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Analytical Methods

Selenium containing conducting polymer based pyranose oxidase biosensor for glucose detection

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ABSTRACT

A novel amperometric pyranose oxidase (PyOx) biosensor based on a selenium containing conducting polymer has been developed for the glucose detection. For this purpose, a conducting polymer; poly(4,7-bis(thieno[3,2-b]thiophen-2-yl)benzo[c][1,2,5] selenadiazole) (poly(BSeTT)) was synthesized via electropolymerisation on gold electrode to examine its matrix property for glucose detection. For this purpose, PyOx was used as the model enzyme and immobilised via physical adsorption technique. Amperometric detection of consumed oxygen was monitored at −0.7 V vs Ag reference electrode in a phosphate buffer (50 mM, pH 7.0). K_M^{app}, I_{max}, LOD and sensitivity were calculated as 0.229 mM, 42.37 nA, 3.3×10^{-4} nM and 6.4 nA/mM cm^2, respectively. Scanning electron microscopy (SEM), Electrochemical Impedance Spectroscopy (EIS) and cyclic voltammetry (CV) techniques were used to monitor changes in surface morphologies and to run electrochemical characterisations. Finally, the constructed biosensor was applied for the determination of glucose in beverages successfully.

1. Introduction

The development of conducting polymer based biosensors has rapidly increasing in the fields of biological analysis, health care and food processing industries for the detection of various analytes (Cesarino, Moraes, Lanza, & Machado, 2012; Gerard, Chaubey, & Malhotra, 2002; Singh, Chaubey, & Malhotra, 2004). Sensor performance depends largely on the surface properties, interaction between the enzyme molecule and electrode surface and protection of three dimensional structure of enzyme molecule. Therefore, conducting polymers were emerged as one of the most fascinating transducers due to their simple preparation (Chaubey & Malhotra, 2002; Malhotra & Chaubey, 2003; Soylemez, Kanik, Nurioglu, Akpinar, & Toppare, 2013). Conducting polymer based biosensors bring simple, accurate, reliable and low-cost determination of various analytes and act as a very effective analytical tool in the food quality control, selectivity and high sensitivity (Gvozdenovic et al., 2011; Kesik et al., 2013; Ramanavicius, Ramanaviciene, & Malinauskas, 2006). Furthermore, they function as a three dimensional matrix for biomolecule deposition. Their charge transfer ability serves as excellent matrices for biomolecules providing enzyme mimic environment (Tuncagil, Ozdemir, Odaci Demirkol, Timur, & Toppare, 2011; Türkarslan, Kayahan, & Toppare, 2009).

The flavoenzyme pyranose 2-oxidase (PyOx, glucose 2-oxidase, pyranose: oxygen 2-oxidoreductase, EC 1.1.3.10) is a type of oxido-reductase enzyme. PyOx catalyses C-2/C-3 oxidation of numerous sugars to their corresponding dicarbonyl derivatives (aldos-2-uloses or glycosid-3-uloses), coupled to the reduction of FAD (Halada, Leitner, Sedmera, Halt Rich, & Volca, 2003; Marešová, Palyzová, & Kyslík, 2007). Exhibiting a high affinity to its corresponding 2-keto sugars, with accompanying generation of hydrogen peroxide, PyOx was used in various biotechnological applications in carbohydrate chemistry. The working principle of the biosensor is based on the following:

$$\text{d-glucose} + O_2 \rightarrow \text{2-dehydro-d-glucose} + H_2O_2$$

Glucose oxidase (GOx) is a type of oxido-reductase enzyme that catalyses the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone. GOx shows high resistance to adverse microenvironment conditions such as denaturing agents and acidic medium. (Uzun et al., 2013). Although PyOx and GOx have a similar mechanism, PyOx has several important advantages. The particular

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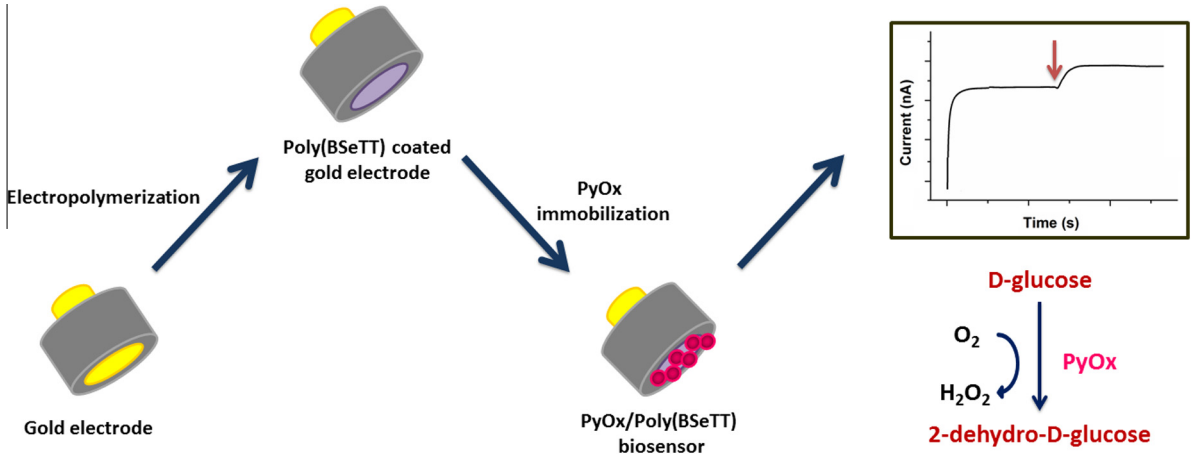

Scheme 1. The typical representation of construction of the proposed biosensor.

advantages in biosensor construction are as follows: its excellent stability and high affinity for D-glucose (km ~1 mM), ability to oxidise efficiently various sugars provide a better alternative than GOx. Moreover, its sensitivity is twice compared to GOx (Odaci, Telefoncu, & Timur, 2008). In the fabrication of biosensors, determination of suitable matrix and immobilisation strategies are the most significant factors. Surface properties of the biosensor also exhibit crucial importance for the successful immobilisation. A suitable matrix should be biocompatible, non-toxic to biorecognition element and durable. In this work, immobilisation of PyOx was performed via physical adsorption within the conducting polymer of 4,7-bis(thieno[3,2-b]thiophen-2-yl)benzo[c][1,2,5]selenadiazole (poly(BSeTT)). Due to the presence of aromatic units in the polymer backbone, the immobilisation was achieved with the help of π–π stacking interactions of the polymer and enzyme molecules. These strong interactions stabilize tertiary structure of proteins effectively (Uzun et al., 2013). For this purpose, BSeTT was synthesized and electrochemical polymerisation of the monomer was performed on gold electrode. After preparation of the gold electrodes, PyOx was immobilised onto the Selenium (Se) containing polymer using glutaraldehyde as the cross linking agent for the construction of glucose biosensor. Due to the biocompatible properties of Se moiety, a robust, high sensitive and long life biosensor can be achieved easily. Due to the unique redox property of Selenium, Se-containing polymers led researchers to explore many opportunities in various applications. Moreover, it was reported that Se shows a protective role against oxidative stress (Valencia-Rodriguez et al., 2012). Hence, Se containing conducting polymers help developing efficient, rapid and high accuracy glucose biosensors. A preparation of proposed biosensor was depicted in Scheme 1. Optimisation and characterisation studies were performed to achieve the best results in fabrication of the biosensor. The application of biosensor was tested via determining glucose in beverages.

2. Material and methods

2.1. Materials

Pyranose oxidase (PyOx; pyranose: oxygen 2-oxidoreductase, E.C.1.1.3.10, from *Coriolus* sp. (10.8 U/mg solid), glucose, $NaClO_4$, $LiClO_4$ were purchased from Sigma–Aldrich and used with no further purification. Dichloromethane (DCM), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). For enzyme immobilisation, a phosphate buffer solution (pH 7.0) consisting of 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company) was used. As the substrate, a glucose solution was prepared by dissolving 0.18 g of glucose in 10 mL pH 7.0 buffer solution. All chemicals were of analytical reagent grade.

2.2. Apparatus

For amperometric measurements, a Palmsens potentiostat (Palm Instruments, Houten, The Netherlands) was used. Electropolymerisation was performed with Voltalab 50 potentiostat. All electrochemical measurements were performed in a three-electrode cell consisting of gold electrode (BaSi (AUE) 1.6 mm diameter) as the working electrode. A platinum wire as the counter electrode, and a Ag wire as the pseudo reference electrode were employed. Amperometric measurements were performed in a three-electrode system. In amperometric analyses, the data were given as the average of three measurements and standard derivations were recorded as ±SD. All measurements were performed at ambient conditions (25 °C). For surface investigation of the biosensor, scanning electron microscope (SEM) (JEOL JSM-6400 model) was used. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Spectrospin Avance DPX-400 Spectrometer. Chemical shifts were given in ppm downfield from tetramethylsilane. HRMS study was done with a Waters SYNAPT MS system. Electrochemical Impedance Spectroscopy (EIS) was performed with a GAMRY Reference 600 (GAMRY Instruments Inc., Pennsylvania, USA).

2.3. Synthesis of the monomer BSeTT

Synthesis and characterisation of the monomer, BSeTT was carried out according to a previously described method (Toksabay, Hacıoğlu, Unlu, Cırpan, & Toppare, 2014).

After bromination of benzothiadiazole, the product, 4,7-dibromo-2,1,3-benzothiadiazole, was reduced to achieve 3,6-dibromobenzene-1,2-diamine. To obtain 4,7-dibromobenzo[c][1,2,5]selenadiazole, the diamine product was reacted with selenium dioxide. Subsequently, the product was coupled with stannylated thienothiophene using Stille coupling reaction. Then, the product was subjected to column chromatography to afford dark purple solid to have the monomer 4,7-di(thieno[3,2-b]thiophen-2-yl)benzo[c][1,2,5]selenadiazole (BseTT). Scheme S1 shows synthetic route of the BSeTT.

2.4. Biosensor preparation

Before the experiments, the gold electrode surface was initially polished with alumina powder and was conditioned in 0.5 M H_2SO_4 solution by cycling the potential between 0 and +1.5 V until

a reproducible voltammetric response was obtained. The electrochemically prepared polymer was constructed on bare gold electrode. Electrochemical polymerisation on the electrode surface was carried out with cyclic voltammetry scanning the potential between 0.0 and 1.3 V up to 10 cycles (at a scan rate of 100 mV s^{-1}) as shown in Fig. 1A. As seen from the figure, while the polymerisation proceeds during the scan, the increase in the current density shows the successful deposition of the polymer on the working electrode. The deposited charge increases with the increasing cycles. After the polymerisation, polymer film was rinsed with distiled water to get rid of impurities.

Pyranose oxidase (PyOx) was immobilised onto the conducting polymer using glutaraldehyde. 10 μL of PyOx solution (50 mM pH 7.0 sodium phosphate buffer solution containing 6.5 U PyOx) were spread over the polymer coated electrode surface and 5 min later, 5 μL glutaraldehyde (GA) solution was added on the electrode surface. Then the electrode was left to dry for 3 h at room temperature. The electrodes were stored in a refrigerator at 4 °C when not in use. Before use, it was washed with distiled water to remove un-bound enzyme molecules. Hence, conducting polymer modified biosensor was achieved successfully.

2.5. Amperometric biosensor measurements

Amperometric determination of the biosensor response was achieved in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.0) by applying constant potential at −0.7 V and following the decrease in oxygen level as a result of enzymatic reaction. Before each measurement, enzyme electrodes were left in 10 mL phosphate buffer (pH 7.0). After the background current reached a steady state, glucose as a substrate was injected in the working buffer solution and then current change was monitored.

3. Results and discussion

3.1. Optimisation studies

Development of an effective immobilisation of biomolecules is very essential during biosensor construction in order to obtain sensitive and robust biosensor. For this reason, optimisation studies are very crucial. To determination of optimum conditions for the constructed biosensor, several parameters such as pH, enzyme amount, cycle number were investigated.

Firstly, optimum polymer thickness was determined by tuning the scan number during electropolymerisation. Thickness of the polymer film was adjusted by the duration of electropolymerisation. In order to investigate the effect of polymer layer thickness, monomer was polymerised on the gold electrode with different scan numbers (15, 30, 45 and 60 scans) and their biosensor response to the substrate were compared by keeping the other parameters constant (Fig. 1B). The highest signal was recorded for the biosensor coated with 30 scans deposition which corresponds to 15.6 nm (equivalent of 0.7 mC charge) in thickness. Thickness of the polymer layer is very important to arrange satisfying immobilisation matrix. Electrochemically generated polymers are used to enhance for three dimension structure of the enzyme molecules as well as providing an excellent environment for the enzyme immobilisation. Optimum thickness of the polymer

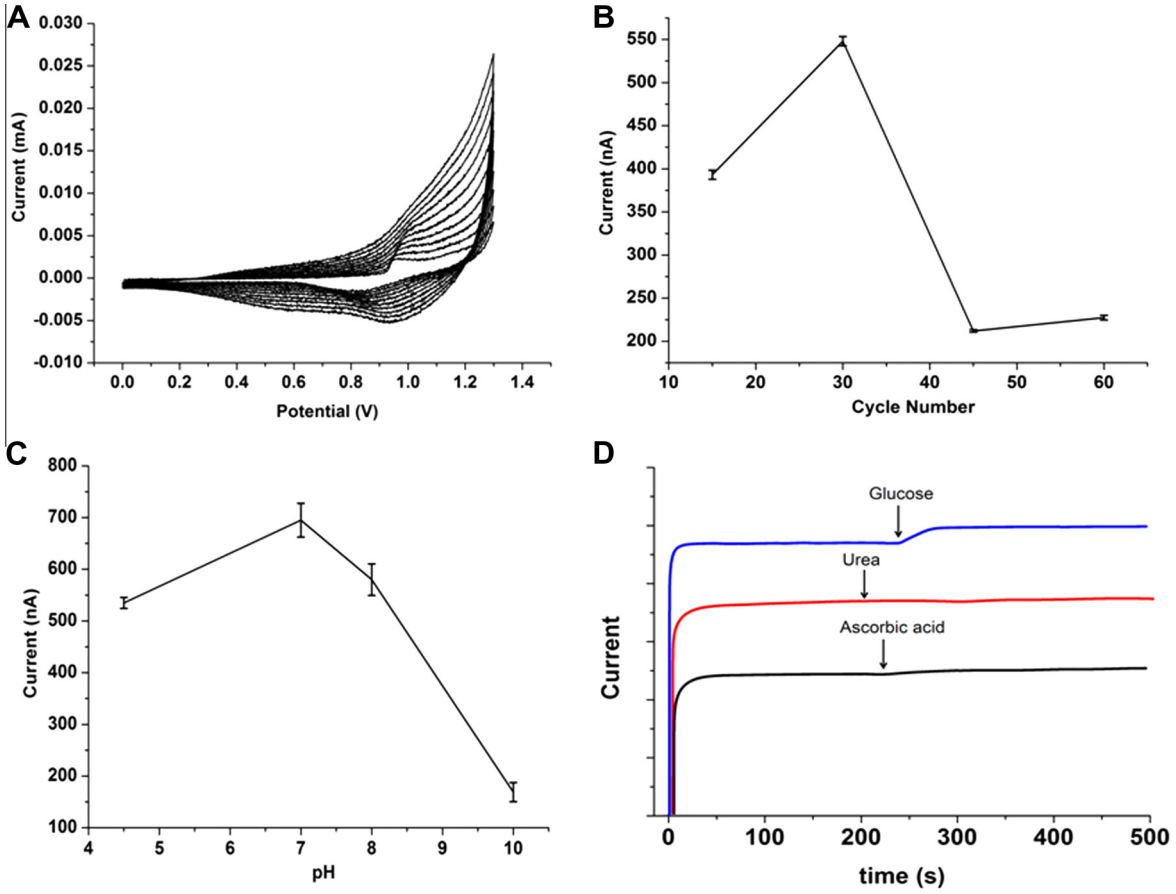

Fig. 1. (A) Repeated potential-scan electropolymerisation of BSeTT on gold electrode (up to 10 cycles), effect of (B) scan number, (C) pH, (D) interferents on the biosensor response (in phosphate buffer, 50 mM, pH 7.0, 25 °C, −0.7 V, [glucose]: 0.5 mM). Error bars show standard deviation of three measurements.

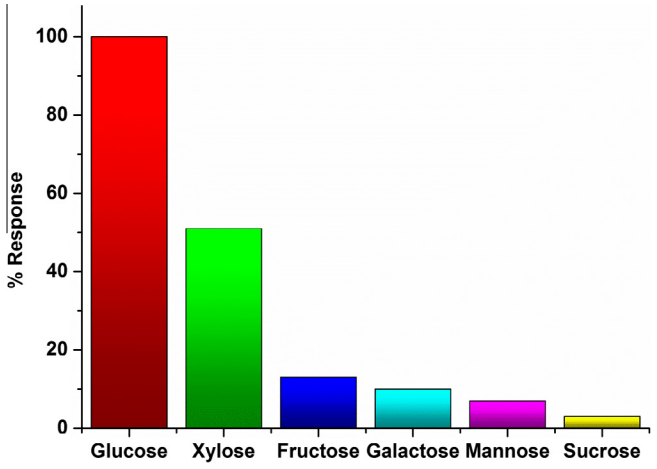

Fig. 2. Carbohydrate analysis using PyOx biosensor (phosphate buffer: 50 mM, pH 7.0, −0.7 V.

brings the better morphology and film quality for generation of biosensor. Therefore, optimum cycle number was chosen as 30 scans and used for subsequent experiments.

The effect of amount of PyOx on the amperometric response was also investigated in the presence of 0.5 mM glucose. Different amounts of the enzyme 0.4 mg (4.32 U), 0.6 mg (6.48 U), 0.7 mg (7.56 U) and 0.8 mg (8.64 U) were immobilised on the modified electrode surface and optimum amount of enzyme was found as 0.6 mg. As illustrated in Fig. S1, optimum biosensor response was recorded with the optimum amount of enzyme.

The effect of pH on the stability of the constructed biosensor was investigated using 0.5 mM glucose as the substrate. The activity of enzymes is directly affected by the pH of the reaction medium. For this reason, the effect of pH on the amperometric biosensor response was examined using 50 mM buffer solutions in a range of pH 4.5–10.0 (sodium acetate buffer at pH 4.5, sodium phosphate buffer at pH 7.0 and 8.0; sodium bicarbonate at pH 10.0, 25 °C.) in the presence of glucose (0.5 mM). During the experiments freshly prepared enzyme electrodes were used. As shown in Fig. 1C, optimum enzyme activity was found at pH 7.0. Therefore, for further experiments, pH 7.0 sodium phosphate buffer was used as the working buffer solution.

In addition, investigation of effects of interferents such as ascorbic acid and urea (between 0.1 and 1 mM) were studied as the substrates in a reaction medium containing 50 mM, pH 7.0 phosphate buffer solution by applying −0.7 V. As shown in Fig. 1D no significant response was recorded for the interferents. Hence, proposed biosensor was very suitable for the glucose determination.

For determination of substrate specificity of the proposed biosensor various sugars (0.5 mM in 10 mL phosphate buffer solution) such as xylose, fructose, galactose, mannose and sucrose were investigated. The amperometric response of glucose was accepted as 100% and biosensor responses obtained for the other substances were correlated accordingly (Fig. 2). The results exhibited good correlation with the other biosensors reported in literature (Liden, Volc, Marko-Varga, & Gorton, 1998; Odaci et al., 2008; Ozdemir, Yeni, Odaci, & Timur, 2010; Timur, Yigzaw, & Gorton, 2006).

3.2. Surface characterisation

The surface morphology of polymer film before and after PyOx immobilisation was monitored by SEM technique. In Fig. 3A, the image was taken after 30 cycles of electrocopolymerisation on gold electrode. The morphology of the Se containing conducting polymer, poly(BSeTT) (Fig. 3A), has a uniform cauliflower-like structure. In Fig. 3B, after biomolecule immobilisation, surface morphology was greatly changed. This change may be due to the orientation of enzyme molecule while protection of their active three dimension conformation. Due to the well interaction and suitable organisation of conducting polymer and enzyme molecule, enzyme molecules cover the electrode surface properly. Furthermore, the difference in morphology confirmed the successful biomolecule immobilisation.

3.3. Electrochemical characterisation

Cyclic voltammetry (CV) studies were carried out in 5 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl to characterise the electrode surface after each layer construction (Fig. 4A). In case of the bare electrode (gold) 85 μA of current was observed. When poly(BSeTT) was coated on the bare electrode, the increase in peak currents (271 μA) can be attributed to the increase of effective surface area due to the electrodeposited polymer layer. Poly(BSeTT) provides a conductive pathway for electron transfer and promote electron-transfer reactions. On such surface, PyOx was immobilised and the decrease of oxidation current (189 μA) confirmed the effective attachment of the biomolecule on the electrode. This was the case owing to the insulating character of the biological molecules.

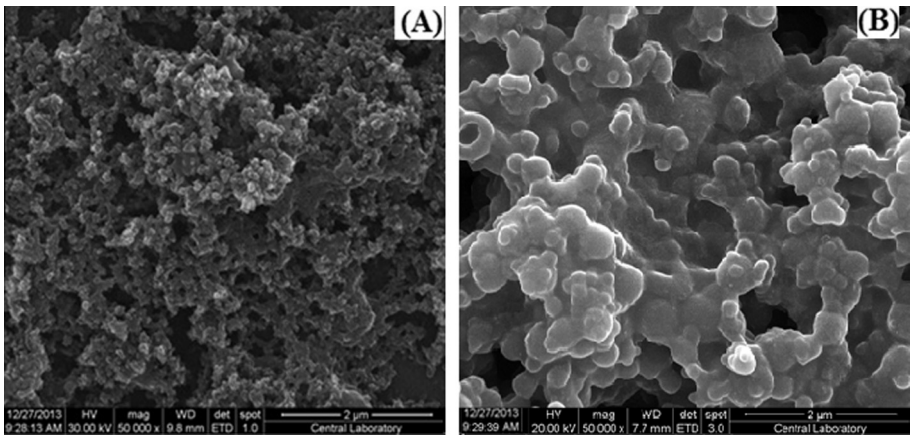

Fig. 3. Surface characteristics of (A) conducting polymer (poly(BSeTT)) (B) PyOx immobilised conducting polymer (poly(BSeTT)) via SEM images.

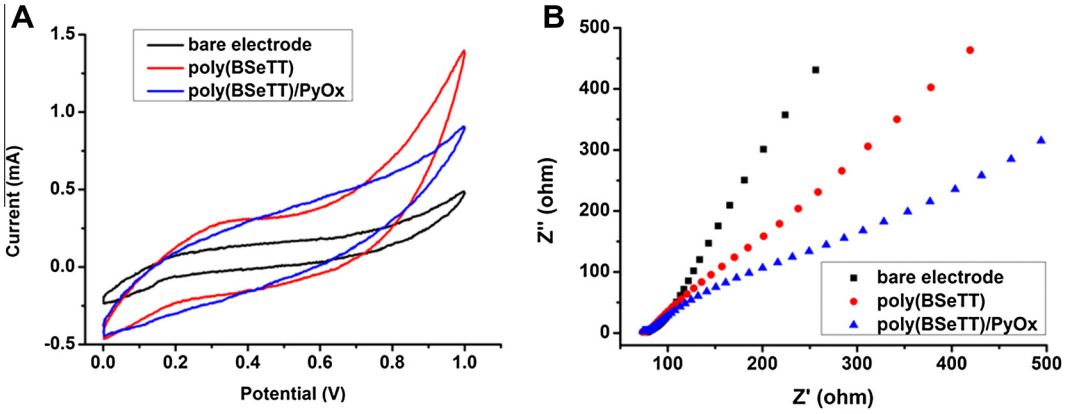

Fig. 4. (A) Cyclic voltammograms and (B) typical Nyquist plots resulting from the bare electrode, poly(BSeTT), poly(BSeTT)/PyOx in 5.0 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl.

The average value of the electroactive surface area was calculated according to the Randles–Sevcik equation (Bard & Faulkner, 2000):

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} \gamma^{1/2} C$$

where n is the number of electrons participating in the redox reaction, A is the area of the electrode (cm^2), D is the diffusion coefficient of the molecule in solution ($cm^2\ s^{-1}$), C is the concentration of the probe molecule in the bulk solution ($mol\ cm^{-3}$), and γ is the scan rate ($V\ s^{-1}$). According to the equation, the increase in the peak currents can be attributed to an increase in the effective surface area. The electroactive surface area for bare electrode, poly(BSeTT) and poly(BSeTT)/PyOx modified electrodes were 0.076, 0.241, 0.168 cm^2, respectively.

Electrochemical Impedance Spectroscopy (EIS) was carried out to characterise the interface properties of the modified electrodes at the surface during the fabrication process of the biosensors (Lisdat & Schäfer, 2008; Randviir & Banks, 2013). Electron transfer between the solution species and the electrode surface occurs by tunneling through the barrier. In a Nyquist plot, the semicircle portion corresponds to the electron-transfer resistance at the higher frequency range which controls the electron transfer kinetics of the redox probe at the electrode surface. The semicircle diameter equals to the electron transfer resistance. Such resistance controls the electron-transfer kinetics of the redox probe at the electrode interface. Moreover, linear part of the plot at lower frequency range represents the diffusion limited process. EIS study was performed on the modified electrodes in 5.0 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl solution with a frequency range 1 Hz–200 kHz via applying 5 mV. Fig. 4B illustrates typical Nyquist plots obtained for bare electrode; (a) poly(BSeTT), (b) poly(BSeTT)/PyOx using $Fe(CN)_6^{3-/4-}$ as the redox probe. It can be seen that a very small interfacial resistance was exhibited. After coating the electrode surface with poly(BSeTT), a very small interfacial resistance was revealed and the semicircle diameter decreased due to excellent electrocatalytic activity even the presence of an additional layer onto the bare electrode. This indicates that conducting polymer coating makes high electron conduction pathways between the electrode and solution. After PyOx was immobilised onto the coated electrode surface, the semicircle diameter increased considerably since the layer blocked the redox probe. Moreover, since most biological molecules were poor electrical conductors at low frequencies, this increase in charge transfer resistance was the direct evidence of successful immobilisation of enzyme on the modified transducer surface. These results were consistent with cyclic voltammetry studies (Fig. 4A).

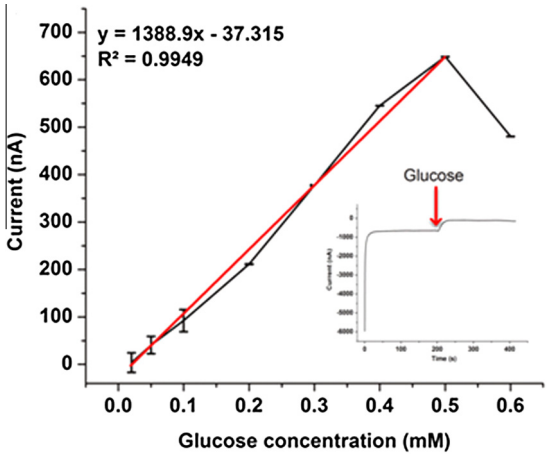

Fig. 5. (A) Calibration curve for glucose (in 50 mM phosphate buffer, pH 7.0, 25 °C, −0.7 V). Error bars show standard deviation of three measurements (A typical amperometric response to 0.5 mM glucose in phosphate buffer, 50 mM, pH 7.0 given as inset), (B) some characteristics of the proposed biosensor.

3.4. Analytical characterisation

After all optimisation studies, calibration curve for glucose was constructed (Fig. 5A). The relationship between the concentration of the substrate and the current change was obtained in 0.02–0.5 mM concentration range using a calibration curve. This is represented by an equation ($R^2 = 0.9949$) given in Fig. 5. When the concentration of glucose was higher than 0.5 mM, substrate saturation was observed. Hence, a perfect linearity was obtained between 0.02 and 0.5 mM glucose concentration. Moreover, limit of detection (LOD) was calculated as 3.3×10^{-4} nM for glucose as S/N = 3 criteria. K_M^{app} and I_{max} values which were determined from the Lineweaver–Burk plot and sensitivity values were calculated as 0.229 mM, 42.37 nA and 6.4 nA/mM cm^2, respectively. Hence, with the help of the modified polymer matrix, immobilised enzyme molecules exhibit higher affinity toward the glucose. Biosensor repeatability was tested by eight successive measurements in the same day with 0.5 mM glucose solution. Standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.365% and 0.1%, respectively. A newly prepared PyOx biosensor at optimum conditions (pH 7.0, 50 mM phosphate buffer) was used for the determination of the operational stability of biosensor. In a period of 5 h, 8 measurements were done using 0.5 mM glucose as the substrate. 11% activity decrease was found in biosensor

Table 1
Glucose detection in various beverages. (All measurements were conducted three times; standard deviations were calculated.)

Sample	Glucose content		Relative error (%)
	Product label (mM)	Poly(BSeTT)/PyOx biosensor (mM)	
C® coke	0.311	0.308	0.97
I® ice tea	0.194	0.198	−2.02
S® milk	0.125	0.122	2.45

response. Among several PyOx biosensor studies reported previously in the literature, operational and shelf life stabilities were found to be superior (Odaci, Telefoncu, & Timur, 2010; Ozdemir et al., 2010). One can notice that use of a Se containing conducting polymer contributes to the improvement of operational stability. Emre et al. reported that Se containing conducting polymer improves the biosensor performance (Bilge Emre et al., 2011). Moreover, Se acts as an antioxidant that prevents the cell degeneration of tissues; providing biocompatible environment for biosensor construction (Valencia-Rodriguez et al., 2012). With this information, a transducer surface with conducting polymer modification is an excellent platform for the enzyme immobilisation to obtain a fast response and increasing the shelf-life of biosensor. Hereby, a Se containing conducting polymer was used to construct a PyOx biosensor. This biosensor represents a promising analytical tool for food analysis due to its high stability and excellent sensitivity compared to several PyOx biosensors.

3.5. Detection of glucose content in beverages

The proposed biosensor was tested to analyse the glucose concentration in coke, ice tea and milk under optimised conditions. The target samples were injected into the reaction cell instead of glucose without any pretreatment and amperometric responses were recorded (the experiments were repeated three times for each sample). Then, the concentration of samples was calculated using a calibration graph (Fig. 5). The results were compared with glucose concentrations reported by product label of the samples in order to confirm the accuracy of the biosensor (Table 1). As illustrated in Table 1, the results are in considerably good agreement, proving the reliability and accuracy of the biosensor system. Thus, it is an accurate method for glucose test without any matrix effect in real samples. Moreover, this target system is applicable for real time analysis; therefore it is more favourable for routine analysis due to serving several advantages like simple measurement procedure, short response time, easy to fabricate and sufficient sensitivity and selectivity.

4. Conclusions

Novel selenium containing conducting polymer based PyOx biosensor was fabricated to determine glucose content. For the construction of biosensor, physical adsorption technique was used through the enzyme molecule and polymer. The surface morphology was confirmed by SEM images. Also, electrochemical properties were characterised by EIS and CV techniques. The fabricated biosensor shows very good kinetic parameters such as K_M^{app}, I_{max}, low LOD and high sensitivity. After optimisation studies, the biosensor was successfully applied for the determination of glucose in the beverages showing the satisfactory results. Hence, the proposed sensing system becomes an important tool for glucose test for real time analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.065.

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