A Novel Conducting Polymer for Biosensor Applications

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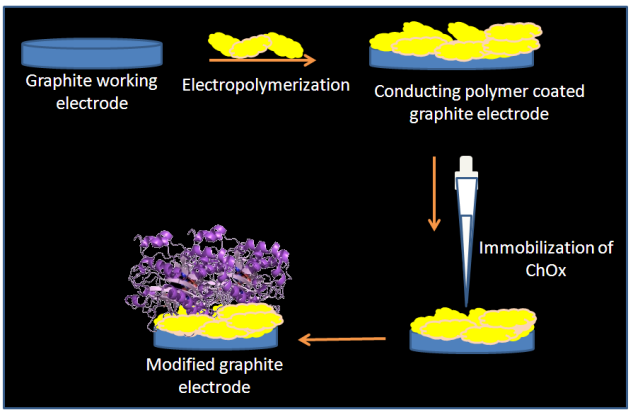
ABSTRACT

A new cholesterol biosensor based on a novel conducting polymer was developed. For this purpose, 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole, a novel monomer, was synthesized and tested as a platform for biomolecule immobilization. This novel monomer was coated on a graphite electrode by an electropolymerization technique. Cholesterol Oxidase (ChOx) was immobilized on the conducting polymer coated surface. Due to the presence of aromatic units and fluorine substitution on the polymer backbone, H-bonding between polymer and enzyme molecules is generated. Amperometric response was measured as a correlation of concentration of cholesterol, at -0.7 V vs. Ag/AgCl in phosphate buffer (pH 7.0). Consequently, optimum conditions of this constructed biosensor was determined and K_m^{app}, I_{max}, LOD and sensitivity values were investigated.

Keywords: conducting polymer, cholesterol biosensor, cholesterol oxidase.

1 INTRODUCTION

Biosensors have got more attention among researches as a worthy analytical device which converts a biological response into an electrical signal. Conducting polymers have been used for a transducer in biological sensors due to their attractive properties such as strong biomolecular interactions. Conducting polymers act as three dimensional platforms for immobilized enzymes [1]. Cholesterol is a molecule found in mammalian cell membrane. Development of reliable methods of cholesterol determination is very important in clinical and food analysis since high values of cholesterol is related to various clinical disorders such as arteriosclerosis [2], diabetes [3] and hypertansion [4]. In this study, a newly synthesized conducting polymer of , 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole was used as the support material to immobilized cholesterol oxidase. Hence, a new type of amperometric cholesterol biosensor based on conducting polymer was achieved. Electrochemical characteristics and optimum values of the biosensor were also investigated. Scheme 1 depicts the general construction of biosensor.



Scheme 1: The representation of construction of the biosensor.

2 EXPERIMENTAL

2.1 Materials

Cholesterol oxidase (E.C.1.1.3.6) (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, Triton-X 100, sodium perchlorate ($NaClO_4$) and lithium perchlorate ($LiClO_4$), benzothiadiazole, sodium borohydride ($NaBH_4$), bromine (Br_2) and ceric ammonium nitrate, $NH_4Ce(NO_3)_6$, (CAN) were purchased from Sigma-Aldrich and used with no further purification. Dichloromethane (DCM), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany).

2-Propanol (Merck) was used as received to prepare the cholesterol stock solution (0.005 M) at room temperature via gently mixing to obtain a clear solution. For enzyme immobilization, 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) were used. All chemicals were of analytical reagent grade.

2.2 Apparatus

For the amperometric measurements, Ivium CompactStat (The Netherlands) potentiostat and a cell equipped with three electrodes were used. Electropolymerization was performed with a Voltalab 50 potentiostat. Graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13 % porosity) was used as the working electrode, platinum (BASi platinum electrode) was used as the counter electrode and Ag/AgCl reference electrode (3M KCl filled) (BASi reference electrode) was used as the reference electrode.

2.3 Synthesis of 2-(4-fluorophenyl)-4,7-di(thiophene-2-yl)-1*H*-benzo[*d*]imidazole monomer

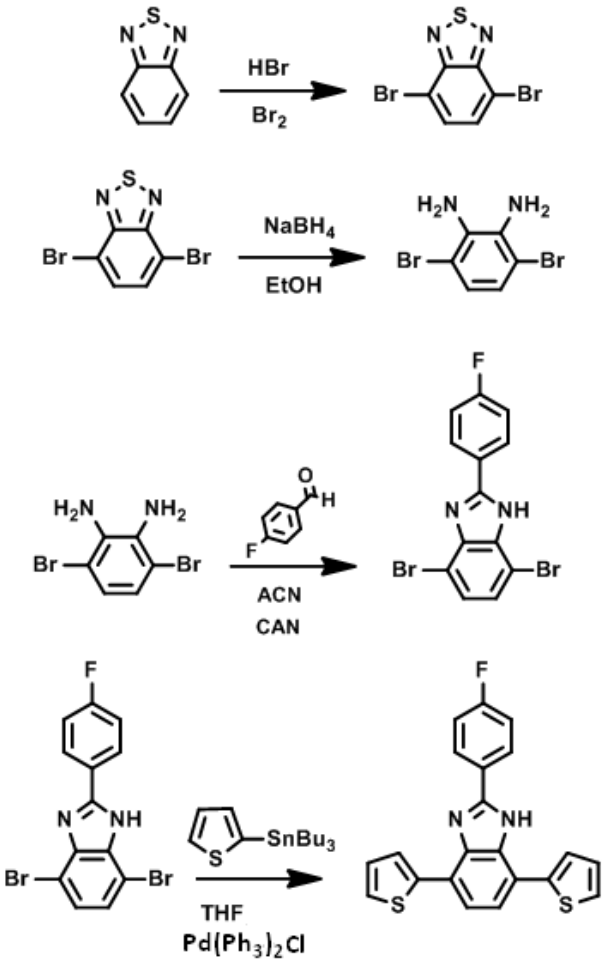
The first step of the synthesis is bromination of benzothiadiazole. For bromination, HBr was added to benzothiadiazole (3.67 mmol) in a round bottom flask and a solution containing Br_2 in HBr was added drop wise to the mixture very slowly. After complete addition of Br_2, the solution was refluxed for 6 h and cooled to room temperature. Excess Br_2 was consumed with saturated solution of $NaHSO_3$. As a result of this reaction, 4,7-dibromo-2,1,3-benzothiadiazole was achieved.

Next, 4,7-dibromo-2,1,3-benzothiadiazole (1.03 mmol) was dissolved in EtOH. To this mixture, sodium borohydride (0.04 mol) was added drop wise in ice bath, and the mixture was stirred at room temperature. Afterwards, the solvent was evaporated completely, and the extraction was performed firstly with ether and water, secondly with ether and brine. 3,6-Dibromo-1,2-phenylenediamine was obtained.

3,6-Dibromo-1,2-phenylenediamine (1.50 mmol) was dissolved in acetonitrile (ACN). Hydrogen peroxide was added to the mixture directly. The aryl aldehyde (1.50 mmol) was added drop wise. After that, $NH_4Ce(NO_3)_6$, used as catalyst, was added to this mixture. After addition of the catalyst, the mixture was refluxed at room temperature under Argon atmosphere to obtain 4,7-dibromo-2-(4-fluorophenyl)-1*H*-benzo[*d*]imidazole.

As the final step, coupling reaction was performed. A tributyl(thiophen-2-yl)stannane (2.70 mmol) was added to the 4,7-dibromo-2-(4-fluorophenyl)-1*H*-benzo[*d*]imidazole. After that, a dried tetrahydrofuran (THF) (0.49 mmol) and dichlorobis(triphenylphosphine)-palladium(II) as a catalyst was added. The mixture was refluxed and mildly stirred under argon atmosphere. Solvent was evaporated under vacuum and the product was purified by silica gel column chromatography as a yellow solid as 2-(4-fluorophenyl)-4,7-di(thiophen-2-yl)-1*H*-benzo[*d*]imidazole.

The synthetic pathway was depicted step by step in Scheme 2.



Scheme 2: Synthetic route to 2-(4-fluorophenyl)-4,7-di(thiophen-2-yl)-1*H*-benzo[*d*]imidazole.

2.4 Electrochemical studies of the novel monomer

Before electropolymerization, spectroscopic grade graphite rods were polished on emery paper and washed with distilled water to clean the surface of the electrodes.

To study the electrochemical properties of a monomer, cyclic voltammetry (CV) is the most widely used technique. It is a type of potentiodynamic electrochemical measurement. It is applied for observing the redox properties of the electroactive species. In CV, a changing potential is applied to electrodes and this plotted versus time with a scan rate. The three-electrode system was used for cyclic voltammetry studies. These are counter (CE), reference (RE) and working electrodes (WE). As a counter electrode Pt electrode was used and as a reference electrode Ag/AgCl reference electrode was used. Furthermore graphite was used as a working electrode. With cyclic voltammetry, electrochemical polymerization of monomer can be investigated by observing the redox sites of polymer.

Polymerization of newly synthesized monomer was performed potentiodynamically on graphite electrode using 0.1 M sodium perchlorate ($NaClO_4$) and lithium perchlorate ($LiClO_4$) containing 5:95 DCM (dichloromethane):ACN (acetonitrile) solution with repeated scan intervals between 0.0 and 1.85 V with respect to Ag/AgCl reference electrode with a scan rate of 100 mV/s.

In the first cycle of during the electropolymerization in the cyclic voltammogram, monomer oxidation was observed at 1.0 V, additionally polymer oxidation and polymer reduction were observed at 0.62 V and 0.86 V, respectively. While continuing the electropolymerization, the increase in current density in the resultant cyclic voltammogram shows the successful polymerization and film deposition onto the graphite electrode surface.

	$E_{p,a}$ (V)	$E_{p,c}$ (V)
Monomer	1.0	-
Polymer	0.62	0.86

Table 1: Redox potentials of the polymer.

Moreover, a cyclic voltammogram for the resultant 10-cycled conducting polymer film was also recorded in monomer-free solution containing 0.1 M sodium perchlorate ($NaClO_4$) and lithium perchlorate ($LiClO_4$) as supporting electrolytes containing 5:95 DCM (dichloromethane):ACN (acetonitrile). A quasi-reversible oxidation and reduction couple for the polymer was observed which is depicted in Figure 1 showing the electroactivity of the resultant conducting polymer. This confirms the availability and appropriateness of the novel polymer for the electrochemical applications such as electrochemical biosensors.

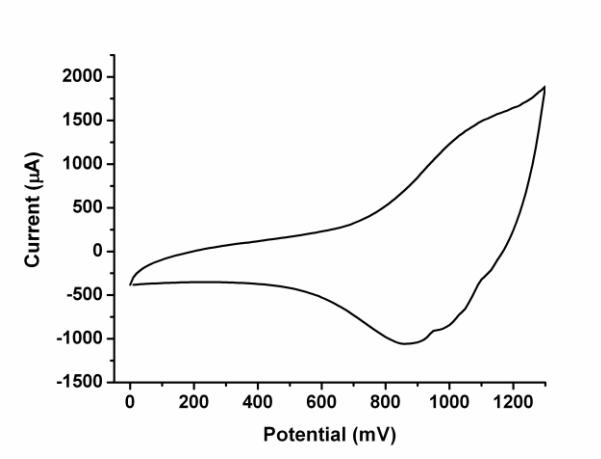


Fig 1: Single scan voltammogram for polymer in monomer free, 0.1 M sodium perchlorate ($NaClO_4$) and lithium perchlorate ($LiClO_4$) ACN solution.

2.5 Preparation of the conducting polymer based cholesterol biosensor

After electropolymerization of the monomer on the graphite electrode, immobilization of the enzyme molecules was achieved.

For immobilization of enzyme, a suitable amount of ChOx solution (26 U in 50 mM phosphate buffer, pH 7.0) was spread over the polymer coated electrode and after two minutes later, glutaraldehyde (1%, in 50 mM phosphate buffer, pH 7.0) was added and electrode was waited at ambient conditions to dry.

2.6 Measurements

Amperometric studies were carried out at ambient conditions in 10 mL phosphate buffer solution in open atmosphere. Firstly, baseline current became constant, cholesterol as the substrate was added to the reaction cell; current changed in a very short time (response times were 3-4 s) and reached a steady state. The biosensor response values were recorded as the current signal (µA). The electrodes were washed with distilled water after each measurement. Buffer solution was refreshed after each measurement. Figure 2 shows a typical amperometric current response.

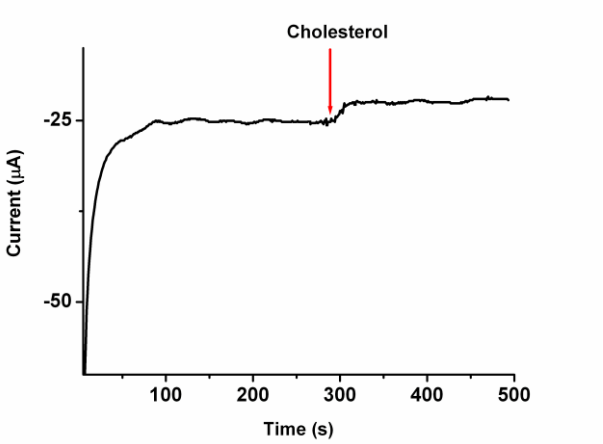


Figure 2: Amperometric response of the constructed biosensor to 25 µM cholesterol solution in 10 mL pH 7 50 mM phosphate buffer solution.

3 RESULTS AND DISCUSSION

3.1 Optimization studies

Since biomolecular activity was affected by various conditions, the biosensor was optimized to improve its

properties. Firstly, bioactive layer was optimized to enhance the interactions between the enzyme molecule and surface of the conducting polymer. To maintain the immobilization successfully and fixed strongly to enzyme on the polymer, film thickness of the polymer is important. 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). Optimum thickness was determined as 30-cycled polymer. It shows the effectiveness of the novel conducting polymer.

Moreover, enzyme amount during the immobilization was also optimized. For this, different amounts of cholesterol oxidase such as 0.8 mg (21 U), 1.0 (26 U) and 1.2 mg (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 highest amperometric results were obtained with the biosensor containing 1.0 mg cholesterol oxidase and optimum amount of enzyme was found as 1.0 mg.

Furthermore, the pH of the solutions in enzymatic reactions is really important due to their fragile biological activity. For this reason, the pH of the reaction medium was optimized in a range of pH 5.5-10. pH 7 was found as optimum one and used for further studies.

In addition, crosslinker amount is really important in biosensor construction. Glutaraldehyde (GA) was used as the crosslinker. Nevertheless, the aromatic groups of the polymer and aromatic units of the enzyme molecules are in good interaction, the use of crosslinker enhances the compact structure of the enzyme molecules on the polymer. In addition to mentioned π-π stacking interactions, the presence of fluorine substitute on the polymer backbone also forms possible H-bonding with the related units of the enzymes which strengthen the immobilization. With the help of these non-covalent interactions, there is no need to use any entrapment materials such as membranes. The enzyme molecules were successfully immobilized on the functional polymer. Crosslinker GA also provides stronger enzyme structures. GA amount used in immobilization was optimized. 0.5 %, 1.0 %, 1.5 % and 2.5 % GA values were evaluated and 1 % GA was found as the optimum value.

4 CONCLUSION

A novel polymer film was synthesized successfully for the construction of a cholesterol oxidase biosensor. The amperometric biosensor based on conducting polymer was fabricated by using adsorption technique. Under the light of this optimum values a biosensor was constructed. With this biosensor a calibration curve for cholesterol was prepared. The fabricated biosensor exhibits excellent kinetic parameters such as K_m^{app}, I_{max}, low LOD and high stability.

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