a graphite electrode is presented. It was aimed to propose a newly synthesized copolymer with enhanced biosensing properties as a novel sensor for the quantification of ethanol. The conjugated copolymer (TBeSe-co-P3CA) was prepared through electrochemical polymerization by potential cycling. After polymer modification, alcohol oxidase (AOx) was immobilized on a modified electrode surface for ethanol sensing. In the analytical investigation, the calibration plot is linear above large concentration range (0.085 to 1.7 mM), where sensitivity is around 16.44 $\mu\text{A}/\text{mMcm}^2$ with a very low detection limit (LOD) of 0.052 mM based on the signal-to-noise ratio in short response time. Moreover, interfering effect of some possible compounds were examined and the capability of the biosensor in estimating ethanol content in commercial alcoholic beverages was also demonstrated. The results showed satisfactory accuracy of the developed sensor and confirm the proposed sensor has a potential for ethanol quantification compared to the currently used techniques.

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1. Introduction

The detection and quantification of ethanol in food analysis is a hot topic since currently available methods such as colorimetry [1], spectrophotometry [2], chemiluminescence [3] and chromatographic methods [4] are expensive, time-consuming methods and need comprehensive sample pretreatment and bulky instrumentation. To overcome such problems and meet the needs of the sensor technology, enzyme amperometric biosensors have been proposed in food-quality monitoring and biotechnological applications which provide highly specific, rapid and repetitive assays and require low-cost transducers [5–7]. Alcohol biosensors have been considered as an ethanol sensing device in beverages using alcohol oxidase (AOD; EC1.1.3.13), mostly preferred enzyme in the development of alcohol biosensors, catalyzing the oxidation of low molecular weight alcohols to aldehydes in the presence of molecular

oxygen. To provide high selectivity for ethanol, biosensors should fulfill several requirements: They should be robust, stable, reproducible and interference free. The unique and fascinating properties of conjugated polymers (CPs) have produced tremendous motivation among scientist to explore the possibilities of using them in biosensing applications. CPs were recognized as attractive materials having wide applications in various fields like biosensors, electrochromic devices, organic light emitting diodes, and smart windows [8–10]. In addition to the promising applications mentioned above, unique properties of CPs potentially result in a great architecture for simple biosensor development. Moreover, the biocompatible and appropriate surface morphology of polymers offer high enzyme loading and effective contact between the deeply buried active sites of enzymes and the electrode, resulting in dramatically improved sensor performance. Moreover, selenium containing polymers led researchers to explore many opportunities in various applications. It was reported that selenium containing conjugated polymers help developing rapid and high accuracy electrochemical biosensors. Additionally, since selenium unit is biocompatible and improves the transfer of charge which is most desirable in enzyme immobilization selenium containing conducting polymer based platform reveals a high sensitivity for the detection of the desired analytes [6].

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One of the key factors in developing amperometric biosensors is the connection of the biological component (enzyme) to the electrode surface as a transducer. Recently, major developments have been devoted to improve biosensor characteristics by integrating various nanostructured and polymeric materials into the sensing system design. In literature, several types of ethanol sensors were designed and tested for ethanol sensing. Literature is highly oriented toward the finding of uncomplicated procedures in a convenient way. Brett et al. utilized an electrochemical biosensor based on carbon nanotube-modified carbon film electrodes for ethanol quantification. They highlighted the sensitivity obtained with the newly designed surface architecture was higher than that of any nanomodified sensors [11]. Similarly, Pingarron and co-workers exploited a colloidal gold (Aucoll)–multiwalled carbon nanotubes (MWCNTs) composite electrode using Teflon as a binder for ethanol detection. It was reported that such a sensor can detect the ethanol up to 1 mM in commercial beers [12]. In another work, Ju et al. developed a stable poly(thionine)–CNF/AOD biocomposite film on GCE and alcohol oxidase was modified on the prepared surface. It displayed good analytical characteristics as well as excellent reproducibility and stability [13]. A similar ethanol sensor was developed by immobilizing alcohol oxidase (AOx) through polyamidoamine (PAMAM) dendrimers on a cysteamine-modified gold electrode surface for ethanol analysis in various alcoholic beverages [14]. Moreover, an ethanol biosensor was proposed by Dzyadevych et al. [15]. In this aspect, alcohol oxidase was immobilized in resydrol polymer for ethanol detection and the biosensors showed good analytical characteristics such as reproducibility, operational and storage stability. It has been also tested for ethanol detection in real alcoholic beverages. Our group also fabricated a conjugated polymer based ethanol sensing platforms for alcohol oxidase immobilization [16–18]. The main goal of the design of ethanol biosensor is to find the best biosensor performance and also create a proper method as an alternative to other traditional methods. In the present work, fabrication and utilization of a novel conjugated polymer based biosensing electrode for the detection of ethanol is described. For this reason, two different monomers were utilized to obtain a polymeric film on the electrode surface for electrochemical sensing experiments. Electrochemical properties of the polymers and biosensor were evaluated for different aspects. There are no reports offering this type of biosensor using (4,7-di(thiophen-2-yl)benzo[c][1,2,5]selenadiazole-co-1H-pyrrole-3-carboxylic acid) (TBeSe-co-P3CA) polymer for detection of ethanol. TBeSe-co-P3CA polymer was for the first time used for ethanol detection in this work. To the best of our knowledge, the biosensor fabricated in this study showed one of the lowest LOD and K_M^{app} values

among all the enzyme-containing conjugated polymer-based ethanol biosensor in the literature. The concept provides a useful way for easy (one-step) preparation of the polymer modified electrode transducer for a wide range of sensing applications. All these properties offer a good promise for practical ethanol analysis in beverages. The sensing performances, such as sensitivity, calibration behavior, selectivity, and stability of the device are also evaluated and compared with the performance of available ethanol biosensors. Construction route for the ethanol biosensor is illustrated in Scheme 1.

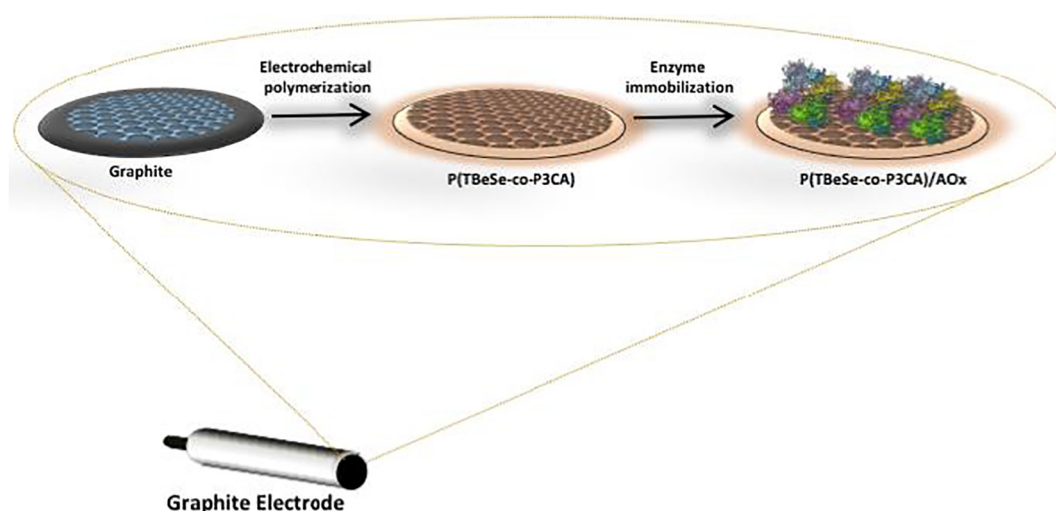
2. Experimental

2.1. Chemicals and instrumentation

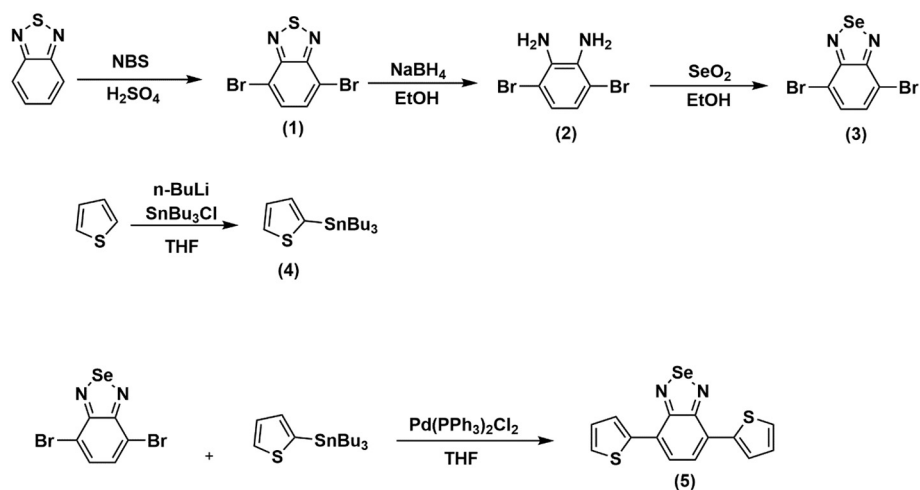
All chemicals used for monomer synthesis and electrochemical polymerization were purchased from Sigma Aldrich and used without further purification. For biosensor construction, alcohol oxidase (AOx, from *Pichia Pastoris*, 1.1.3.13.1, 250 U/mg solid) was purchased from Sigma-Aldrich. N-Hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Fluka (Buchs, Switzerland) and Sigma, respectively. JEOL JSM-6400 models SEM was utilized for surface morphologies of the fabricated biosensor. In order to determine the spectroelectrochemical properties of the polymer films Jasco V-770 UV-Vis-NIR spectrophotometer was used.

Amperometric measurements were recorded with a computer-controlled potentiostat/galvanostat Palm Instrument (PalmSens, Houten, The Netherlands). Electrochemical polymerizations of the (4,7-di(thiophen-2-yl)benzo[c][1,2,5]selenadiazole) (TBeSe) and 1H-pyrrole-3-carboxylic acid (P3CA) were performed by GAMRY Reference 600 Potentiostat/Galvanostat. Graphite electrodes for electrochemical measurements were firstly polished with an emery paper. Then, the electrodes were rinsed with distilled water. The amperometric measurements were evaluated by applying a constant potential of -0.7 V versus pseudo Ag wire electrode in a stirred solution of phosphate buffer saline (PBS) for different concentrations of substrate (ethanol). All electrochemical studies were performed with a three-electrode system, based on a graphite working electrode (type RW001, 3.05 mm diameter and 13% porosity; 3 mm diameter), a pseudo Ag wire reference electrode, and a platinum wire as the counter electrode.

The working cell filled with 10 mL buffer solution was used in electrochemical biosensing experiments under controlled magnetic stirring. Convective transport during the amperometric measurements was



Scheme 1. Schematic of sensing device containing graphite electrode, a network of polymer and AOx.



Scheme 2. Synthetic roadway of the monomer (TBeSe).

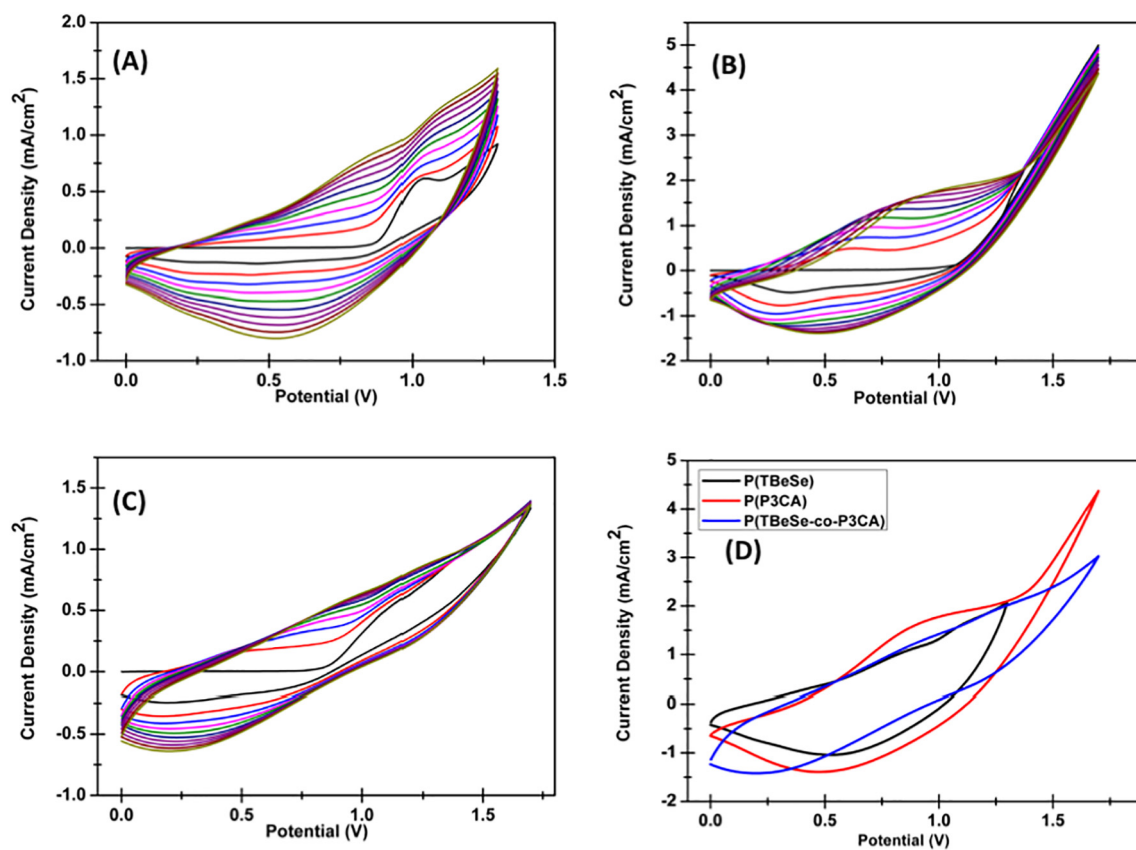
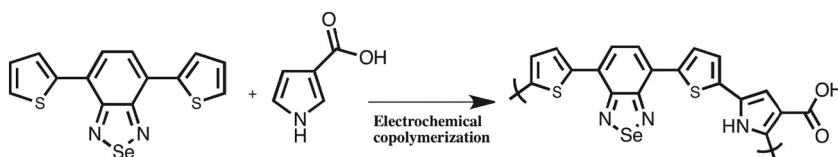


Fig. 1. Electropolymerization of the (A) P(TBeSe), (B) P(P3CA), (C) P(TBeSe-co-P3CA) (up to 10 cycles) and (D) Cyclic voltammograms of the related polymers in 0.1 M supporting electrolyte containing DCM: ACN solution.

provided using magnetic stirrer. A new buffer solution was used for each assay during electrochemical measurements and all experiments were performed at room temperature. After the background current

was allowed to a constant level, a certain amount of ethanol was added to the buffer solution. The results (the difference between the before and after substrate addition) were evaluated as a sensing response.



Scheme 3. Schematic illustration of the electrochemical copolymerization of the related monomers.

The results were reported as the average of three measurements and standard deviations were calculated as $\pm$ SD.

For sample application part, commercial rum (37.5% vol.), vodka (37.5% vol.) and raki (45% vol.) were purchased from a local supermarket. Before analysis of the samples, the beverages were diluted by a dilution factor of 1/10.

2.2. Synthesis of (4,7-di(thiophene-2-yl)benzo[c][1,2,5]selenadiazole) (TBeSe)

Compounds 1, 2, 3 and 4 were obtained according to previously reported syntheses [19–21]. 4,7-Dibromo-2,1,3-benzoselenadiazole (0.50 g, 1.40 mmol), tributyl(thiophen-2-yl)stannane (2.19 g, 5.87 mmol) and $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (49.1 mg, 0.107 mmol) were dissolved in a freshly distilled THF (15 mL). The solution was heated to reflux under the argon atmosphere at 125 °C for 2 days. The solvent was removed from the solution using a rotary evaporator and the resulted product (5) was purified by column chromatography eluting with hexane/methylene chloride (2:1) to afford a dark red crystalline solid. Yield: 65% (Scheme 2).

^1H NMR (400 MHz, CDCl_3): 8.05 (d, $J = 5.1$ Hz, 2H), 7.85 (s, 2H), 7.49 (dd, $J_1 = 3.9$ Hz, $J_2 = 1$ Hz, 2H), 7.25 (d, $J = 5.2$ Hz, 2H),

^{13}C NMR could not be obtained due to difficulties in solubility.

GC–MS (ESI) for $\text{C}_{14}\text{H}_8\text{N}_2\text{S}_2\text{Se}$ calculated 347.93, found 347.90.

2.3. Modification of graphite electrode for biosensor application

TBeSe-co-P3CA film was electrodeposited onto a previously cleaned graphite electrode via cycling potential from 0 to 1.7 V in an equimolar 0.1 M sodium perchlorate (NaClO_4) and lithium perchlorate (LiClO_4) supporting electrolyte containing DCM:ACN solution at a scan rate of 0.1 Vs^{-1} in 20 cycles unless otherwise stated. Under the same electrochemical conditions, homopolymers of TBeSe and P3CA were performed with a repeated scan interval between 0 and 1.4 V and 0 and 1.7 V, respectively. Biosensor performances of the homopolymers were also compared with a biosensor obtained with TBeSe-co-P3CA film. After electropolymerization, the electrodes were rinsed with distilled water. In order to prepare biosensor sensing to ethanol alone, 4 μL of an AOx solution was mixed with 3 μL of EDC (0.4 M) and 3 μL of NHS (0.1 M) and 10 μL of the resulted mixture was carefully spread on the working electrode. The electrodes were left for 90 min. at a room temperature of 25 °C. Then, the biosensor was kept in the refrigerator for 24 h at +4 °C to access a good crosslinking ability. The biosensor was washed with distilled water just before the measurement to remove any weakly or unbounded species from the surface.

3. Results and discussion

3.1. Fabrication and electrochemical characterization of the (4,7-di(thiophene-2-yl)benzo[c][1,2,5]selenadiazole-co-1H-pyrrole-3-carboxylic acid) (TBeSe-co-P3CA) film by electropolymerization

Electrocopolymerization of the monomers was carried out using cyclic voltammetry. Electrocopolymerization was achieved with different ratios of comonomers such as (4:3; 5:1; 2:5; 1:4; 0:1 and 1:0), (M:M, TBeSe: P3CA). Electropolymerization of 4:3 (M/M) TBeSe: P3CA containing copolymer was performed in 4.0 mM TBeSe, 3.0 mM P3CA and equimolar 0.1 M NaClO_4 , LiClO_4 supporting electrolyte containing 5:95 (v/v) DCM:ACN solution with repeated scan intervals between 0.0 and 1.70 V versus Ag wire pseudo-reference electrode (Fig. 1). Polymerization of TBeSe and P3CA was also performed on ITO-coated glass slides using the same experimental conditions. After the copolymerization step (TBeSe-co-P3CA) (as illustrated in Scheme 3), polymer redox potentials were changed, indicating the formation of the copolymer. Although electrocopolymerization was achieved in different molar ratios of comonomers, the best biosensor performance was obtained for

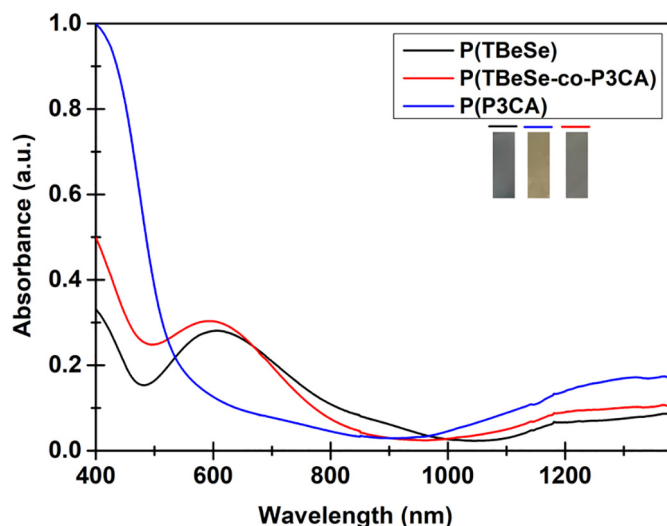


Fig. 2. Absorbance spectra of the polymers (color of the films were given as inset).

4:3 mM ratio copolymerization and it was used for further experiments. Apart from this ratio, it is difficult to get a reliable and reasonable signal for sensor experiments.

Absorption spectra of P(TBeSe-co-P3CA) and homopolymers were recorded for further investigation of the copolymer formation (Fig. 2). Comparing λ_{max} values of homopolymer (P(TBeSe (615 nm) and P3CA (absorbs in UV-region))) with the copolymer (590 nm), there is a slight change in the absorption with a shift to the lower wavelengths.

The electrochemical behaviors of bare graphite electrode (black curve), P(TBeSe-co-P3CA) (red curve) and P(TBeSe-co-P3CA)/AOx (blue curve) modified graphite electrodes were studied in 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution containing 0.1 M KCl at a scan rate of 100 mVs^{-1} (Fig. 3). Modification of the electrode surface with P(TBeSe-co-P3CA) leads to an increase in the peak current of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (ipa: 0.21 mA) compared to the bare electrode (ipa: 0.14 mA). This could be due to high surface coverage for biomolecule deposition. Moreover, the immobilization of an insulating biomolecule on the polymer modified surface decreases the electroactivity of the corresponding electrode (ipa: 0.16 mA), but it still pursues its electron transfer efficiently. Randles-Sevcik equation [22] was utilized to find the effective surface

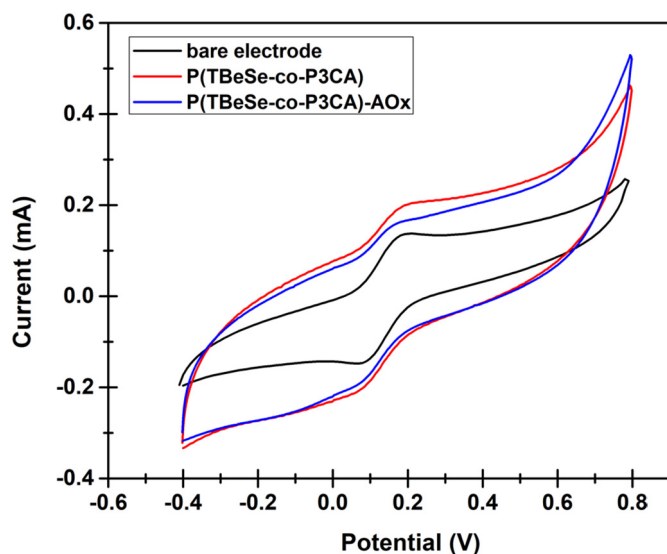


Fig. 3. CVs of bare graphite electrode, P(TBeSe-co-P3CA), P(TBeSe-co-P3CA)/AOx in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ containing 0.1 M KCl and pH 7.0 PBS.

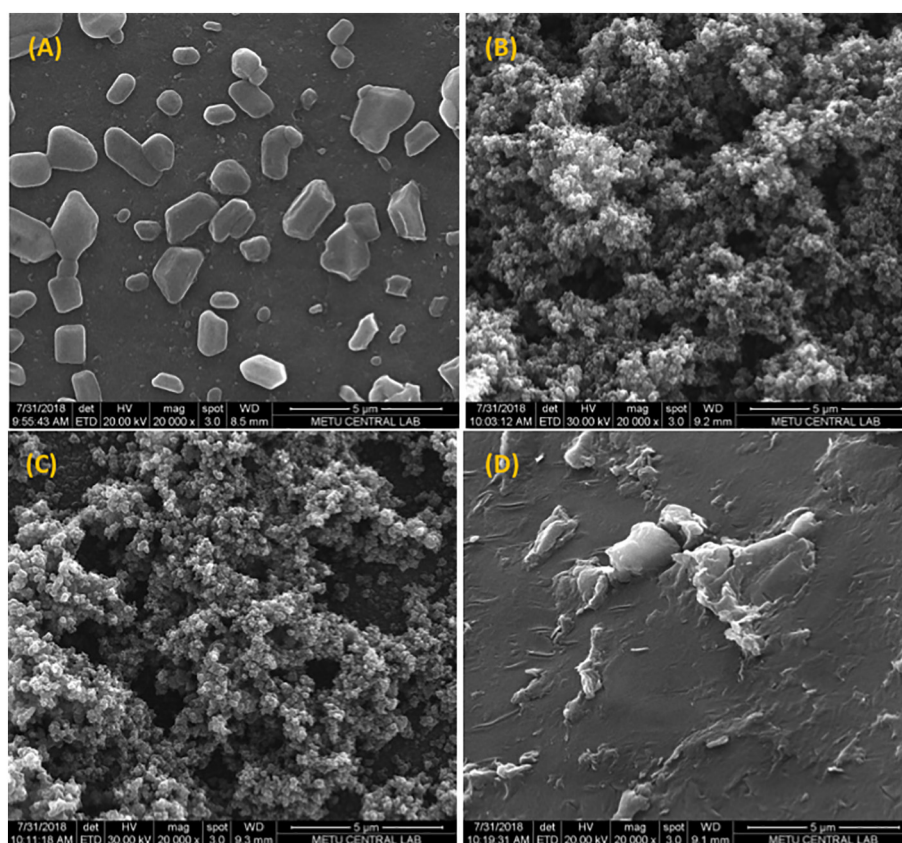


Fig. 4. SEM images of (A) only P(P3CA) coated electrode surface, (B) only P(TBeSe) coated electrode surface, (C) copolymer P(TBeSe-co-P3CA) coated electrode surface and (D) P(TBeSe-co-P3CA)/AOx modified electrode surfaces.

area of the bare, P(TBeSe-co-P3CA) and P(TBeSe-co-P3CA)/AOx modified electrodes. The values were calculated as 0.124, 0.184 and 0.142 cm², respectively.

3.2. Surface characterization of the biosensor

The surface morphology of P(P3CA), P(TBeSe), P(TBeSe-co-P3CA) and P(TBeSe-co-P3CA)/AOx were studied by SEM. As confirmed by SEM image in Fig. 4A, a total homogenous coating of the bare electrode by electrochemical deposition of the P(P3CA) could not be achieved (The SEM image is correlated with amperometric results since non-homogenous surface brings unstable sensor signals). On the contrary, electrochemical deposition of the P(TBeSe) brought a highly homogenous cauliflower-like structure with high porosity, which is a typical network for conducting polymers (Fig. 4B). Although there are no significant morphological differences between those films after copolymerization step (Fig. 4C), main differences for both surfaces were summarized in the electrochemical characterization part. More importantly, an obvious difference was observed between P(TBeSe-co-P3CA) and P(TBeSe-co-P3CA)/AOx surfaces (Fig. 4D), which proves the successful deposition of biomolecule onto the polymer coated surface. This result is also in accordance with the results of the above-explained CV experiments.

3.3. Optimization studies of the biosensor

The effect of the important parameters on the performance of P(TBeSe-co-P3CA)/AOx biosensors were examined in the present work. These parameters were evaluated by following four steps; P(TBeSe-co-P3CA) layer thickness optimization on graphite electrode, enzyme (AOx) amount and the pH value of the buffer solution. Firstly, the

influence of P(TBeSe-co-P3CA) layer on the biosensor response of ethanol was explored. The polymer was coated on the working electrodes varying scan numbers between 5 and 20 (5, 10, 20, 30 and 40). The results showed that the response reached a maximum value at 20 cycles of polymer coating on the surface (Fig. 5A). With changing cycle number of the layer below or above 20, the lower responses were obtained. Therefore, polymer coated 20 cycles was adopted for further studies. The second optimization was achieved for enzyme amount. Since the charge transfer in bioelectrochemical reaction was affected by the biomolecule amount, the optimization of biomolecule amount on the electrode surface of biosensors is fairly important. For this reason, biosensors were prepared using varying amounts of AOx from 2 Units to 8 Units. Current vs ethanol concentration graphs are created in Fig. 5B. When enzyme content of the biosensor is more than 4.5 U, a thick and complex bioactive layer is being formed at the electrode surface and the diffusion of the substrate from solution to the electrode is becoming difficult. The similar low responses were obtained with lower than 4.5 U enzyme content. As a result, inadequate enzyme loading caused low sensitivity of the biosensor. Therefore, 4.5 U of AOx is determined to be an optimum amount of enzyme. As the final step, the optimization of the pH value of the buffer solution was performed preparing different buffer solutions having the pH values as 6.0, 6.5, 7.0, 7.5 and 8.0 (Fig. 5C). The highest sensitivity sensor is obtained using pH 7.0 buffer.

3.4. Analytical characterization of biosensor for ethanol detection

The good electrochemical performance of P(TBeSe-co-P3CA)/AOx led us to construct a representative amperometric biosensor based on the substrate of ethanol. Typical calibration curves of the biosensor for the determination of ethanol are shown in Fig. 6. Linear part of the

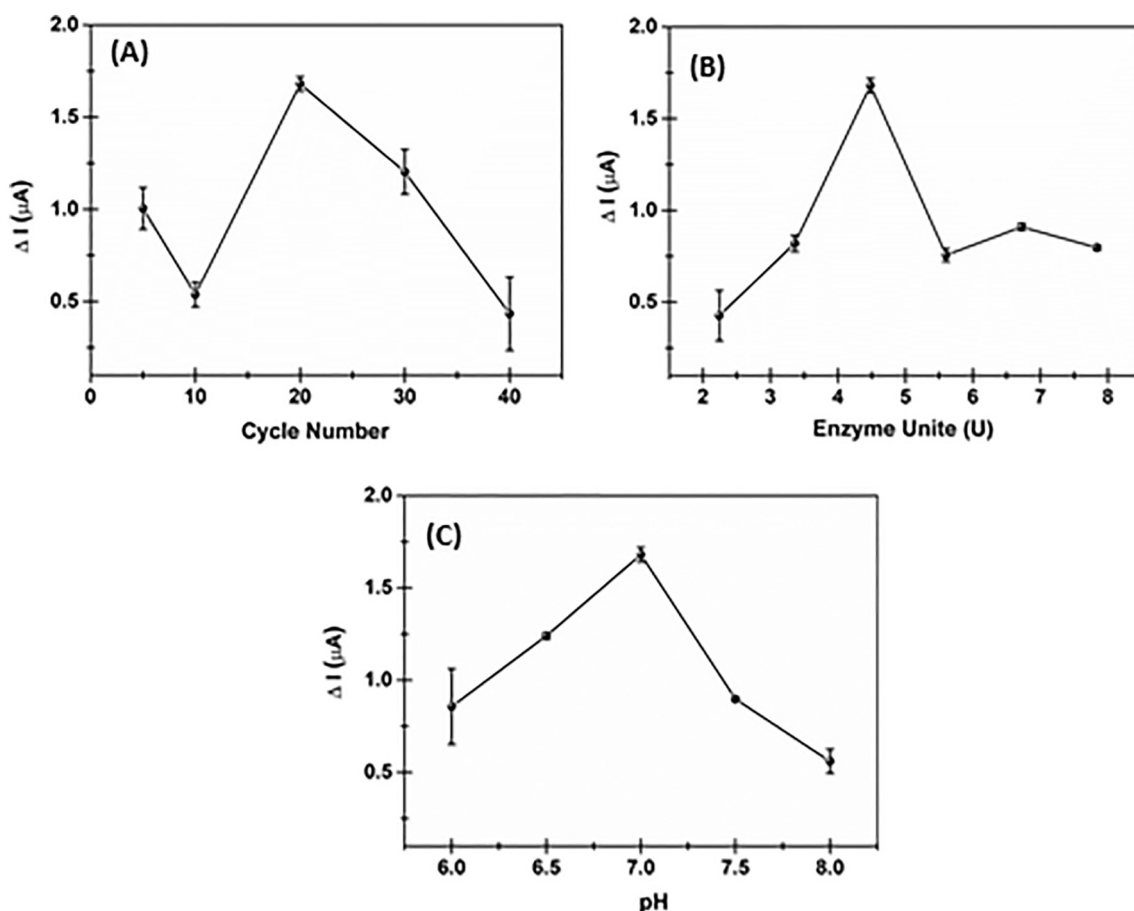


Fig. 5. Average responses of biosensor toward ethanol for optimization of (A) cycle number, (B) enzyme activity (U) and (C) pH.

calibration curve is described by the equation $y = 1.0386x + 0.1347$ (for P(TBeSe-co-P3CA)/AOx biosensor; $R^2 = 0.9984$) where y is the current when the response reaches the steady-state value (μA) and x is the concentration of ethanol (mM). In addition, the limit of detection (LOD) value of the P(TBeSe-co-P3CA)/AOx biosensor was calculated based on $S/N = 3$ criteria. The sensitivity of the biosensor and K_M^{app} value (Michaelis-Menten constant) were determined as $16.44 \mu A/mMcm^2$ and 0.37 mM, respectively. To the best of our knowledge, estimated

K_M^{app} value of the proposed sensor is the smallest one among the enzymatic sensor based literature examples [16–18,23–26]. Since the value of K_M^{app} is highly significant for developing enzymatic analytical systems (an indicator of the substrate affinity) obtaining lower K_M^{app} values for sensing experiments is a desired situation.

The influence of electrode surface modification on the amperometric response of an enzyme sensor was also assessed. For this purpose, the system was considered for three biosensors, one of which contains a P (P3CA) polymer with AOx and whereas another biosensor contains a P(TBeSe) polymer with AOx and the last one contains both P(TBeSe) and P(P3CA) polymers with AOx. The sensors were prepared by electrochemical polymerization on a graphite electrode as described previously. After enzyme immobilization step, relative response values of P (TBeSe)/AOx, P(P3CA)/AOx and P(TBeSe-co-P3CA)/AOx enzyme sensors were recorded at optimum conditions. When comparing the response of modified electrodes, herein, it was devoted most of the work to copolymer based ethanol biosensor as it is more stable and sensitive sensing layer. Through the use of copolymerization sensing response of the biosensor was increased three fold. All these experimental results reveal that the biosensor prepared with copolymerization brought better sensing results.

Moreover, in the literature, P(P3CA) was used as a sensing layer for different purposes. For instance, electrochemical polymerization of pyrrole-3-carboxylic acid on electrochemically over-oxidized pencil graphite electrode (p(P3CA)/EOPGE) surface was achieved for dopamine detection [27]. In that work, it was reported that the electrode prepared with P3CA revealed superior voltammetry performances compared to bare PGE. The presence of the conjugated polymer in the sensing layer improved the sensitivity and selectivity of the desired biosensor. Similarly, in another work, Ozcan and Ilkbas developed a poly

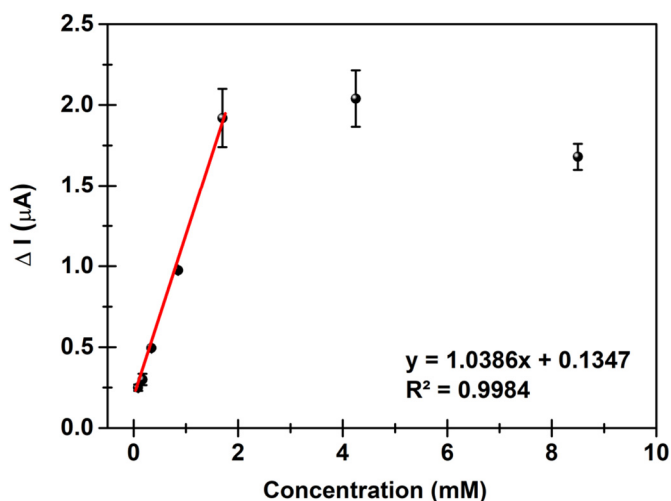


Fig. 6. Amperometric response of the alcohol sensors to increasing ethanol concentrations in 50 mM pH 7.0 PBS.

Table 1
Comparison of biosensor examples for AOx (NR: Not reported).

Biosensor architectures	LOD (mM)	Sensitivity	K_m^{app} (mM)	Reference
Poly(MCCH)/AOx	0.131	$4.79 \mu\text{A mM}^{-1} \text{cm}^{-2}$	8.74	[16]
Poly(TiFc-co-BEDOA-6-poly(L-Boc))/AOx	0.28	$12.52 \mu\text{A mM}^{-1} \text{cm}^{-2}$	2.67	[18]
Hydrogel/AOx/platinum electrode	NR	10.6 nA/mM	NR	[32]
Poly(neutral red) (PNR)/AOx	44	84 nA/mM	2.4	[25]
P(TBeSe-co-P3CA)/AOx	0.052	$16.44 \mu\text{A mM}^{-1} \text{cm}^{-2}$	0.37	This work

(pyrrole-3-carboxylic acid) modified pencil graphite electrode (p(P3CA)/PGE) for the determination of serotonin (SER) in blood serum and urine samples [28]. It was highlighted that simple preparation route of the poly(pyrrole-3-carboxylic acid) films are the central point for the fabrication of the desired sensor. In another work, poly(pyrrole-3-carboxylic acid) film was covered on a gold-coated grating substrate for the detection of human IgG. In that, authors reported that created poly(pyrrole-3-carboxylic acid)-based sensor is a sensitive tool to improve the binding of biological molecules [29]. Additionally, the presence of the carboxylic group in the P(P3CA) structure allowed the covalent immobilization of the enzyme with the electrode surface resulting in a proper biomolecule localization on the transducer surface. Because of these reports, herein P3CA was selected as a comonomer for both above mentioned properties and its functional carboxylic groups to bind with enzyme properly for produce an ethanol sensing. Although they also provide good stability and high immobilization density, herein the biosensor prepared with homopolymer of P(P3CA) did not show a good biosensing ability without copolymerization with P(TBeSe). Moreover, since thiophene-based building blocks have good stability, high contrast and fast switching times, thiophene and its derivatives as donor units and benzoselenadiazole as acceptor units were used in various electrochemical application areas [30]. Thiophene also enables strong and broad absorption, suitable molecular energy levels to ensure good charge separation properties [20,31]. Hence, herein it was aimed to combine P3CA with TBeSe to create TBeSe-co-P3CA film for ethanol detection. To the best of our knowledge P(TBeSe) monomer and P(TBeSe-co-P3CA) films were used for the first time as an immobilization platform. The properties of the biosensors reported in this paper compare favorably with that reported in recent literature. The detailed references based on ethanol sensing cited in Table 1. Herein, we propose a sensor design with a simple electrochemical synthesis route acting as a great connector between the biomolecule and the transducer which eliminates the need for additional matrices and possible interferences.

Since the biosensor selectivity is an important factor when working with most of the samples (blood serum, beverages etc.), evaluation of the possible interfering substances is a mandatory step in the biosensor

construction. Herein, the selectivity of the developed P(TBeSe-co-P3CA)/AOx bioelectrode for the determination of ethanol was also evaluated by considering glucose, ascorbic acid, citric acid, urea as the potential interfering reactants. These substances are commonly found in samples, and their oxidation is possible via applying the potential to an amperometric transducer. In this work, using the designed sensor, a negligible interfering effect was noticed for ethanol sensing (Fig. 7). This is probably due to the properly selected surface design and low operating potential where the interfering compounds are not easily oxidized. These results led us to conclude that this biosensor can be suitable for use both blood serum samples and ethanol content quantification of ethanol. Stability and repeatability of the biosensor were diagnosed by the recording of consecutive amperometric measurements on the series of using the same sensor. For this experiment, seven consecutive measurements were taken and the results were compared with each other. The standard deviation (S.D) and relative standard deviation (RSD) values were calculated as 0.061 and 1.07%, respectively. The results reveal that the sensor has a reproducible signal capacity with high accuracy.

3.5. Detection of ethanol in alcoholic beverages

Applicability of the biosensor was tested by determining the alcohol content in several alcoholic beverages. Three different commercially-produced rum, vodka and raki were used. Before testing it, the samples were diluted with 50 mM PBS (pH 7.0) to fit their final concentration within the linear range of the biosensor and the results were reported using amperometric measurements. As displayed in Table 2, the ethanol content in the three “alcoholic” beers using the proposed biosensor showed very good agreement with the ethanol content provided by the producer. These results clearly indicate the constructed biosensor is an accurate way to test for alcohol in real samples.

4. Conclusions

This work reports the development of a copolymer-based electrochemical ethanol biosensor for the detection of ethanol in alcoholic products. A good sensing ability biosensor was fabricated using (4,7-di(thiophene-2-yl)benzo[c][1,2,5]selenadiazole-co-1H-pyrrole-3-carboxylic acid) (TBeSe-co-P3CA) polymeric film on graphite electrode for alcohol oxidase immobilization. The procedure for sensor fabrication is quite simple and gives an opportunity to get almost the same sensor ability that matches the aim of the biosensor design. In this novel biosensor architecture, carboxyl groups, which are arising from the P3CA, were used for the covalent linkage between the amino groups of the enzyme and transducer surface to get a robust biosensor. For comparison,

Table 2
Results for ethanol analyses in beverages by enzyme electrode.

Sample	Ethanol content (mM)		Percent error (%)
	Product label	P(TBeSe-co-P3CA)/AOx	
B@Rum	0.64	0.59	−7.8
Y@Raki	0.77	0.79	2.59
I@Vodka	0.64	0.58	−9.37

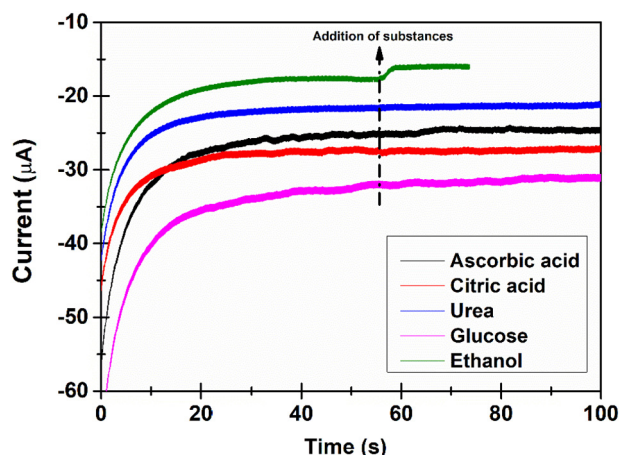


Fig. 7. Effect of interfering substances on biosensor performance (0.5 mM substances).

three different enzyme electrodes (P(TBeSe), P(P3CA) and P(TBeSe-co-P3CA)) were constructed and it was found that copolymer based electrode has the best biosensing properties without using any additional membrane and additives. The detailed surface property was characterized using SEM and CV techniques. The sensor shows great sensing ability with good sensitivity ($16.44 \mu\text{A}/\text{mMcm}^2$), low K_M^{app} value (0.37 mM) and LOD (0.052 mM) values. All these results reveal that a newly designed sensor has a good capability for ethanol sensing with high accuracy results.

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