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Amperometric cholesterol biosensor based on reconstituted cholesterol oxidase on boronic acid functional conducting polymers

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ABSTRACT

An amperometric cholesterol biosensor was fabricated by electropolymerization method of conducting polymers on pencil graphite electrode (PGE) after which apo-cholesterol oxidase (apo-ChOx) was immobilized onto a flavin adenine dinucleotide (FAD) monolayer by reconstitution method. In this study two well-known and widely used materials in the field of biosensors thiophene-3-boronic acid and 3-aminophenyl boronic acid were electropolymerized onto PGE and modified with cholesterol oxidase. Electrochemical performance of modified electrodes and their properties were compared. Based on the results, thiophene-3-boronic acid based electrode shows sensitivity 10 times higher response than that of one based on 3-aminophenyl boronic acid. The effect of pH and temperature were assessed as well the reusability and stability of the fabricated biosensor were discussed.

1. Introduction

Heart disease, coronary artery disease, hypertension, cerebral thrombosis, arteriosclerosis are some of the most alarming diseases amongst clinical disorders related to abnormal cholesterol level in blood, and give rise to public concern, necessitating prevention, detection and regular monitoring of abnormal cholesterol level in the modern world [1]. In order to constantly measure presence, absence or concentration of these specific substances, developing a compacted analytical device that employs biological elements as the analyte sensor [2] and by integrating them within a physico-chemical transducer [3] is crucial in the diagnosis and prevention of a huge number of disorders.

Cholesterol oxidase (ChOx), FAD-containing flavo-enzyme which catalyzes the dehydrogenation of C(3)—OH of the cholestan system, is commonly isolated from *streptomyces hygroscopicus* (SCO) and *brevibacterium sterolicum* (BCO), and is the most commonly used enzyme in fabrication of cholesterol biosensor [1]. ChOx from both SCO and BCO exist in monomeric form and contain FAD as a prosthetic group which is noncovalently linked in ChOx from SCO, whereas covalently linked in BCO via an 8-histidinyl-FAD residue [4,5]. Therefore, ChOx from BCO is more attractive for biosensor application as a receptor due to its high ability of covalent binding. The enzymatic reactions occurring, when using ChOx as a receptor, are that the cholesterol is catalyzed by cholesterol oxidase in the presence of oxygen and in the same time hydrogen peroxide is produced (Eq. (1)), and after applying suitable potential to the system the electro-oxidation of hydrogen peroxide is detected (Eq. (2)), which can be demonstrated as follows [6]:

$$\text{Cholesterol} + O_2 \rightarrow \text{Cholest}-4-\text{en}-3-\text{one} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (2)$$

In order to fabricate an efficient amperometric biosensor, interaction between enzyme and electrode is crucial, which is usually achieved using conducting polymers (CP). Reversible interchange between redox states in CP allow changes in its properties such as polymer conformation, doping level, conductivity etc., which gives CP suitability in electrochemical devices application [7,8]. Polypyrrole [9], polyaniline (PANI) [10], diaminonaphthalene [11], poly(vinylferrocenium) [12] and poly(thiophen-3-boronic acid) (PTBA) [13], are some of the commonly studied and noteworthy CPs which are used in different type of amperometric biosensors. Some conductive polymers like PTBA and PANI can be easily synthesized in an aqueous solution and that can have good environmental stability and applicability, as seen in the

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literature reported by Huang et al., are reporting preparation of bio-nanocomposite of platinum nanoparticles using thiophen-3-boronic acid [14], Singh et al. have reported fabrication of cholesterol biosensor by covalent immobilization of ChOx and Cholesterol esterase on electrochemically deposited PANI matrix [15], further on Cui et al., reported an electro-polymerization of thiophen-3-boronic acid film (PTBA) in ionic liquid for the development of a hydrogen peroxide biosensor [16], Khan et al., publishes a study on developing a cholesterol biosensor via covalent immobilization of ChOx on electrochemically prepared PANI film in presence of TX-100 (non-ionic surfactant) which resulted in improving biocompatibility and make better conformation for effective immobilization of ChOx [17], Şenel et al., reported a study on amperometric biosensor for glucose detection based on thiophene-3-boronic acid [13], etc.

Although, characteristics of this conducting polymer provide enormous opportunities for binding of biomolecules, increasing their biocatalytic properties, rapid mass transfer, and direct communication to produce a range of analytical signals [7], desired efficiency will not be seen if immobilized enzyme on electrodes surface results in random orientation of redox center [18,19]. Wilner's group proposed a solution to the problem above by stripping the FAD redox center of glucose oxidase (GOx) and D-amino acid oxidase, and then reconstituting apo-enzymes with the modified cofactors [20], which was shown to be quite an efficient and effective method in enhancing the sensitivity of biosensors, which was later applied on couple different enzymes such as; D-amino acid oxidase [20], glucose dehydrogenase [21], horseradish peroxidase [22], and cholesterol oxidase [18].

Herein, we report a comparison study on the development of cholesterol biosensor using reconstituted ChOx as a recognition element, onto an electro-polymerized pencil graphite electrode with PTBA and 3-aminophenyl boronic acid (PABA). The biosensor is based on modified electrode using the reconstitution method after which their electrochemical performances were investigated. Electrode fabrications were performed by electro-polymerization of thiophene-3-boronic acid and its *co*-polymer thiophene on pencil graphite electrode after which FAD (flavin adenine dinucleotide) was immobilized and finally, reconstitution of apo-ChOx on to FAD monolayer. Optimum experimental conditions for pH efficiency, temperature and stability of the biosensor were assessed.

2. Experimental part

2.1. Materials

Cholesterol oxidase from Brevibacterium sp., isopropylalcohol, Triton X-100, 3-aminophenyl boronic acid, lactic acid, ascorbic acid, urea, uric acid and glucose were purchased from Sigma-Aldrich, *flavin adenine dinucleotide* sodium salt (FAD) was obtained from Sigma Chemical Co., and cholesterol from Alfa Aesar Germany. All other chemicals were of analytical grade and were used without further purification.

2.2. Electropolymerization of thiophene-3-Boronic acid and 3-aminophenyl Boronic acid

Pencil graphite electrodes were washed with ethanol then with distilled water in order to eliminate the adsorbed organic material. Electrochemical polymerization was carried out by using thiophene-3-Boronic acid and 3-aminophenyl boronic acid by careful immersion of PGE in a

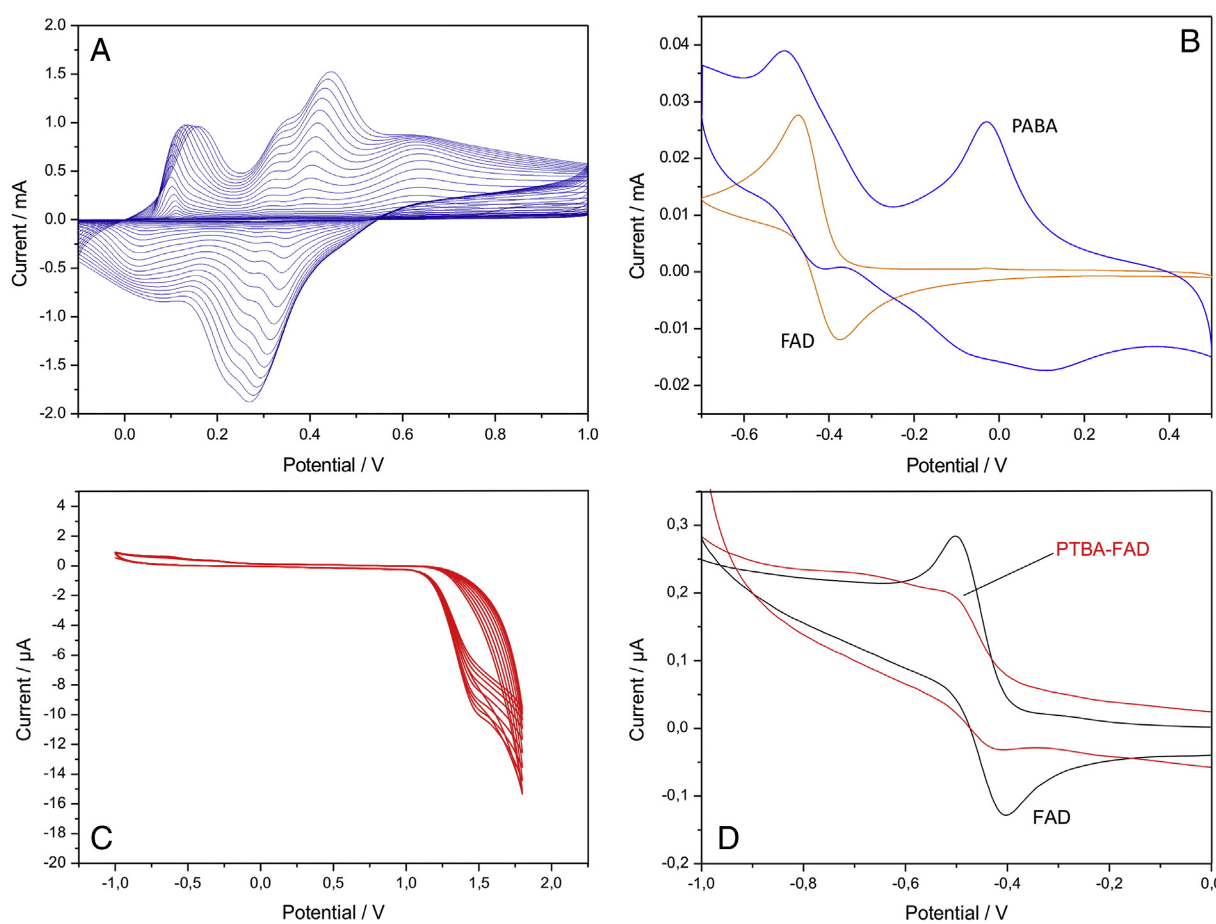

Fig. 1. A) Cyclic voltammogram of 3-aminophenyl boronic acid in 0.2 M NaF in 0.5 M HCl B) Cyclic voltammogram of a solution of FAD and FAD-PABA film electrode in 50 mM PBS (pH 7.0) C) Cyclic voltammogram of thiophene-3-boronic acid in 0.1 M TBAP-acetonitrile mixture scan rate 100 mV/s. (D) Cyclic voltammogram of a solution of FAD and FAD attached PTBA film electrode in 50 mM PBS (pH 7.0) scan rate 100 mV/s.