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Highly sensitive amperometric detection of bilirubin using enzyme and gold nanoparticles on sol–gel film modified electrode

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A B S T R A C T

We describe the development of a simple and highly sensitive electrochemical (amperometric) sensing of bilirubin based on bilirubin oxidase (BOx) incorporated into the gold nanoparticles (AuNPs). This nano-electrode platform with self-assembled enzyme is highly sensitive toward the electrochemical oxidation of bilirubin and increased the bilirubin concentration linearly from 1 to 5000 μM with a correlation coefficient of 0.9960, and an apparent Michaelis constant ($K_{M,app}$) of 44 ± 0.4 μM. Using an amperometric method, the detection limit for bilirubin at the enzyme-modified electrode was 1.4 nM (signal-to-noise ratio = 3). The modified electrode retained a stable response for 2 days while losing only ca. 3.4% of its initial sensitivity during a 10 days storage period in 0.2 M phosphate buffer solution (pH = 8.4) at ≤4 °C. The practical application of the modified electrode was demonstrated by measuring the concentration of bilirubin in blood serum sample.

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1. Introduction

Bilirubin is a tetrapyrrole compound that is formed from the breakdown of heme in red blood cells and presents in the blood as an unconjugated (free) form [1–3]. Normally, it is conjugated with albumin to form a water-soluble complex and is excreted from hepatocytes into bile mainly as bilirubin glucuronides [4–6]. Bilirubin is rarely excreted from urine in its true form, but with liver dysfunction, conjugated bilirubin can be excreted with urine which is a symptom of clinical jaundice [1,4–6]. Normal levels of bilirubin in blood serum of adult range from 10^{-5} M to 10^{-6} M [1]. When the amount of bilirubin in the body exceeds the binding capacity of albumin, extra free bilirubin binds and deposits to various tissues, especially in the brain with deleterious effects [1,4–6]. Its deposition and accumulation in tissues can cause disorders in the metabolism of bilirubin and lead to hepatitis, jaundice or kernicterus [7–9]. This may cause cell death in various tissues and possibly lead to brain neuron damage causing mental disorder (e.g., mental retardation, learning disability and deafness), cerebral palsy or even death (especially in the case of babies) [9–11]. Neonatal jaundice is extremely common as almost every newborn develops an unconjugated serum bilirubin level of >30 μmol/L during the first week of life, and is reportedly more common in East Asians [12]. Transcutaneous bilirubinometry which is portable and non-invasive cannot be relied on for accurate measurements of serum bilirubin in infants with jaundice and can be only used as a screening tool [13]. Thus, the accurate determination of bilirubin is clinically important.

In the past few years, numerous methods have been developed for the detection of bilirubin in clinical samples and the most common detection methods are the direct spectroscopic measurement [14] and the diazo reaction [15]. However, the direct spectroscopic measurement of bilirubin suffers from the interference from other heme proteins and the accuracy in determination of the bilirubin concentration based on diazo reaction is compromised, partly as the reaction rate is pH dependent [16]. Other analytical methods, such as polarography [17] and fluorometry [18] have also been well known for the bilirubin detection analysis. These methods are also less selective compared to the diazo reaction [15]. Alternatively, there have been extensive attempts to obtain more accurate and simple routine analytical methods [19–21] including various enzymatic systems for the determination of bilirubin in analytical electrochemistry [22–29]. Importantly, the high over-potential required for oxidation is a major concern with electrochemical methods while using multilayered, polymer film–Mn(II) complex and AuNPs–MWCANT modified electrodes. For instance, the oxidation of bilirubin occurred at 0.6, 0.4 and 0.45 V for multilayered, polymer film–Mn complex and AuNPs–MWCNT modified electrodes, respectively [23,28,29]. Sensitivity of the enzyme electrode mainly depends on the number of bilirubin oxidase layers attached

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to the electrode surface, further the amperometric current response was negligible at high blood serum concentration [23] and the stability of AsOx largely depends on the PEI coating in the case of polymer-coated electrode [28]. Thus, the development of a simple and highly sensitive detection platform for the determination of bilirubin is much needed.

The sol–gel technology provides a versatile way to prepare a 3-D silicate network through the hydrolysis and condensation of silicon alkoxide precursors [30]. The sol–gel-derived three-dimensional (3-D) network is particularly attractive in the development of sensing devices [31–34], because the network exhibits tunable porosity, high thermal stability and chemical inertness. Gold nanoparticles (AuNPs) have emerged as a promising nanomaterial and their widespread applications in the fields of electronics, catalysis and biosensors [35–43]. In the recent past AuNPs-based materials have been used for the immobilization of enzymes such as ascorbate oxidase, glucose oxidase, alcohol dehydrogenase and bilirubin oxidase for the development of biosensors [44–49] and biofuel cell [50] applications. In the present investigation, the AuNPs platform can provide a suitable environment for the self-assembling of BOx enzyme and the enzyme/nanoparticles based biosensing electrode displays excellent performance in terms of operating potential, detection limit, stability and reproducibility with respect to the existing enzyme, polymer and nanomaterials based electrodes.

2. Experimental

2.1. Chemicals

Bilirubin, bilirubin oxidase (bilirubin:oxygen oxidoreductase, EC 1.3.3.5, lyophilized powder, 17 units/mg) from *Myrothecium verrucaria,* hydrogen tetrachloroaurate trihydrate ($HAuCl_4{\cdot}3H_2O$), (3-mercaptopropyl)-trimethoxysilane (MPTS) and blood serum sample were purchased from Sigma–Aldrich. All other chemicals used in this investigation were of analytical grade and were used without further purification. The 0.2 M phosphate buffer solution (PBS, pH = 8.4) was prepared from Na_2HPO_4 and NaH_2PO_4 and a freshly prepared solution of bilirubin was used in all the experiments. Double-distilled water was used to prepare all the experimental solutions.

2.2. Instrumentation

Electrochemical measurements were performed using two-compartment, three-electrode cell with a polycrystalline Au working electrode, a Pt wire auxiliary electrode and a saturated calomel reference electrode. Cyclic voltammograms (CVs) were recorded using a computer-controlled CHI660C electrochemical analyzer (CH Instrument, USA). The electrochemical impedance measurements were performed using the BOx on AuNPs modified electrode in the presence of 1 mM of $K_3Fe(CN)_6$ in 0.2 M PBS using an alternating current voltage of 5 mV. The electrode formal potential was 0.22 V and the frequency range was from 1 Hz to 100 kHz. Scanning electron microscopic measurements were performed with a JEOL JSM-6700F field emission scanning electron microscope (FESEM). A magnetic stirrer was used to provide constant stirring during the amperometric experiment.

2.3. Preparation of colloidal gold nanoparticles (AuNPs)

All glasswares used in the following procedures were cleaned in a bath of freshly prepared 1:3 HNO_3/HCl, rinsed thoroughly with doubly distilled water and dried in hot air oven. (*Caution: aqua regia is a powerful oxidizing agent and it should be handled with extreme care*). The colloidal AuNPs were prepared by citrate reduction of $HAuCl_4$ according to the procedure described in the literature [20]. In a typical synthesis of ∼12 nm-diameter AuNPs, 18.75 mg of $HAuCl_4$ in 62.5 mL of distilled water (0.88 mM) was brought to a vigorous boil with stirring in a round-bottom flask fitted with a reflux condenser and 6.5 mL of 1% (w/v) sodium citrate solution was then rapidly added to the flask. The solution was boiled for another 15 min, during which time the solution changed from pale yellow to deep red. The solution was allowed to cool to room temperature with continuous stirring. The suspension was stored at 4 °C until further use, conditions under which the nanoparticles are stable for several months. The resulting solution of AuNPs was examined using a UV–vis spectrum, which showed a strong surface plasma resonance band at 519 nm, typical characteristic feature of monodispersed AuNPs indicating that the as-synthesized AuNPs have the average diameters of ∼12 nm-diameter.

2.4. Preparation of MPTS sol–gel

The MPTS sol was prepared by dissolving MPTS, methanol and water (as 0.1 M HCl) in a molar ratio of 1:3:3 and stirring the mixture vigorously for 30 min [22,46].

2.5. Self-assembling of AuNPs on MPTS sol–gel network

The polycrystalline Au electrode of geometrical surface area 0.07 cm^2 was polished repeatedly with alumina (0.06 μm) and sonicated in water for 10 min. The well-polished electrode was then subjected to electrochemical pretreatment by cycling the potential between −0.2 and 1.5 V in 0.05 M H_2SO_4 at a scan rate of 100 V s^{-1} for 10–15 min or until a voltammogram characteristic of a clean polycrystalline Au electrode was obtained. The cleaned Au electrode was thoroughly rinsed with water and ethanol and was soaked in 0.5 mL of MPTS sol–gel for 20 min. The MPTS sol–gel chemisorbs on the polycrystalline Au electrode and exists as a 3-D silicate network [46,51]. The resulting MPTS sol–gel modified electrode was thoroughly rinsed with water to remove the physically adsorbed MPTS sol–gel and immersed into colloidal AuNP for 8 h. The thiol groups are distributed throughout the MPTS sol–gel network, the GNP can be conveniently self-assembled on the thiol groups present both inside and on the surface of the network.

2.6. Enzyme electrode preparation

6 μL of bilirubin oxidase (BOx, 50 units) in 0.2 M PBS was dispensed carefully onto the conducting surface of a freshly prepared, inverted Au/MPTS/AuNPs electrode and allowed for 2 h to dry to a film at ≤4 °C. To prevent enzyme loss, the electrode surface was carefully covered with a small eppendorf tube. The resulting modified electrode was stored at ≤4 °C in 0.2 M PBS (pH = 8.4) while not in use. Hereafter, the BOx self-assembled AuNPs will be referred to as Au/MPTS/AuNPs/BOx electrode. The amperometric biosensor for the sensing of bilirubin was fabricated as illustrated in Scheme 1. To obtain the FESEM image of the enzyme assembly on the AuNPs, a MPTS sol–gel film modified on the gold coated silicon wafer (cover slip) was used instead of a polycrystalline Au electrode. All electrochemical experiments were performed in nitrogen atmosphere. The electrochemical cell was covered by the dark-emery paper to preventing the loss of enzyme activity. The PBS (0.2 M, pH = 8.4) was used as a supporting electrolyte in all biosensor experiments. All the experiments were repeated at least four times and reproducible results were obtained. The calibration plots for the amperometric

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