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A cholesterol biosensor based on a bi-enzyme immobilized on conducting poly(thionine) film

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ABSTRACT

A simple and cheap cholesterol biosensor was designed by immobilizing cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) onto a poly(thionine)-modified glassy carbon electrode (GCE/PTH). Being mediated by hydroquinone (HQ), the immobilized HRP exhibited excellent electrocatalytic activity in reducing H_2O_2, which was produced from cholesterol by the enzymatic reaction of ChOx. The linear detection range for cholesterol was 25–125 μM, with a detection limit ($S/N = 3$) and a sensitivity of 6.3 μM and 0.18 μA/cm^2/μM, respectively, under optimal conditions. The highly reproducible and sensitive GCE/PTH/ChOx/HRP sensor exhibited an interference-free signal for cholesterol detection with excellent recoveries for real sample analysis.

1. Introduction

Cholesterol is one of the essential structural constituents of cell membranes and exists in egg yolk, dairy products, goat meat etc. [1,2]. Determination of cholesterol is of great importance in clinical analysis because the concentration of blood cholesterol is one of the major parameters for the diagnosis of a number of disorders such as coronary heart disease, anaemia, nephrosis, arteriosclerosis, hypertension and myocardial infarction [3–5]. Generally, spectrophotometry, colorimetry, and high performance liquid chromatography (HPLC) have been applied for the determination of cholesterol [6,7]. Among them, HPLC method has significantly higher sensitivity than spectrophotometric method for the detection of cholesterol [8,9]. However, most of these methods involve complicated steps and generally it takes quite a long time for a single analysis [10]. Additionally, some of them exhibited obvious disadvantages in specificity and selectivity due to interference caused by unstable and corrosive reagents [7].

Electrochemical biosensors are much more suitable with respect to their good selectivity, reproducibility, and fast response [11–13]. Receptors in a biosensor for a biological recognition can be constructed via immobilizing specific enzyme on special matrix such as conducting polymers (CPs), hydrogel, sol–gel deposited thin films, lipid bilayer membranes, Langmuir–Blodgett films, self-assembled monolayer, and many composite-type matrix [14–21]. Among them, CPs have many advantages because of their feasibility to control the amount of immobilized enzyme by controlling film thickness [22,23] as well as the efficient charge transfer, homogeneity in electropolymerization, and the strong adherence to electrode surface [24,25]. Among CPs, poly(thionine) (PTH) is a potential electrode modifier for the development of various electrochemical sensors, which can be electrochemically synthesized from the organic redox dye thionine [13,24,26]. The electrochemical synthesis of PTH can be varied with the experimental conditions such as media (pH), solvent, substrate, and the applied potential [26–28]. The common approach for the immobilization of enzymes on PTH films is the use of linker molecules such as chitosan and glutaraldehyde for the development of cholesterol and glucose biosensors [29,30]. Meanwhile the pendant amine ($-NH_2$) groups on the PTH films enabled to immobilize the enzymes and biomarkers directly on PTH films via covalent linkage. Consequently, horseradish peroxidase (HRP) and probe DNA were immobilized onto PTH-modified glassy carbon electrode (GCE/PTH) for the construction of hydrogen peroxide (H_2O_2) and DNA hybridization sensor, respectively [31,32]. To the best of author knowledge, no reports are available for the direct immobilization of cholesterol

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oxidase (ChOx) on PTH-modified GCE (GCE/PTH), followed by the covalent attached of HRP onto GCE/PTH/ChOx for the detection of cholesterol. In this study, a bi-enzymatic cholesterol biosensor was developed by immobilizing ChOx and HRP onto GCE/PTH. Hydroquinone (HQ) was used as a mediator to promote the electron transfer between the enzyme and the electrode as reported elsewhere [33,34]. In comparison to the physical entrapment or the electrochemical deposition of enzymes in CPs films [14,22], the present approach preserves a more facile access from substrate to the immobilized enzymes and facilitates macromolecular interaction, by which the enzymatic activity was corroborated to obtain an excellent reproducibility and stability [35]. Additionally, the use of the immobilized HRP and the HQ redox mediator is expected to increase the selectivity of the sensor by lowering the H_2O_2 reduction potential. It also helps to maintain the enzymatic activity of ChOx by minimizing the accumulation of H_2O_2 at the interface [33,34]. The electrochemical characteristics and optimum variables of this biosensor for cholesterol detection were further investigated to quantify the amount of the free cholesterol in human serum.

2. Experimental

2.1. Chemicals

ChOx (EC 1.1.3.6, from brevibacterium) with a specific activity of 50 units/mg protein, HRP (E.C.I. 11.1.7) with a specific activity of 250–330 units/mg solid, cholesterol (water soluble), thionine, NaH_2PO_4, Na_2HPO_4, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), human serum samples (From human male AB plasma, hemoglobin, ≤20 mg/dL) were purchased from Sigma–Aldrich. All reagents were obtained in analytical grade and used without further purification. Double-distilled water obtained from a Milli-*Q* water purifying system (18 MΩ cm) was used throughout the experiments. Phosphate buffer solution (PBS) was prepared as described previously [36]. The solutions of ChOx and HRP were freshly prepared in PBS (50 mM, pH 7.0). Cholesterol stock solution (5 mM) was prepared in PBS (pH 7.0) and was stored at 4 °C. All experiments were carried out at room temperature.

2.2. Instrumentation

A CHI430A electrochemical workstation (CH instruments, Inc. USA) was used for all the electrochemical experiments. A conventional three-electrode system was used with glassy carbon electrode (GCE, 3 mm diameter) or modified GCEs, a platinum wire, and a Ag/AgCl (*aq*, saturated KCl) electrode as working, counter, and reference electrodes, respectively. Electrochemical impedance spectroscopic (EIS) measurements were carried out with a three-electrode cell in 5 mM $K_3[Fe(CN)_6]_{(aq)}$ solution containing 1 M KCl. EIS spectra were obtained at +0.30 V (the formal redox potential of $[Fe(CN)_6]^{3-}$) in the frequency range of 10^5–0.1 Hz with the ac amplitude of 5 mV (IM6ex, Zahner-Elektrik GmbH & Co. KG). Simulation of EIS spectra with the equivalent circuit model was performed by using Z-view software (version 3.1, Scribner Associates Inc., U.S.A.). Electron spectroscopy for chemical analysis (ESCA) was performed using a Sigma-Probe (Thermo VG, UK) spectrometer with monochromatic Al-*K* as the X-ray source. The X-ray power was 100 W and the vacuum in the sample chamber was 3.8×10^{-9} mbar.

2.3. Preparation of the GCE/PTH/ChOx/HRP sensor

Scheme 1A shows the schematic construction of the sensor matrix. First, GCE/PTH was prepared according to our previous report [31]. Prior to immobilization of ChOx on GCE/PTH, 1 mL of ChOx (100 μg/mL) was kept for 4 h at room temperature with EDC and NHS (20 mM each) to activate the carboxyl group of ChOx. Then the GCE/PTH electrode was immersed into a ChOx solution for 6 h at 4 °C for immobilization. After immobilization of ChOx, the GCE/PTH/ChOx electrode was washed with PBS (pH 7.0) to remove the unbound ChOx. Finally, EDC and NHS treated HRP (1 mg/mL in PBS, pH 7.0) was immobilized onto GCE/PTH/ChOx to prepare the GCE/PTH/ChOx/HRP sensor, of which the electrode structure is similar to the choline biosensor described in the literature [35]. When not in use, the sensor was stored in PBS (pH 7.0) at 4 °C in order to minimize the enzyme degradation and the loss during measurement [3,33].

3. Results and discussion

3.1. Characterization of the electrode surface

The immobilization of ChOx onto PTH by covalent link was confirmed by C 1s and N 1s in the ESCA as shown in Fig. 1A and B. Compared with PTH, the appearance of a new peak at 288.43 eV in PTH/ChOx, attributable to N—C=O group [37], indicated the formation of amide (—CO—NH—) bond between the amine group ($—NH_2$) of PTH and the carboxylic group (—COOH) of ChOx (Fig. 1A). Furthermore, it was strongly supported by the significant enhancement of the peak height for N—C bond at 400.42 eV [38] after the anchoring of ChOx (Fig. 1B). The charge transfer resistance (R_{CT}) obtained from fitting results of EIS spectra were 142.5, 1564.7, 1792.6, and 2178.9 Ω for polished GCE, GCE/PTH, GCE/PTH/ChOx, and GCE/PTH/ChOx/HRP, respectively (Fig. 1C). The enhancement of R_{CT} by the stepwise electrode modifications were attributed to the enhancement of the degree of hindrance in the electron transfer to the redox species in solution across the interfaces [33]. It clearly suggested the sequential immobilization of ChOx and HRP onto GCE/PTH. Cyclic voltammograms (CVs) was further used to study the changes of the electrode behavior after each modification steps and the results were in good agreement with those obtained from EIS (Fig. S1).

3.2. Electrochemical response of cholesterol at GCE/PTH/ChOx/HRP sensor

CVs were collected in PBS (pH 7.0) containing 0.5 mM HQ to validate the function of GCE/PTH/ChOx/HRP sensor for cholesterol detection (Fig. 1D). HQ in the solution clearly showed its diffusional redox waves in the absence of cholesterol. However, the reduction current increased in the presence of 0.1 mM cholesterol, while the corresponding oxidation current decreased. Meanwhile, the redox behavior of 0.5 mM HQ at GCE/PTH/ChOx and GCE/PTH/HRP sensors in presence and absence of 0.1 mM cholesterol did not induce any noticeable variation of both oxidation and reduction currents of HQ (Fig S2), which clearly indicates that both GCE/PTH/ChOx and GCE/PTH/HRP sensors mediated by HQ could not catalyze the oxidation of cholesterol [33]. This clearly demonstrated that HQ could effectively shuttle electrons between the redox center of HRP at GCE/PTH/ChOx/HRP sensor as indicated in the Scheme 1B. As we can see from Scheme 1B, ChOx is attached onto PTH by the covalent bonding between the amine ($—NH_2$) group of PTH and the —COOH group of ChOx. H_2O_2 is produced from the cholesterol as a result of the ChOx enzymatic reaction [39] to generate cholestenone. The produced H_2O_2 reduces to H_2O by the oxidative enzyme activity of the immobilized HRP. The oxidized HRP (HRP_{ox}) can be regenerated with the aid of the oxidation of HQ to bezoquionone (BQ), which can be electrochemically reduced back to HQ at the electrode [40]. Therefore, the concentration of cholesterol is directly proportional