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Design of an electrochemical biosensing system for xanthine detection and a study on binding interaction of ketoconazole with xanthine oxidase

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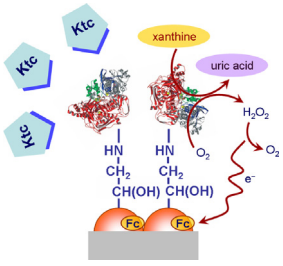
HIGHLIGHTS

- XO was covalently immobilized on poly(glycidyl methacrylate-*co*-vinylferrocene).
- Physicochemical parameters was determined by monitoring response current at 0.6 V.
- Ktc–XO interaction was studied by spectrofluorometric and electrochemical methods.
- Binding interaction of Ktc with XO was confirmed by SDS-PAGE and native-PAGE analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Xanthine oxidase (XO) was successfully immobilized by covalent attachment on poly(glycidyl methacrylate-*co*-vinylferrocene) (P(GMA-*co*-VFc)), a redox copolymer containing pendant epoxy and ferrocene moieties, for the evaluation of both the biosensing properties and the effect of the interaction of ketoconazole (Ktc) with the immobilized XO. The binding interaction between Ktc, a drug used to treat fungal infections, and the immobilized XO on P(GMA-*co*-VFc) was also studied by fluorescence spectroscopy technique. The binding capacity of the drug was determined using a calibration curve equation that was drawn at excitation wavelength of 300 nm using fluorescence spectroscopy. The interaction ability was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE analysis. The enzyme electrode exhibited a linear range from 2.7×10^{-3} to 0.55 mM with a sensitivity of 19.42 μA mM^{-1} cm^{-2} and a detection limit of 8×10^{-4} mM for the detection of xanthine. The activation energy (E_a) and the apparent Michaelis–Menten constant (K_{mapp}) values were found to be 12.30 kJ mol^{-1} and 0.38 mM, respectively.

1. Introduction

Xanthine oxidase (XO) plays an important role as the terminal enzyme of purine catabolism in human and mammalian life, catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. XO is a flavoprotein that contains molybdenum, iron and labile sulfur. The enzymatic reaction, occurs between xanthine (or hypoxanthine) and XO, is based on a mechanism involving nucleophilic attack on substrate by the Mo–OH group at the active site of XO [1,2]. Xanthine, a major metabolite in the degradation of adenine nucleotide, can serve as a marker of many clinical disorders, including perinatal asphyxia, adult respiratory distress syndrome, cerebral ischemia, tumor hyperthermia and pre-eclampsia [3,4]. For the diagnosis and the treatment of various

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diseases, the amount of xanthine in the blood and the tissue samples should be easily analyzed. The level of xanthine is also used in the food industry as an indicator for evaluating the freshness of fish products due to the accumulation of xanthine in the tissues after the death of fish. Thus, the sensitive and selective determination of xanthine has considerable importance in clinical analysis as well as food quality control [5].

In recent years, there are some studies reported on the identification of the interaction of drug with proteins and enzymes by using fluorescence spectroscopy and electrochemical technique [6–8]. The combination of these methods provides a fast and sensitive identification of the interaction. Fluorescence spectroscopy is very useful tool for the protein studies. The fluorescence character of the proteins comes from the intrinsic fluorescence of two amino acids which are tyrptophane and tyrosine [7,9]. However, when a small molecule such as ligand and drug has an emission, the identification of the interaction between these compounds and protein (or enzyme) immobilized on a matrix surface is also managed via fluorescent character. For example, our research group have studied on the interaction between human serum albumin and several fluorescent drugs such as Ktc and donepezil by using both fluorescence spectroscopy and electrochemical techniques [10–12]. In addition to studies performed with fluorescence spectroscopy, the electrochemical system is also used for the determination of the protein binding by modified the different types of electrodes. The binding capacity and the binding mechanism of the biomolecule on the supporting material and the interaction of the biomolecule with small molecule (e.g., drug molecule) is determined by monitoring the electrochemical response for each process step [13–16]. Particularly, the combination of the spectroscopic and electrochemical approach can provide new insights, which will lead to further understanding of the mechanism of interaction between a variety of pharmaceuticals and protein (or enzyme).

In this paper, we present a new xanthine biosensor based on the immobilization of XO on a redox copolymer (poly(glycidyl methacrylate-*co*-vinylferrocene)-P(GMA-*co*-VFc)) coated platinum electrode. The redox copolymer contains pendant epoxy and ferrocene groups. The epoxy and ferrocene moieties of P(GMA-*co*-VFc) were employed for direct covalent immobilization of XO and electron transfer mediator for the fabrication of xanthine biosensor, respectively. The characterization and the experimental conditions related to the preparation of the immobilization of XO with P(GMA-*co*-VFc) film on platinium electrode (XO/P(GMA-*co*-VFc)/Pt) were studied in detail. The effect of applied potential, polymeric film thickness, temperature and xanthine concentration on the response of XO/P(GMA-*co*-VFc)/Pt were evaluated. The performance of the biosensor, i.e., linear range, response time, sensitivity, reproducibility, and stability was also described. Besides, the interaction of ketoconazole (Ktc) with the immobilized XO was studied by spectrofluorometric and electrochemical methods. The percentage of Ktc interacted with the immobilized XO was determined at excitation wavelength of 300 nm, and increased gradually with increasing of the amount of immobilized XO. However, this increase in the percentage of Ktc did not indicate a significant effect on the response current of XO/P(GMA-*co*-VFc)/Pt.

2. Material and methods

2.1. Reagents

Xanthine oxidase (XO) (EC 1.17.3.2), xanthine, vinylferrocene (VFc, 97%), ketoconazole (Ktc) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide solution (30%, extra pure), *N*,*N*-dimethylformamide were obtained from Merck (Darmstadt, Germany). Glycidyl methacrylate (GMA, 97%) was purchased from sigma (St. Louis, MO, USA) and purified by distillation under reduced pressure. α,α'-Azobisisobutyronitrile (AIBN, 98%) (Sigma, St. Louis, MO, USA) was purified by recrystallization from methanol. The enzyme solution was prepared by dissolving of XO in 0.01 M phosphate buffer solution (PBS) of pH 7.4, and stored at 4 °C. All other chemicals were of analytical grade and deionized water (Millipore Milli-Q Plus water purification system) was used for preparing all of the solutions.

2.2. Apparatus and instrumentations

All the electrochemical measurements were performed with a CHI-660 C electrochemical workstation (CH Instruments Co., USA). A conventional three electrode system was used with platinum electrode (area: 0.0314 cm^2) as working electrode, a platinum wire as auxiliary electrode and an Ag/AgCl (3 M KCl) electrode as reference electrode. Prior to each experiment, Pt electrode was polished with 0.05 μm alumina powder, and then ultrasonicated in deionized water. Scanning electron microscopy (SEM) images were obtained by using EVO-LS 10 (Carl Zeiss, Germany). UV–vis absorption and fluorescence measurements were recorded on a Shimadzu 2001UV spectrophotometer (Shimadzu Co., Japan) and a Perkin-Elmer LS-55 fluorescence spectrometer (Perkin-Elmer Inc., USA), respectively. The average molecular weights and the molecular weight distributions of P(GMA-*co*-VFc) was determined on an Agilent 1100 GPC Instrument (Agilent Tech., USA) consisting of a pump, a refractive index detector and two Waters Styragel columns and using THF as eluent at a flow rate of 0.5 mL min^{-1} at 23 °C. IR spectra were recorded on a Perkin-Elmer spectrum 100 FT-IR spectrometer (ATR) (Perkin-Elmer Inc., USA).

2.3. Synthesis of P(GMA-co-VFc)

P(GMA-*co*-VFc) was prepared by conventional free-radical copolymerization GMA and VFc monomers initiated by α,α′-azobisisobutyronitrile (AIBN) as reported previously [17] (Scheme 1). The copolymerization conditions and the composition data of P(GMA-*co*-VFc) prepared in this study were given in Table 1.

2.4. Preparation of XO electrode

The proposed procedure for the preparation of P(GMA-*co*-VFc) coated Pt electrode, the immobilization of XO on the electrode and the enzymatic reaction on the electrode surface is shown in Scheme 1. The copolymer film was prepared by the drop-coating method on the surface of Pt electrode with 5 μL of 1 mg mL^{-1} P(GMA-*co*-VFc) solution in N,N-dimethylformamide(DMF) and dried in air at room temperature. The copolymer coated Pt electrode was immersed in 0.01 M PBS (pH 7.4) for 1 h, and then kept in 2 mg mL^{-1} XO in 0.01 M PBS (pH 7.4) for the enzyme immobilization under stirring for 24 h at 4 °C. Finally, the biosensor (defined as XO/P(GMA-*co*-VFc)/Pt) was washed lightly with 0.01 M PBS to remove the unbound XO and stored at 4 °C in 0.01 M PBS prior to use.

2.5. Interaction of Ktc with XO

Different amounts of P(GMA-*co*-VFc) copolymer were mixed with a concentration of 0.075 mg mL^{-1} XO in 0.01 M PBS, pH 7.4. Each mixture was tumbled for 24 h at 4 °C at a P(GMA-*co*-VFc) concentration range of 1–20 mg mL^{-1} [18]. The XO bounded copolymer (XO/P(GMA-*co*-VFc)) was centrifuged at 6500 rpm for 10 min and separated from the supernatant. XO/P(GMA-*co*-VFc) washed lightly with 0.01 M PBS to remove the unbound XO, and then kept for the binding interaction of ketoconazole (Ktc). The