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Construction of an amperometric bilirubin biosensor based on covalent immobilization of bilirubin oxidase onto zirconia coated silica nanoparticles/chitosan hybrid film

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ABSTRACT

A method is described for the construction of a highly sensitive electrochemical biosensor for the detection of bilirubin. The sensor is based on covalent immobilization of bilirubin oxidase (BOx) onto zirconia coated silica nanoparticles (SiO_2@ZrONPs)/chitosan (CHIT) composite electrodeposited onto Au electrode. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infra-red spectroscopy (FTIR), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The biosensor showed optimum response within 2 s at pH 8.5 (0.1 M Tris–HCl) and 35 °C, when operated at 20 mV s^{-1}. The biosensor exhibited excellent sensitivity (detection limit as 0.1 nM), fast response time and wider linear range (from 0.02 to 250 μM). Analytical recovery of added bilirubin was 95.56–97.0%. Within batch and between batch coefficients of variation were 3.2% and 3.35% respectively. The enzyme electrode was used 150 times over a period of 120 days, when stored at 4 °C. The biosensor measured bilirubin levels in sera of apparently healthy and persons suffering from jaundice, which correlated well with a standard colorimetric method (r=0.99).

1. Introduction

Bilirubin (formerly known as hematoidin) is a yellow colored breakdown product of normal heme catabolism. Heme is a principal component of hemoglobin, found in red blood cells. The normal levels of serum bilirubin are as follows—direct (also called conjugated) bilirubin: 0–0.3 mg/dL and total bilirubin: 0.3–1.9 mg/dL. Bilirubin is excreted in bile and urine, and its elevated levels in serum indicate jaundice, brain damage thalassemia, sphaerocytosis, Gilbert syndrome, heamolytic uremic syndrome and sickle cell anemia. It is responsible for yellow color of bruises, urine (via its reduced breakdown product, urobilin) discoloration in jaundice and brown color of feces (via its conversion to stercobilin). Bilirubin is generated by the action of biliverdin reductase on biliverdin, a green tetrapyrrolic bile pigment and also a product of heme catabolism. Bilirubin, when oxidized, reverts to biliverdin again. This cycle has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant (Baranano et al., 2002; Liu et al., 2008). Thus, determination of bilirubin in biological fluid is of great importance in the differential diagnosis of jaundice. Serum bilirubin levels are also clinically determined as part of the routine in newborn nurseries. This is because high concentrations of bilirubin in the blood may cause brain damage or even death, specifically in the case of babies (Maisels, 2009). Therefore, a cost effective measurement of bilirubin level is essential for the diagnosis and medical management of jaundice patients. Various methods have been reported for the measurement of bilirubin, the most common detection methods are the direct spectroscopic measurement (Doumas et al., 1973) and the diazo reaction (Bergmeyer et al., 1985). However, the direct spectroscopic measurement of bilirubin suffers from interference by other heme proteins, while the diazo reaction is pH dependent (Li and Rosenzweig, 1997). Other analytical methods for bilirubin determination such as polarography (Wang et al., 1985), and fluorometry (Koch and Oakingbe, 1981) require costly equipment, time consuming sample preparation and skilled persons to operate, and thus are not suitable for routine use. Nevertheless, biosensing methods are comparatively more simple, rapid and sensitive than these methods and require no sample pre-treatment. Electrochemical amperometric bilirubin biosensors employing bilirubin oxidase (BOx) are based on either the measurement of decreasing level of molecular oxygen (Wang and Ozsoz, 1990) or oxidation of hydrogen peroxide (Fortuney and Guilbault, 1996) or the mediated electron transfer by the Mn(II) ion using a conductive poly-terthiophene–Mn(II) complex (Rahmana et al., 2008). However these biosensors

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also have certain disadvantages such as low detection limit due to poor electron flow and lower storage stability of the enzyme electrode.

Recently, nanomaterials have been employed to improve the analytic performance of biosensors. Coated nanoparticles are essentially defined as the particles containing a core and a shell, having dimensions in the nanometer range. Composite nanoparticles exhibit improved physical and chemical properties over their single-component counterparts, and hence are potentially useful in a wide range of applications. These composite nanoparticles exhibit a variety of novel properties e.g., optical, electrical, catalytic, magnetic, and mechanical. Zirconia coated silica nanoparticles have been used as an efficient catalyst.

Chitosan (CHIT) is an important biopolymer for immobilization of biomolecules, due to its excellent film-forming ability, high permeability, mechanical strength, non-toxicity, biocompatibility, low cost and easy availability. Further, $-NH_2$ groups of CHIT provides hydrophilic environment for the biomolecules (Wang et al., 2010). We describe herein a novel approach of immobilizing bilirubin oxidase onto SiO_2@ZrONPs/CHIT modified Au electrode, its characterization and application for amperometric determination of bilirubin.

2. Experimental section

2.1. Materials

Bilirubin oxidase (BOx) from Sigma-Aldrich, St. Louis, USA, tetraethylorthosilicate (TEOS) from Fluka, Mumbai, India, and potassium chloride (KCl), bilirubin, chitosan and zirconium oxide nanoparticles (ZrONPs) from SISCO Research Laboratory, Mumbai, India were used. Double distilled water (DW) was used throughout the experimental studies.

2.2. Assay of free BOx

The assay of free BOx was carried out as described by Satyapal and Pundir (1993) with modifications. The assay was based on the quantification of H_2O_2, generated from oxidation of bilirubin catalyzed by BOx, using a color reaction consisting of the 4-aminophenazone, phenol and peroxidase as chromogenic system. The reaction mixture containing 0.7 ml of Tris–HCl buffer pH 8.5 (0.1 M), 0.1 ml of bilirubin solution (34.21 μM), and 0.1 ml of BOx solution (5 U/ml) was incubated at 37 °C for 10 min. Color reagent (1.0 ml) was added to the reaction mixture and kept at 37 °C in dark to develop the pink color. After 15 min, A_{520} was read and H_2O_2 concentration in the reaction mixture was interpolated from the standard curve between A_{520} vs. H_2O_2.

One unit of enzyme is defined as the amount of enzyme required to catalyze the formation of 1.0 μmol of H_2O_2 from oxidation of bilirubin per min/ml under standard assay conditions.

2.3. Preparation of SiO_2@ ZrONPs

SiO_2@ZrONPs were prepared by the precipitation method (Stober et al., 1968). To a mixture of TEOS (2.0 ml) and ethanol (20 ml) in a 50 ml beaker, NH_4OH (4.0 ml) was added dropwise. The mixture was stirred for 8 h and centrifuged at 4000 rpm for 5 min. The white colored silica oxide nanoparticles (SiO_2NP) generated were dispersed into DW (10 ml). ZrONPs (200 μl) suspension was added to this SiO_2NP suspension and its pH was brought to pH 10–10.5, by adding NH_4OH. The zirconia coated silica nanoparticles (SiO_2@ ZrONPs) formed were kept at 40 °C for drying. The characterization of zirconia coated silica nanoparticles was carried out by recording its UV and visible spectra in a UV–visible spectrophotometer, X-ray diffraction pattern in an X-ray diffractometer (XRD) and transmission electron micrograph in a transmission electron microscope.

2.4. Electrodeposition of SiO_2@ZrONPs/CHIT hybrid film onto Au electrode

The surface of Au electrode was polished with alumina slurry (diameter 0.05 μm), followed by washing with DW and sonication in ethanol to remove adsorbed particles and finally washing with DW to remove ethanol. The cleaned electrode was dipped into 25 ml 1 M KCl containing chitosan (0.2%, 200 μl) and SiO_2@ ZrONPs suspension (200 μl) and subjected to 20 successive deposition cycles at −0.1 to 0.2 V using a potentiostat–galvanostat (Fig. 1). The resulting SiO_2@ZrONPs/CHIT modified Au electrode was washed thoroughly with DW to remove unbound matter and kept in a dry Petri-plate at 4 °C.

2.5. Immobilization of BOx onto SiO_2@ZrONPs/CHIT modified Au electrode

SiO_2@ZrONPs/CHIT modified Au electrode was dipped into 1 ml of 2.5% glutaraldehyde in 0.1 M Tris–HCl buffer (pH 8.5), kept at room temperature for 7 h, washed thoroughly with DW, dipped into 1.5 ml of BOx solution (25 U/ml) and kept overnight at room temperature for immobilization. The resulting electrode with immobilized BOx was washed 3–4 times with 0.1 M Tris–HCl buffer (pH 8.5) to remove residual unbound protein. The BOx immobilized onto a SiO_2@ZrONPs/CHIT modified Au electrode retained 83.47% of the initial activity of free enzymes with a conjugation yield of 3.03 mg/cm^2. The resulting BOx/SiO_2@ZrONPs/CHIT/Au electrode was used as working electrode and stored at 4 °C, when not in use. This working electrode was characterized by SEM at different stages of its construction.

2.6. Scanning electron microscopy of enzyme electrode

The SEM images of bare Au electrode and SiO_2@ZrONPs/CHIT/Au electrodes were taken in a scanning electron microscope (Zeiss EV040) at Jawaher Lal Nehru University, New Delhi, on commercial basis.

2.7. Construction and testing of bilirubin biosensor

The enzyme electrode (BOx/SiO_2@ZrONPs/CHIT/Au) as working electrode, Ag/AgCl as reference and Pt wire as counter electrode, were connected through potentiostat/galvanostat. Cyclic voltammogram (CV) of the BOx/SiO_2@ZrONPs/CHIT/Au electrode was recorded

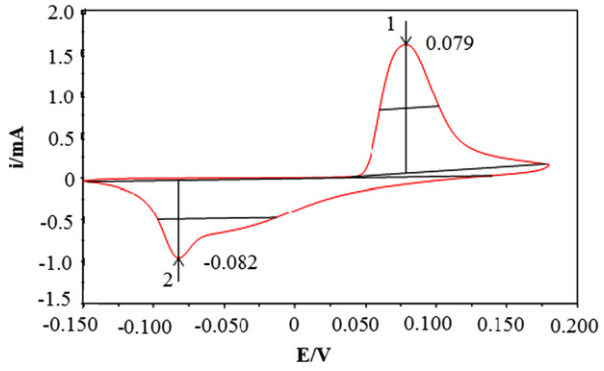

Fig. 1. Cyclic voltammogram for electrodeposition of SiO_2@ZrONPs/CHIT composite film. Supporting electrolyte: 1 M KCl solution; scan rate: 20 mV s^{-1}.