Talanta 118 (2014) 84–89

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a novel biosensor based on a conducting polymer

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ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form
30 September 2013
Accepted 3 October 2013
Available online 10 October 2013

Keywords:
Conducting polymer
Cholesterol biosensor
Amperometric biosensor
Cholesterol oxidase

ABSTRACT

A new type of amperometric cholesterol biosensor was fabricated to improve the biosensor characteristics such as sensitivity and reliability. For this purpose, a novel immobilization matrix 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole (BIPF) was electrochemically deposited on a graphite electrode and used as a matrix for the immobilization of cholesterol oxidase (ChOx). Due to strong π–π stacking of aromatic groups in the structures of polymer backbone and enzyme molecule, one can easily achieve a sensitive and reliable biosensor without using any membrane or covalent bond formation between the enzyme molecules and polymer surface. Moreover, through pendant fluorine group of the polymer, H-bond formation between with enzyme molecules and polymer was generated. Cholesterol was used as the substrate and amperometric response was measured in correlation with cholesterol amount, at −0.7 V vs. Ag/AgCl in phosphate buffer (pH 7.0). Consequently, optimum conditions for this constructed biosensor were determined. $K_M$app, I_{max}, LOD and sensitivity values were investigated and calculated as 4.0 nM, 2.27 μA, 0.404 μM and 1.47 mA/mM cm^2, respectively. A novel and accurate cholesterol biosensor was developed for the determination of total cholesterol in food samples.

1. Introduction

Biosensors have attracted great attention due to their easy recognition of various important analytes in biological systems [1]. A biosensor represents two main parts: (1) a recognition part in which biological component is immobilized on a solid surface and (2) a transducer. Combination of these parts allows measuring a target analyte without using reagents. The aim of the biosensor is to convert a biological event into an electrical signal [2]. Solid support is used to anchoring a sensing molecule on transducer. It should be adaptable to different environments and resistant to a wide range of physiological conditions.

Conducting polymers (CPs) have attracted great interest since they were used for construction of the electrochromic devices [3,4] and as suitable matrices for enzyme molecules due to enhanced sensitivity, versatility of biosensors for the detection of desired substances [5]. In fabrication of efficient cholesterol biosensors conducting polymers are the most studied materials for matrix preparation [6–10]. Due to their good electrochemical and physical properties, they can be used as immobilization matrices in the preparation of biosensors [11,12]. Besides, CPs afford increased surface area, thickness control, produce a long-life biosensor, as well as enhanced electron transfer during the electrochemical reactions on the electrode surface [13].

There are several problems as regards to loss of enzyme on a support surface and maintenance of enzyme stability and shelf life of the biosensors. In order to overcome these problems, several enzyme immobilization methods such as adsorption, covalent bonding, entrapment and cross-linking have been used to fabricate desired biosensors [14]. The procedure of biomolecule immobilization on conductive surfaces remains as a fundamental step for the production of mechanically durable and stable biosensors. To improve the performance of the biosensor, it is preferable to find a managable immobilization method and a stable material that can maintain the biocatalytic activity of the biomolecules. Physical adsorption is favored by many researchers due to the simple adsorption of the enzyme molecules onto the number of CPs where it is fixed on the surface by hydrogen bonding and Van der Waals forces [15,16]. By the help of these bonding forces, entrapment of enzymes in conducting polymers provides the localization of biologically active molecules on electrodes of any size or three-dimensional geometry to fabricate amperometric biosensors [17]. This method has been used for the preparation of many biosensors [18–20].

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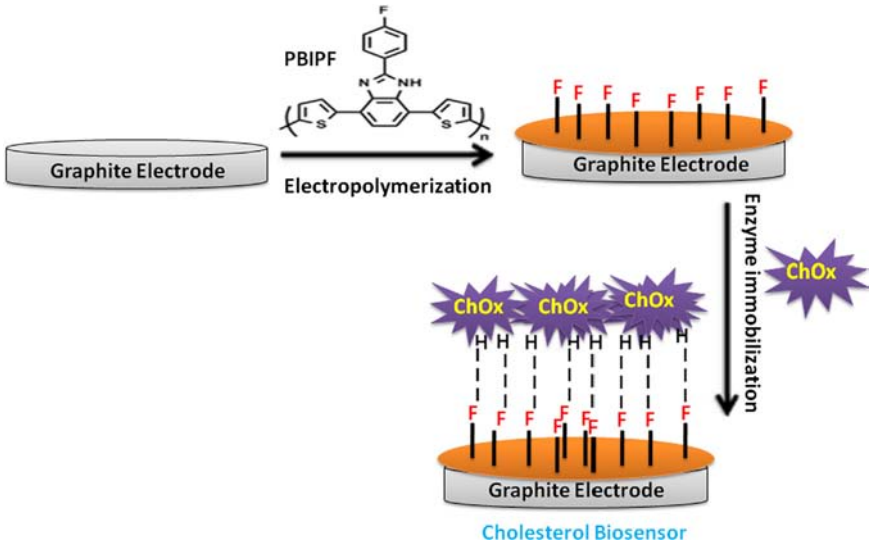
http://dx.doi.org/10.1016/j.talanta.2013.10.007



Scheme 1. A schematic representation of the proposed biosensor.

Cholesterol oxidase (ChOx) which catalyzes the oxidation of cholesterol by molecular oxygen to 4-cholesten-3-one and hydrogen peroxide is an industrially important enzyme molecule for clinical determination of cholesterol. Since several clinical disorders such as hypertension, arteriosclerosis and coronary artery disease increase the alarming level, determination of cholesterol in food samples is very important in an industrialized world [2]. Hence, the development of a new sensitive and durable biosensor is an important aspect of biosensor technology.

In this study immobilization of cholesterol oxidase (ChOx) was performed via the adsorption technique onto a conducting polymer of 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole (BIPF). Electrochemical polymerization of BIPF monomer was undertaken by cyclic voltammetry in acetonitrile (ACN)/dichloromethane (DCM) solution. The polymer was chosen as the immobilization matrix since it has strong π–π stacking of aromatic groups and fluorine group which is open to generate hydrogen-bonding. Additionally, use of conducting polymer as the host matrix for immobilization of ChOx enhanced the electron transfer during the enzymatic reactions. Conducting polymer was electrochemically deposited on a graphite electrode surface by cyclic voltammetry technique. Electropolymerization enables to control morphology and thickness of the polymer [6]. Hence, immobilization of biomolecules by electrochemically generated polymers can be achieved succesfully. ChOx was immobilized onto the polymer coated electrode to construct the amperometric cholesterol biosensor. Immobilization of biological molecule onto the solid support is a fundamental step in the development of a stable and robust biosensor. This immobilization was performed by physical adsorption process where the enzyme molecules were adsorbed in the polymer interface due to π–π stacking effect and the hydrogen bond formation between the functional groups of enzyme molecule and fluorine of conducting polymer. Since immobilization matrix has functional fluorine moiety in its structure, it is easy to achieve sensitive and reliable biosensor without using any membranes or covalent bond formation between the enzyme molecules and polymer surface. It was reported that attachment of the enzyme molecules to the functionalized conducting polymers can be obtained by affinity of the functional groups that can selectively interact with tags on the biomolecules [6]. Moreover, taking the advantage of conductivity and redox stability, conducting polymers can be modified for certain purposes. With this motivation, BIPF was designed and synthesized. To incorporate biomolecules and to improve the binding, novel monomer containing particular functional group and aromaticity were proposed. There are limited applications of adsorption technique for conducting polymer and enzyme based biosensors which display the novelty and value of the present work [21–25].

Hence, an efficient, highly sensitive and fast response biosensor was successfully fabricated in this work. A representative preparation for the proposed biosensor is depicted in Scheme 1. The obtained biosensor was characterized by scanning electron microscopy (SEM) technique. Optimization and characterization studies were done and practical application of modified electrode was tested via determining total cholesterol in food samples.

2. Material and methods

2.1. Materials

Cholesterol oxidase (E.C.1.1.3.6) (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, Triton-X 100 and glutaraldehyde were purchased from Sigma-Aldrich and used with no further purification. A solution of cholesterol (0.005 M) was freshly prepared by dissolving cholesterol in 1% (v/v) Triton-X 100 in 2-propanol (Merck). This mixture provides solubility and stability of cholesterol in aqueous solutions at room temperature. To obtain a clear solution it was then diluted with 50 mM PBS (pH 7.0) consisting of 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company) and distilled water. The chemicals used in the synthesis of the monomer were purchased from Sigma Aldrich. All other chemicals were analytical grade. Cholesterol oxidase colorimetric kit was obtained from Human GmbH-65205 (Wiesbaden-Germany).

2.2. Apparatus

All electrochemical measurements were performed using an Ivium CompactStat (The Netherlands) potentiostat in a reaction cell equipped with three electrodes consisting of a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, platinum electrode as the counter electrode and Ag/AgCl electrode (3 M KCl filled) as the reference electrode. Electropolymerization was performed with a Voltalab 50 potentiostat. Measurement of amperometric analyses were calculated as an average of four measurements and standard derivations were given as ± SD. Scanning electron microscope (SEM) (JEOL JSM-6400 model) was used for investigation

of the surface morphology of the constructed cholesterol biosensor.

2.3. Synthesis of 2-(4-fluorophenyl)-4,7-di(thiophen-2-yl)-1H-benzo[d]imidazole (BIPF)

Synthesis of the monomer, BIPF was performed according to previously published procedure [26]. 3,6-Dibromo-1,2-phenylenediamine was synthesized by bromination of 1,3-benzothiadiazole and then reduction by $NaBH_4$. After condensation reaction of this product with 4-fluorobenzaldehyde, 4,7-dibromo-2-(4-fluorophenyl)-1H-benzo[*d*]imidazole was synthesized as the acceptor unit. Finally synthesis of BIPF was afforded by Stille coupling of acceptor unit with tributyl(thiophen-2-yl)stannane.

2.4. Preparation of conducting polymer based biosensor

Before the electropolymerization, graphite rods were polished on emery paper and washed with distilled water. The electrochemically prepared polymer was constructed on cleaned graphite electrode. Polymerization of monomer was performed potentiodynamically on graphite using 0.1 M sodium perchlorate–lithium perchlorate ($NaClO_4$–$LiClO_4$) electrolyte in 5:95 DCM:ACN solution with repeated scan intervals between 0.0 and 1.3 V via cyclic voltammetry with a scan rate of 100 mV s^{-1} for 30 cycles (Fig. 1). After electropolymerization polymer coated electrode was rinsed with distilled water to get rid of the possible organic impurities. Charge involved in the film formation was determined as 0.36 mC/cm^2 and the thickness of the polymer film was estimated as 56 nm. The suitable film was achieved with the mentioned electropolymerization.

ChOx solution (1 mg ie. 26 U) in 5 μL, 50 mM phosphate buffer (pH 7.0)) was immobilized onto the conducting polymer and glutaraldehyde (5 μL, 1%, in 50 mM phosphate buffer, pH 7.0) was spread over the electrode surface as a cross linking agent to prevent the release of the encapsulated enzyme molecules from the polymeric film. Electrode surface was left to dry at room temperature for 2 h. Before use, the enzyme electrode was rinsed with distilled water to remove unbound enzyme molecules and reagents. The modified electrode was stored at 4 °C for overnight.

2.5. Amperometric biosensor measurements

All amperometric studies were carried out at room temperature in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.0) under mild stirring. After each measurement buffer solution was refreshed and electrodes were washed with distilled water and kept in phosphate buffer solution (50 mM, pH 7.0) for 3 min. In amperometric studies, the decrease in oxygen level as a result of enzymatic reaction was monitored at −0.7 V vs. Ag/AgCl and correlated with the substrate concentration. For the determination of working potential in amperometric studies, responses of the constructed biosensor for 25 μM cholesterol were recorded at different potentials (−0.8 V; −0.7 V; 0.0 V and 0.2 V). In cholesterol detection using ChOx as the enzymatic catalyst, the optimal potential range as the most sensitive and interference-free was found at −0.7 V. No meaningful responses were recorded at potentials higher than 0.2 V and lower than −0.8 V.

When the baseline current reached constant, freshly prepared substrate (cholesterol) was added to the medium; the current immediately changed and reached a steady state. The differences between these current values were recorded as the biosensor response. All experiments were carried out at ambient conditions. The reaction medium (buffer solution) was refreshed just before each measurement. During the experiments, substrate solution was stored in dark at room temperature.

3. Results and discussion

3.1. Optimization studies

To obtain satisfactory biosensor responses, biosensor performance must be optimized. All experimental parameters affecting the three dimensional structure of the enzyme molecules were optimized to enhance the interactions between the enzyme molecules and surface of the conducting polymer. The polymer thickness was controlled by adjusting scan number during electropolymerization. In order to investigate the effect of polymer layer thickness, monomer was polymerized on the working electrode with different scan numbers (20, 30, 50 and 70 scans). The stability of the immobilized enzyme molecule on an electrode surface can be affected by the distance between the active side of the enzyme molecule and thickness of the polymeric layer. If the layer is too thick electron transfer between the electrode and enzyme molecules may be prevented causing a lower charge transfer rate. On the other hand, very thin films are unable to protect the enzyme molecule from the environmental effects which may lead to deterioration of the immobilization matrix characteristics. As seen in Fig. 2, 30 cycle deposition was determined as the optimum thickness for the biosensor preparation.

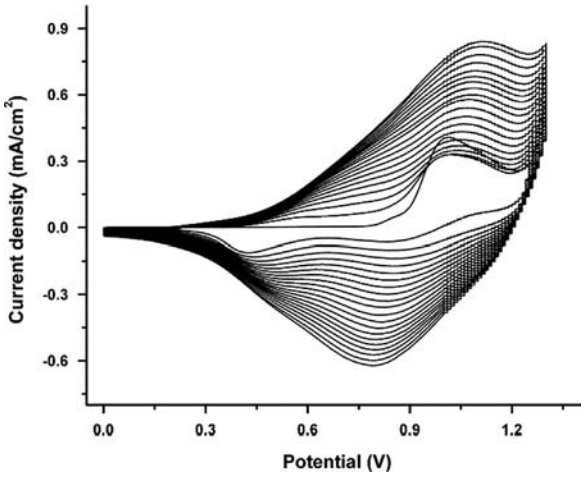


Fig. 1. Repeated potential-scan electropolymerization of BIPF in 0.1 M $NaClO_4$–$LiClO_4$ /DCM/ACN on the graphite electrode (up to 15 cycles).

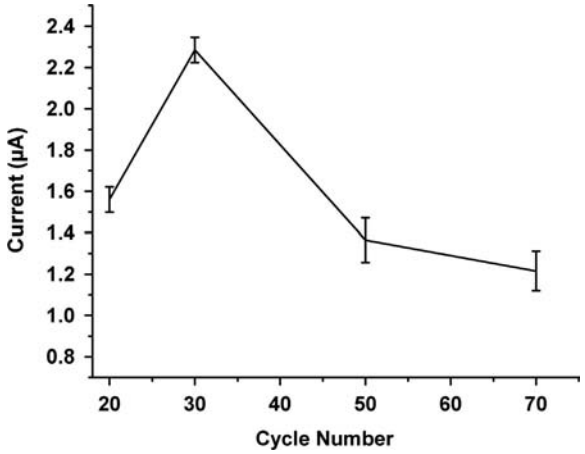


Fig. 2. Effect of cycle number (in phosphate buffer, 50 mM, pH 7.0, 25 °C). The corresponding measurements were performed with 25 μM cholesterol. Error bars show standard deviation (SD) of four measurements.

Without the polymer layer, enzyme molecules could not be fixed onto the graphite surface; hence no current values could be recorded. It was reported that conducting and electrochemically generated polymers improve the properties of constructed biosensor [7,27]. Moreover, polymer membranes can also act as a protective layer for enzyme molecules and screen electrochemically interfering compounds such as ascorbic acid, uric acid from encompassing the electrode surface [28].

To investigate the diffusion capability of the prepared polymeric film, scan rate variation analyses were done. In scan rate studies, current versus scan rate graph showed the linear relationship between scan rates and current for both oxidation and reduction (Fig. 3). The linearity in the peak current shows the processes are non-diffusion controlled and the polymer is well attached to the graphite surface. This means that electron transfer in catalytic reactions on the electrode surface occurs efficiently.

Enzyme amount during the immobilization was also optimized. Different amounts of ChOx from 13 U to 31 U were immobilized on the polymer modified electrode surface and amperometric responses were recorded with respect to 25 μM cholesterol and results were compared. The maximum amperometric responses were obtained with 26 U ChOx. As shown in Fig. 4, efficient connection with enzyme molecules and polymeric layer was not observed at the highest enzyme loading. In lower amounts of ChOx, sufficient responses corresponding to sensitivity could not be recorded. Due to the excess loading in the higher amount of enzyme molecules, enzyme molecules leached from the electrode surface. Moreover, the signals decreased because of the diffusion problems and response times lengthened.

Furthermore, the pH of the solutions in enzymatic reactions was investigated over a pH 5.5–10 with sodium bicarbonate and sodium phosphate buffers. The best result was found as pH 7.0 and for further experiments pH 7.0 phosphate buffer was used as the buffer solution (Fig. 5).

In addition for the biosensor construction, glutaraldehyde (GA) was used as the cross linker. GA has been a critical component to the design of biosensors because of its commercial availability and low cost in addition to its high reactivity. It reacts rapidly with amine groups at the enzyme molecules while maintaining the proper enzyme conformation [29]. Moreover, the aromatic groups of the polymer and aromatic units of the enzyme molecules are in good interaction, the use of GA enhances the compact structure of the enzyme molecules on the polymer. The use of cross linker enhances the compact structure of the enzyme molecules on the polymer. The enzyme molecules were successfully immobilized on the functional polymer. 0.5%, 1.0%, 1.5% and 2.5% GA values were evaluated. 1% GA was found as the optimum value.

3.2. Characterization

Surface morphology of the polymer coated graphite electrode and ChOx immobilized graphite electrode were determined by SEM technique. In Fig. 6(A), the image was taken after 30 cycles of electropolymerization on graphite. From this figure, porous and cauliflower structure of the conducting polymer can be easily seen. The polymer is well-spread over the electrode surface and covers the surface homogeneously. The porous characteristic of the conducting polymer enables the well-immobilization of 3D enzyme molecules. In order to maintain the activities of the enzyme molecules, the 3D structure should be preserved after immobilization. With the successful adsorption on the polymer surface, 3D feature of the protein molecules are protected due to the efficient H-bonding and static interactions in the polymer/enzyme interface. The morphology of the surface changed greatly after immobilization of ChOx in Fig. 6(B). This change can be due to the fact that enzyme molecules are held on the surface of the polymer through the formation of electrostatic interaction and hydrogen bonding between enzyme and polymer. The characteristic structure of the huge enzyme molecules are also easily seen in the figure. The porosity and 3D structure on the electrode

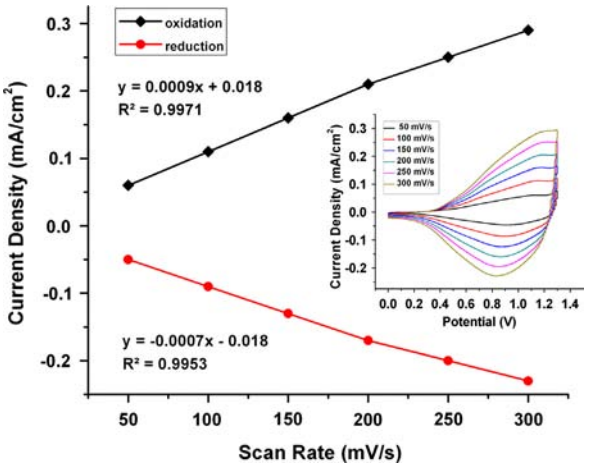


Fig. 3. Scan rate dependence of poly (BIPF) film in 0.1 M $NaClO_4$–$LiClO_4$/DCM/ACN at 50, 100, 150, 200, 250 and 300 mV s^{-1}.

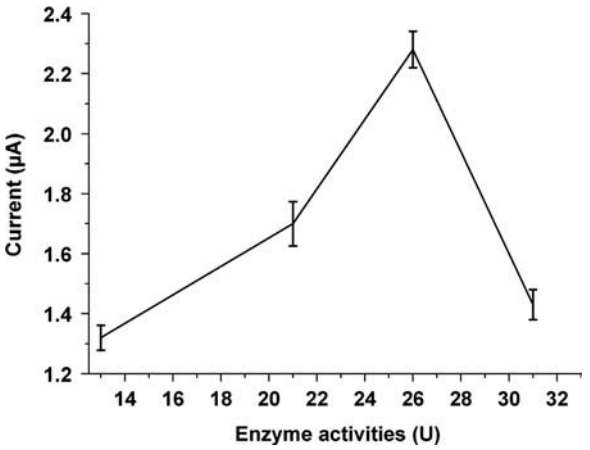


Fig. 4. Effect of loaded enzyme amount (in phosphate buffer, 50 mM, pH 7.0, 25 °C). The corresponding measurements were performed with 25 μM cholesterol. Error bars show standard deviation (SD) of four measurements.

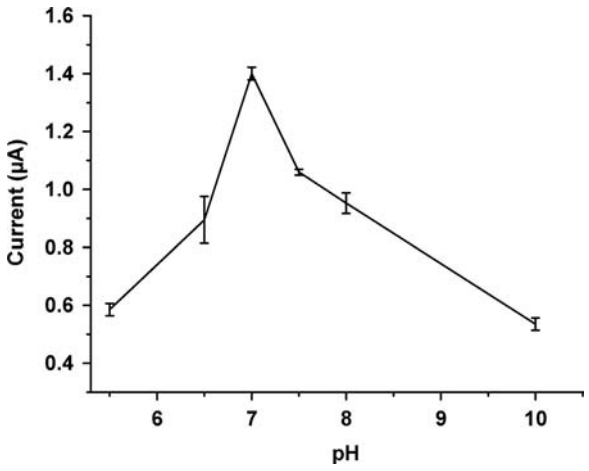


Fig. 5. Effect of pH (50 mM sodium phosphate buffer at pH 5.5; 6.5, 7.0; 7.5 and 50 mM sodium bicarbonate buffer at pH 8.0 and 10.0, 25 °C). The corresponding measurements were performed with 25 μM cholesterol. Error bars show standard deviation (SD) of four measurements.

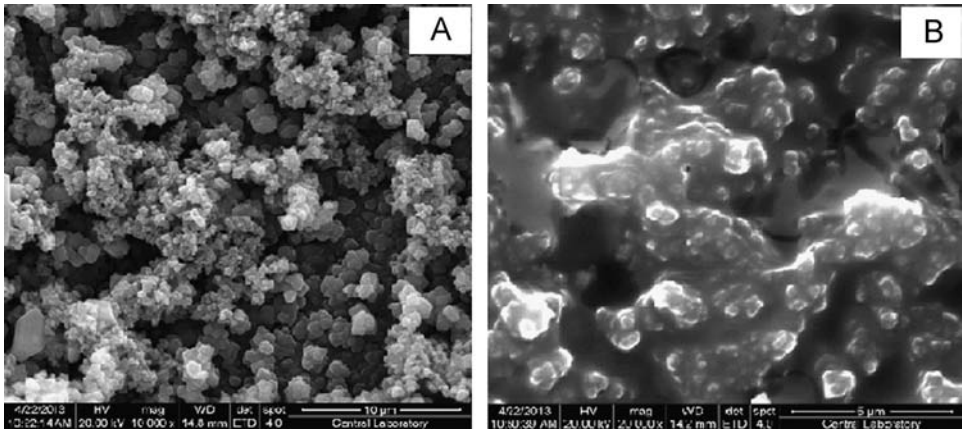


Fig. 6. Surface characteristics of (A) conducting polymer (B) ChOx immobilized conducting polymer coated biosensor SEM images.

Table 1
Some characteristics of the proposed biosensor.

Parameter	
Linear range	0.5–30 μM
$K_M$app	4.0 nM
I_{max}	2.27 μA
LOD	0.4 μM
Sensitivity	1.47 mA/mM cm^2

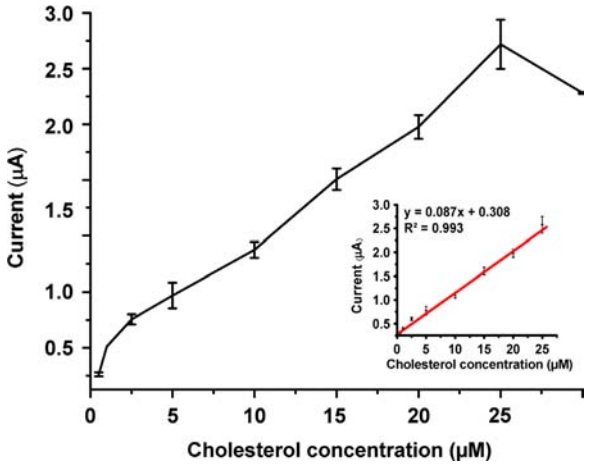


Fig. 7. Calibration curve for cholesterol (in 50 mM phosphate buffer, pH 7.0, 25 °C). Error bars show standard deviation (SD) of four measurements.

surface are still maintained even after the immobilization. This also brings the effective functionality and performance of the enzyme molecules as proved by the amperometric results.

Moreover, three-dimensional structure of enzyme molecules were protected and stabilized during the successful immobilization. This means that satisfactory immobilization can be concluded from the SEM images.

3.3. Analytical properties, repeatability and storage stability of the cholesterol biosensor

Kinetic parameters ($K_M$app, I_{max}), from the Lineweaver–Burk plot and sensitivity were calculated and shown in Table 1. With the optimum conditions, calibration curve for cholesterol was plotted according to the responses of biosensor for different amounts of cholesterol (Fig. 7). The biosensor showed a very low limit of detection (LOD) value which was calculated using S/N (signal-to-noise ratio)=3 criterion.

Reports are available for determination of cholesterol based on different matrices using the immobilization techniques (Table 2). Zhu et al. [30] have recently prepared a cholesterol amperometric biosensor gold nanoparticles (GNPs) and carbon nanotube (CNT) modified electrode. The K_M of this cholesterol biosensor was found to be 0.29 mM. Batra et al. [31] designed at a Zinc oxide (ZnO) based platinum coated silicon (Pt/Si) cholesterol biosensor and calculated the K_M value as 1.08 mM. In the present paper, $K_M$app value of the optimized enzyme biosensor was calculated as 4.0×10^{-6} M, respectively and extremely smaller than the ones reported in earlier studies and the free enzyme. The conformational changes are known to affect an enzyme reaction and nature of immobilization matrix. This shows the effectiveness and success of the immobilization matrix, sensitivity of the biosensor and applicability in other operations. Enzyme activity increases with favorable conformational changes and results in an increased interaction between the substrate and active cite of the enzyme molecule. Hence, obtained lower K_M value is the indication of higher substrate affinity.

To test the repeatability of the cholesterol biosensor response, six successive measurements were obtained in the same day with 25 μM cholesterol solution. Standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.086 and 7.07% respectively.

The lifetime of the modified electrode was determined by measuring the amperometric response during 28 days. During the experiments activity loss of 1.3% was observed in 28 days. This long period shows the satisfactory result of reliability and superiority of the constructed biosensor. The biosensor was kept at +4 °C when not in use.

In order to perform accurate analyses with different kind of samples, measurement medium must not include any interference and biosensor must be specific to the target substrate. So, various possibly interfering substances such as ascorbic acid, urea and glucose were investigated in the poly(BIPF)/ChOx biosensor response since these products can be easily oxidized on the electrode surface. For this reason, ascorbic acid, urea and glucose solutions (1 mM–0.1 M) were injected to reaction cell containing 50 mM PBS (pH 7.0). No significant effect of interfering substances was reported during all experiments. Hence, the constructed biosensor was successfully applied in food samples. Moreover, −0.7 V potential was used in all amperometric measurements in order to prevent the possibility to oxidize various electroactive species in the reaction medium. Hence, this is also an advantage for biosensor design and all these results demonstrate the good selectivity of the proposed electrode for real sample measurements.

Table 2
Various studies from the literature based on conducting polymer containing cholesterol biosensors.

Conducting polymer	Immob. tech.	$K_M$app/V_{max} or I_{max}	Linear range	Ref.
Poly(3,4-ethylenedioxypyrrole)	Entrapment	3.4 mM/34 μAcm^{-2}	NR	[9]
Poly(3,4-ethylenedioxythiophene)	Entrapment	1.3 mM/17.9 μAcm^{-2}	NR	[8]
GNPs/MWCNTs	Adsorption	0.29 mM/NR	0.01–5.00 mM	[30]
ZnO/Pt/Si	Adsorption	1.08 mM/NR	0.12–12.93 mM	[31]
Polypyrrole (Ppy)	Entrapment	9.8 mM/NR	1–8 mM	[32]
ZnO/Chitosan/ITO	Adsorption	8.63 mg/dL/NR	5–300 mg/dL	[33]
poly(3-TPAA)/Pt electrode	Covalent	14.53 mM/NR	0–8 mM	[34]
ZnO/Au electrode	Adsorption	2.57 mM/NR	1.0–15 μM	[35]
BIPF/ChOx	Adsorption	4.0×10^{-6} mM/2.27 μA	0.5–30 μM	This work

NR: Not reported.

Table 3
Determination of cholesterol in food samples by constructed biosensor and reference procedure. [a]

Sample	Reference method (mM)	Constructed biosensor (mM)	Relative error (%)
Liquid oil	0.75 ± 0.12	0.70 ± 0.01	6.7
Margarine	3.66 ± 0.01	3.70 ± 0.04	1.1
Butter 1	2.21 ± 0.04	2.15 ± 0.14	2.7
Butter 2	2.68 ± 0.12	2.45 ± 0.45	8.6

[a] *t*- test with 95% confidence level for 4 degrees of freedom and $N_1=3$, $N_2=3$, the calculated values are smaller than the ones for theoretical *t*-test values.

3.4. Detection of cholesterol in food samples

Previously described method was chosen to determine cholesterol in food samples such as butter, margarine and liquid oil [36]. After suitable treatments, food samples were injected (as the substrate) into 50 mM PBS (pH 7.0) for amperometric measurements and current change was monitored. The food samples were analyzed for cholesterol using cholesterol kit as the reference method. It was clearly shown that values of amperometric and reference method were very close to each other. The analysis time using the commercial kits is not very short and also the kit is very expensive for a practical use. On the other hand, using the biosensor technique, immobilization of enzymes on a suitable matrix reduces the cost of the procedure. Hence, the development of cheap, rapid and accurate biosensors for the detection of cholesterol is important due to the importance of the cholesterol in a human body Table 3.

4. Conclusions

Results clearly suggest that conducting polymer film provides an attractive matrix for effective immobilization of cholesterol oxidase. Strong π–π stacking of aromatic groups in the structures of polymer backbone and especially H-bonding with the polymer and enzyme molecule enhanced the quality of the immobilization. Moreover, combination of organic polymer and enzyme molecules improved the stability of the proposed biosensor. Surface morphology of this constructed biosensor was confirmed by SEM. The fabricated biosensor exhibits excellent kinetic parameters such as $K_M$app, I_{max}, low LOD and high stability. Hence, inexpensive and high sensitive biosensor was developed and the proposed biosensor was successfully applied for the detection of cholesterol in food samples.

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