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Polyphenol biosensor based on laccase immobilized onto silver nanoparticles/multiwalled carbon nanotube/polyaniline gold electrode

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ABSTRACT

Laccase purified from *Ganoderma* sp. was immobilized covalently onto electrochemically deposited silver nanoparticles (AgNPs)/carboxylated multiwalled carbon nanotubes (cMWCNT)/polyaniline (PANI) layer on the surface of gold (Au) electrode. A polyphenol biosensor was fabricated using this enzyme electrode (laccase/AgNPs/cMWCNT/PANI/Au electrode) as the working electrode, Ag/AgCl as the reference electrode, and platinum (Pt) wire as the auxiliary electrode connected through a potentiostat. The biosensor showed optimal response at pH 5.5 (0.1 M acetate buffer) and 35 °C when operated at a scan rate of 50 mV s^{-1}. Linear range, response time, and detection limit were 0.1–500 μM, 6 s, and 0.1 μM, respectively. The sensor was employed for the determination of total phenolic content in tea, alcoholic beverages, and pharmaceutical formulations. The enzyme electrode was used 200 times over a period of 4 months when stored at 4 °C. The biosensor has an advantage over earlier enzyme sensors in that it has no leakage of enzyme during reuse and is unaffected by the external environment due to the protective PANI microenvironment.

Polyphenols, present in a variety of plants, are consumed as important components of both human and animal diets [1–3]. Diets containing an abundance of polyphenolic compounds are protective against a variety of diseases, particularly cardiovascular disease and cancer. Polyphenolic compounds have antioxidant activity [4]. These compounds also affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity, and gene regulation [5]. A number of commonly used methods are available for determination of polyphenolic compounds such as spectrophotometry, gas chromatography, liquid chromatography, and capillary electrophoresis. However, these methods are time-consuming and require sample pretreatment [6]. In addition, the equipment used is expensive and generally unportable. Therefore, there is an interest in developing a simple, sensitive, accurate, and portable system such as a biosensor for determination of phenolic compounds. The development of biosensors was driven by the need for faster and more versatile analytical methods for application in important areas such as clinical, biomedical, environmental, industrial, and pharmaceutical analysis [7]. The advantages of biosensors, relative to chromatographic techniques, are their fast response, cost effectiveness, and simplicity of operation and manufacturing. A judicious choice of the biological receptor and applied potential improves their selectivity and sensitivity. Enzyme immobilization technology is an effective means to improve enzyme stability and perform its reuse [8,9]. Laccase (EC 1.10.3.2, *p*-benzenediol/oxygen oxidoreductase) belongs to a family of multicopper oxidases that catalyze the oxidation of a range of inorganic and aromatic compounds (particularly phenols), with the concomitant reduction of molecular oxygen to water [10–12]. A number of polyphenol biosensors based on laccase immobilized on various supports have been reported [13–15], but there are very few reports on covalent immobilization of enzyme.

Recent studies indicate that silver nanoparticles (AgNPs)[1] exhibit catalytic activity for the reduction of H_2O_2 [16,17]. Electrochemical results showed that the presence of AgNPs was responsible for sensor response, in terms of cathodic current increment, regarding the reduction of H_2O_2 [18]. Carbon nanotubes (CNT) are of special interest due to their ability to promote electron transfer reactions and high thermal capacity [19–21]. Composite materials based on integration of CNT and some other materials possessing properties of the individual components with a synergistic effect have also gained growing interest [22–26]. Because of facile preparation, superior transducing ability, and good environmental stability, polyaniline (PANI), a conducting polymer, has become the most attractive one in the formation of multiwalled carbon nanotubes (MWCNT)-

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[1] *Abbreviations used:* AgNPs, silver nanoparticles; CNT, carbon nanotubes; PANI, polyaniline; MWCNT, multiwalled carbon nanotubes; Au, gold; cMWCNT, carboxylated MWCNT; CV, cyclic voltammetry; Pt, platinum; SEM, scanning electron microscope; MEB, malt extract broth; FTIR, Fourier transform infrared; EIS, electrochemical impedance spectroscopy; Cu, copper; OMC, ordered mesoporous carbon; CV, coefficient of variation.

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based composites. PANI/MWCNT composites synthesized chemically or electrochemically have improved the electrical conductivity, electrochemical capacitance, and electrocatalytic properties of polymers.

In this study, we immobilized laccase (purified from *Ganoderma* sp.) onto AgNPs/PANI/MWCNT/Au (gold) electrode through covalent coupling to construct an enzyme electrode for determination of polyphenols that is likely to overcome the problem of leakage of enzyme. The resulting polyphenol biosensor was employed for amperometric determination of total phenolic content in beverages (tea, coffee, and wine) and pharmaceutical formulations (tablets and intravascular injections).

Materials and methods

Reagents

Sephadex G-100 and DEAE–Sephacel (Sigma–Aldrich, USA), carboxylated MWCNT (cMWCNT, functionalized MWCNT, Intelligent Materials, Panchkula [Haryana], India), and guaiacol and aniline (SRL, Mumbai, India) were used. All other chemicals were of analytical reagent grade. The aniline was purified through vacuum distillation before use.

Apparatus

Cyclic voltammetry (CV) and amperometric measurements were carried out in a potentiostat/galvanostat (model AUT83785, Autolab, Eco Chemie, The Netherlands). A conventional three-electrode system consisting of a modified Au electrode (diameter = 1 mm) as the working electrode, Ag/AgCl as the reference electrode, and platinum (Pt) electrode as the counter electrode was used. All experiments were carried out at room temperature. Scanning electron microscope (SEM, model Joel JSM-6510, Japan) images of electrodes at different stages of construction were taken in the Department of Chemistry at M.D. University (Rohtak, India).

Purification of laccase

The cell-free extract of *Ganoderma* sp. Rckk02 grown in malt extract broth (MEB) [27,28] was used as crude laccase. The crude enzyme was purified using the method of Satyapal and Pundir [29] consisting of the following steps: 0–80% ammonium sulfate precipitation, gel filtration on Sephadex G-100, and ion exchange chromatography on DEAE–Sephacel using a linear gradient of KCl (0.1–0.6 M) at 4 to 10 °C.

Assay of laccase

Assay of laccase was based on the oxidative polymerization of guaiacol [27]. The reaction mixture contained 0.2 μmol of guaiacol, 230 μmol of acetate buffer (pH 5.0), and enzyme in a total volume of 2.5 ml. Change in absorbance of 0.01 min^{-1} ml^{-1} at 470 nm was studied. One enzyme unit was defined as

$$\text{unit activity} = \frac{A_{470}/\text{min} \times \text{total volume of reaction mixture}}{\text{extinction coefficient of guaiacol} \times \text{volume of enzyme}}.$$

Protein content in various enzyme preparations was determined by the Lowry method using bovine serum albumin as standard protein.

Preparation of AgNPs

Silver nanoparticles were prepared as described previously [30]. Here 10 ml of 1.0 mM silver nitrate was added dropwise to 30 ml of chilled 2.0 mM sodium borohydride solution under vigorous stirring. The solution turned light yellow initially, but then turned a brighter yellow when all silver nitrate had been added. The entire addition took approximately 3 min, after which stirring was stopped and the stir bar was removed.

Preparation of AgNPs/cMWCNT/PANI/Au electrode

Prior to the surface modification, Au electrode was cleaned using piranha solution (H_2SO_4/H_2O_2, 3:1) for 20 min and then rinsed thoroughly with distilled water. The electrode was polished with alumina slurry. Then 1 g of MWCNT was suspended in 1 ml of a mixture of concentrated H_2SO_4 and HNO_3 (3:1) and ultrasonicated for 2 h to get a finely dispersed black-colored solution. Then 50 μl of 0.1 M aniline was added to 10.0 ml of 0.1 N HCl, and 1.0 ml of this solution was mixed with 1.0 ml of finely dispersed solution of cMWCNT. cMWCNT and aniline were electrodeposited onto Au electrode by immersing them in a solution of 23 ml of 0.1 M KCl and then by applying 30 polymerization cycles at 0.0–1.5 V as described previously [31]. The resulting cMWCNT/PANI/Au modified electrode was washed thoroughly with distilled water to remove unbound solution and kept in dry Petri plate at 4 °C.

The electrodeposition of AgNPs onto the cMWCNT/PANI modified Au electrode was carried out by immersing the modified electrode into a mixture of 22 ml of 0.1 M KCl and 3 ml of AgNPs colloidal solution and then applying a potential range from −0.2 to +0.4 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 0.1 V s^{-1}. After rinsing with double distilled water, the AgNPs/cMWCNT/PANI/Au electrode was dried in air.

Preparation of enzyme electrode (laccase/AgNPs/cMWCNT/PANI/Au)

The purified laccase was immobilized onto the surface of AgNPs/cMWCNT/PANI/Au electrode through covalent coupling by layering 10 μl of enzyme solution (40 mg ml^{-1} protein) and acetate buffer (pH 5.0) and keeping it undisturbed for approximately 12 h at 4 °C. The electrode was finally washed with acetate buffer (pH 5.0) to remove unbound enzyme. The protein concentration in wash-out solution was determined.

The principle of working of this biosensor was as follows. Guaiacol (standard phenolic substrate) was oxidized to its corresponding *o*-quinone by AgNPs/cMWCNT/PANI/Au electrode-bound laccase and then regenerated through electrochemical reduction of *o*-quinone, thereby forming a bioelectrocatalytic amplification cycle and generating electrons, which were passed to the working electrode from solution (Fig. 1). The current was measured and was directly proportional to the guaiacol concentration.

The fabricated electrode was characterized by SEM and Fourier transform infrared (FTIR) spectroscopy techniques at different stages of its construction. The resulting enzyme electrode was stored in a refrigerator at 4 °C when not in use.

We have recorded FTIR spectra of hybrid material deposited onto gold electrode. For this purpose, the hybrid material was scrapped off the Au electrode, mixed with dried KBr and its pellet was formed by hydraulic pellet press. Then this pellet was kept into the socket of the FTIR spectrometer and its spectra was recorded.

Response measurement of laccase/AgNPs/cMWCNT/PANI/Au

All electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the laccase/AgNPs/cMWCNT/PANI/Au electrode as the working electrode, a Pt wire as the auxiliary electrode, and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode. CV measurements were carried out in a three-electrode cell containing 0.1 ml of guaiacol (10 μM) and 15 ml of acetate buffer (0.1 M, pH 5.0). Current measurements were performed by applying CV