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

Rachna Rawal, Sheetal Chawla, C.S. Pundir *

Department of Biochemistry, M.D. University, Rohtak 124 001, India

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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)-

* Corresponding author.
E-mail address: pundircs@rediffmail.com (C.S. Pundir).

[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

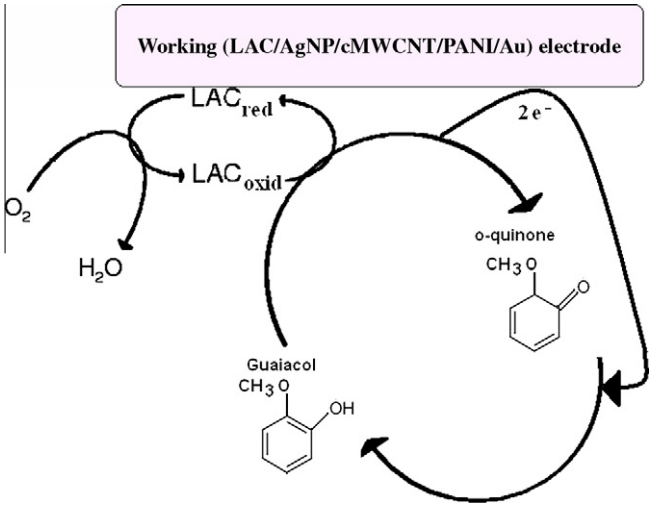


Fig.1. Chemical reaction catalyzed by laccase (LAC).

in the potential range from −0.2 to +1.5 V. To record cyclic voltammograms, the following instrumental parameters were used: step potential of +6 mV and scan rate of 50 mV s^{-1}. All electroanalytical measurements were carried out at room temperature.

Optimization of laccase/AgNPs/cMWCNT/PANI/Au electrode

To determine the optimal working conditions of the enzyme electrode, the pH of reaction buffer (0.1 M) was varied from 3.0 to 7.0 at intervals of 0.5 using acetate buffer in the acidic pH range (pH 3.0–5.0) and phosphate buffer in the pH range of 6.0–7.0. Similarly, to determine the optimal temperature, the reaction mixture was incubated at 15–50 °C at intervals of 5 °C. To determine the optimal response time, the current response was measured from 2 to 12 s at intervals of 2 s. To study the effect of substrate concentration, different guaiacol concentrations, ranging from 0.1 to 500 μM, were used. K_m and I_{max} for guaiacol were calculated from a Lineweaver–Burk plot based on the following equation:

$$\frac{1}{I} = \frac{K_m}{I_{max}}\frac{1}{[S]} + \frac{1}{I_{max}},$$

where K_m is the Michaelis–Menten constant, I_{max} is the maximum current, and S is the guaiacol concentration.

Amperometric measurement of total phenolic content in tea, alcoholic beverages, and pharmaceutical formulations

The biosensor was employed for determination of polyphenols in five commercial brands of tea leaves, alcoholic beverages, and pharmaceutical samples. Samples of tea leaves were prepared by boiling 5 g of dried tea leaves into 20 ml of distilled water and filtering it through a sieve. The alcoholic beverage samples were diluted by 10 times with acetate buffer (pH 6.0). The pharmaceutical tablets (1 g) were grinded in a pestle mortar to powder form and dissolved in 4 ml of acetate buffer (pH 6.0). Total phenolic content in tea leaves, alcoholic beverages, and pharmaceutical samples was determined by the biosensor in the same manner as described above for its response measurement under its optimal working conditions except that guaiacol was replaced by these samples. The current (mA) was recorded, and the amount of total phenolic content was extrapolated from standard curve between guaiacol concentrations and current (μA) prepared under optimal working conditions (Fig. 2).

Determination of total phenolic content in tea, alcoholic beverages, and pharmaceutical formulations by spectrophotometric method

The real sample analysis by standard Folin–Ciocalteu colorimetry [32] was carried out as follows. Sample (20 μl) was added into a 2-ml glass cuvette containing 1.58 ml of water. FC reagent (100 μl) was added to the cuvette and mixed thoroughly by inverting and then incubated for 5 min. Sodium carbonate solution (300 μl) was added, mixed, and incubated for 2 h at room temperature. A_{765} was read against the blank, and total phenolic content in the sample was extrapolated from the standard curve between guaiacol concentration and A_{765}. The values were calculated in guaiacol equivalents using units of μmol guaiacol/g.

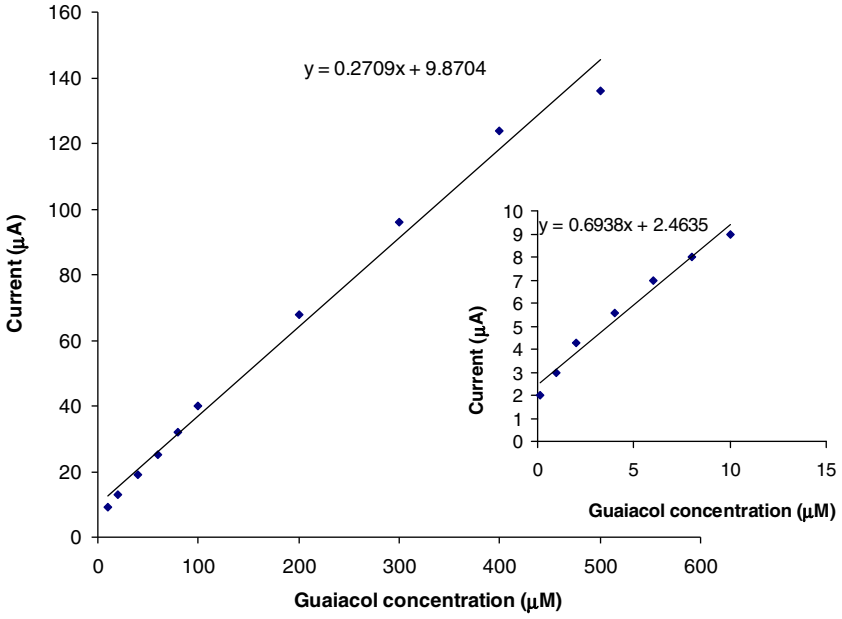


Fig.2. Calibration curve for polyphenol biosensor. Inset: Calibration curve at a concentration below 10 μM guaiacol.

Results and discussion

Electrode surface characterization by SEM and FTIR

The morphology of bare Au electrode, cMWCNT/PANI/Au electrode, and laccase/AgNPs/cMWCNT/PANI/Au electrode was characterized by SEM studies. SEM of the bare Au electrode (Fig. 3A) shows a homogeneous surface. The cMWCNT/PANI composite film shows a net-like structure (Fig. 3B). Film was more uniform and porous, and hence the effective surface area was larger. The SEM images for laccase/AgNPs/cMWCNT/PANI/Au show a regular globular structural morphology (Fig. 3C), indicating the successful immobilization of laccase to the surface of the cMWCNT/PANI layer. Fig. 4 displays the FTIR spectra for cMWCNT/PANI/Au (curve a) and laccase/AgNPs/PANI/cMWCNT/Au (curve b). The FTIR spectrum of electrodeposited cMWCNT/PANI composite (curve a) shows benzenoid and quinoid ring stretching bands (C═C) present at 1447.6 and 1594.2 cm^{-1}. The presence of peaks at 1129 and 3402 cm^{-1} could be attributed to B–N^{+}═Q stretching and –N–H stretching vibrations of PANI in the composite. The peak at

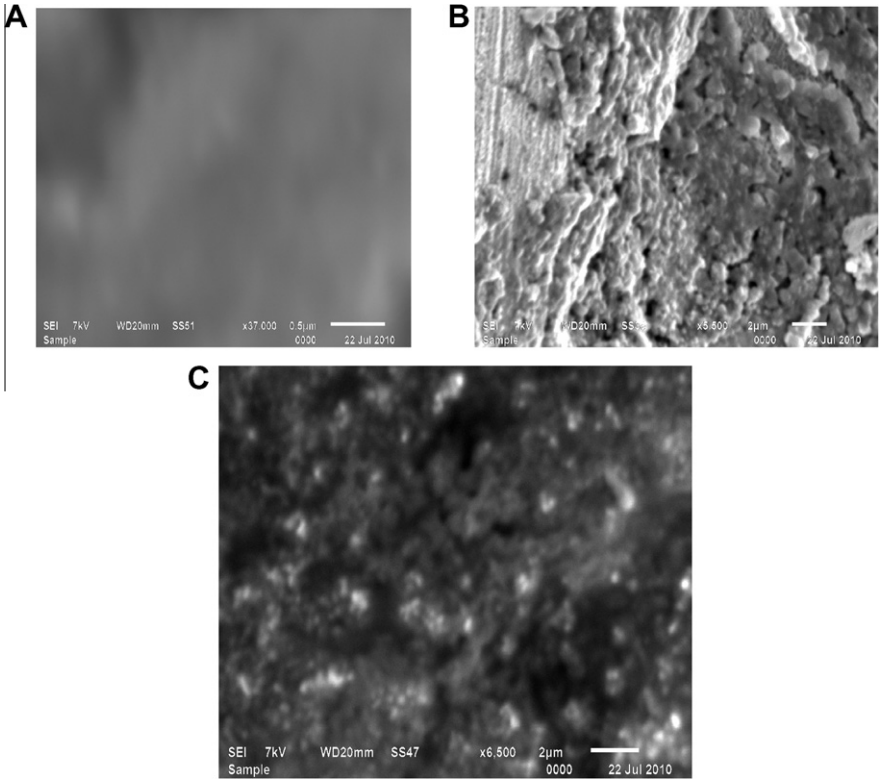


Fig.3. Scanning electron micrographs of bare gold electrode (A), MWCNT/PANI/Au electrode (B), and laccase/AgNPs/cMWCNT/PANI/Au electrode (C).

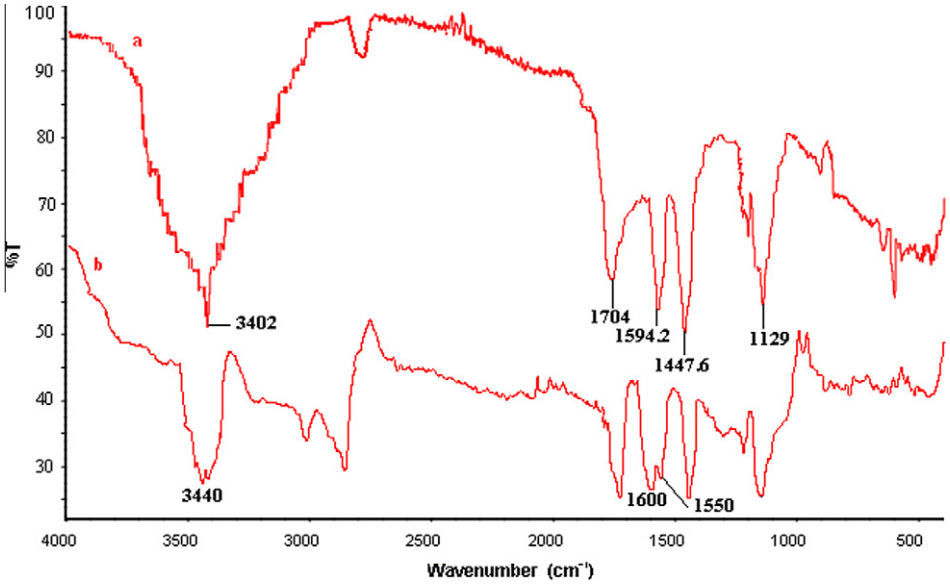
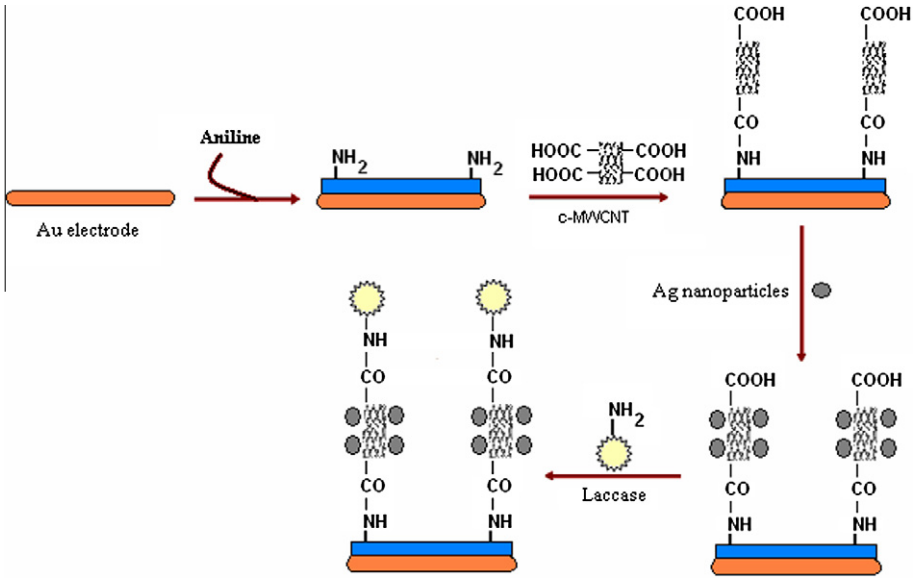


Fig.4. FTIR spectra of (a) cMWCNT/PANI/Au and (b) laccase/AgNPs/PANI/cMWCNT/Au electrode.

1704 cm^{-1} is attributed to –C═O stretching vibrations and indicates the presence of carboxyl group (–COOH). The presence of these peaks reveals the existence of both PANI and cMWCNT on the Au electrode. The Lac/AgNPs/cMWCNT/PANI/Au electrode (curve b) showed characteristic peaks at 1550 and 1600 cm^{-1} attributed to the primary and secondary amide linkages between $–NH_2$ on the surface group on the surface of enzyme molecules and free –COOH groups of MWCNT.

The fabrication of the polyphenol biosensor based on AgNPs onto cMWCNT/PANI modified Au electrode is summarized in Scheme 1. First, PANI was electrodeposited onto Au electrode, and with free $–NH_2$ groups at its ends it gets linked to –COOH groups of cMWCNT through amide bond (–CO-NH–). Silver nanoparticles are then electrochemically deposited onto the surface of PANI/cMWCNT. The electrodeposition method was selected to produce AgNPs on electrode surfaces because this method is easy to



Scheme 1. Chemical sequence of electropolymerization of AgNPs/cMWCNT/PANI onto Au electrode and chemical reaction of immobilization of enzyme on modified electrode.

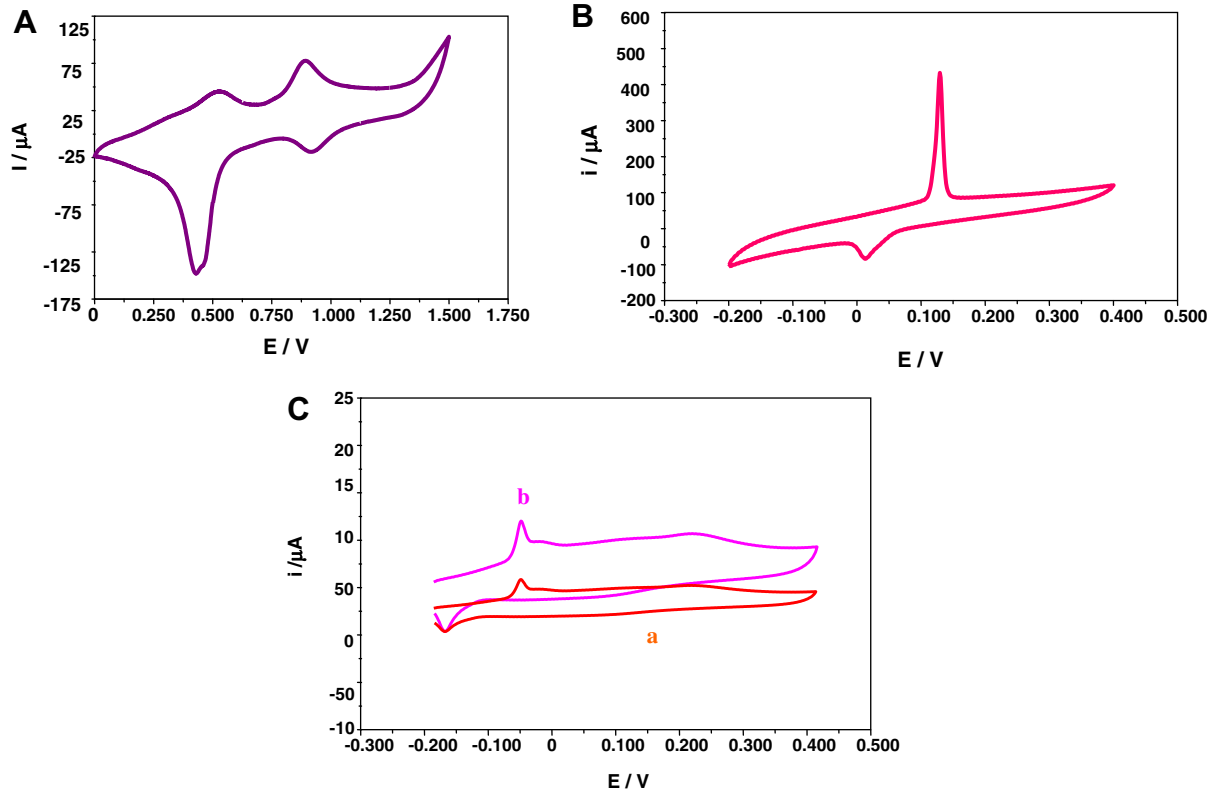


Fig.5. (A and B) Cyclic voltammograms of electrochemical deposition of PANI/cMWCNT onto Au electrode (A) and AgNPs deposition onto cMWCNT/PANI-coated Au electrode (B). (C) CV curves of laccase/AgNPs/cMWCNT/PANI/Au electrode in 0.1 M acetate buffer (pH 6.0) without 2 µM guaiacol solution (curve a) and with 2 µM guaiacol solution (curve b). Supporting electrolyte: 0.1 M KCl solution; scan rate: 50 mV s^{-1}.

carry out and the layer thickness can be controlled. Laccase is immobilized covalently as $-NH_2$ of enzyme forms an amide bond (–CO-NH–) with the –COOH group of cMWCNT. Our results show that AgNPs and cMWCNT provided a remarkable synergistic effect toward the reduction of guaiacol.

To evaluate the charge transfer properties on the surface of the modified electrodes, the CV technique was employed using KCl as electrolyte. Cyclic voltammograms recorded in 0.1 M KCl solution and 0.1 M acetate buffer (pH 5.0) are shown in Fig. 5. Fig. 5A and B display the cyclic voltammograms of cMWCNT/PANI/Au modified electrode and AgNPs/cMWCNT/PANI/Au modified electrode, respectively. CV of cMWCNT/PANI/Au revealed two redox peaks for oxidation of aniline to radical cation and then to radical dication [33]. The electrodeposition of AgNPs onto cMWCNT/PANI modified Au electrode surface leads to increased current intensity as a result of increased electroactive area (Fig. 5B). The preservation of a quasi-reversible electrode process and the large increase in peak currents for the nanocomposite film modified electrode proved that AgNPs exerted an obvious improvement effect on cMWCNT/PANI/Au electrode properties.

Response toward guaiacol at the laccase/AgNPs/cMWCNT/PANI/Au electrode

To evaluate the catalytic activity of laccase at the modified electrode, it was characterized by a cyclic voltammogram in the presence of guaiacol in the potential range from −0.2 to +0.4 V. Fig. 5C shows cyclic voltammograms of the laccase/AgNPs/cMWCNT/PANI/Au electrode in an unstirred 0.1 N KCl solution and 0.1 M acetate buffer (pH 5.5) without guaiacol solution (curve a) and with guaiacol solution (curve b) at a scan rate of 50 mV s^{-1}. It was observed that with the addition of 2 μM guaiacol, the oxidation current increased while reduction current decreased. A well defined oxidation peak (0.22 V) was observed, which clearly indicate the catalytic properties of modified electrode. Thus, for further amperometric study of Lac/AgNP's/cMWCNT/PANI/Au electrode, a potential of 0.22 V was applied.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) provides useful information on the impedance changes of the electrode surface during the fabrication process. The semicircular portion at higher frequencies corresponds to the electron transfer-limited process, and its diameter is equal to the electron transfer resistance, which controls the electron transfer kinetics at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process [34]. Fig. 6 displays the Nyquist plots of the EIS of cMWCNT/PANI/Au electrode (curve a), AgNPs/cMWCNT/PANI/Au electrode (curve b), and laccase/AgNPs/cMWCNT/PANI/Au electrode (curve c) in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1)] in the 0.1 M KCl, at the polarization potential of 0.2 V in the frequency range 0.1–10^4 Hz. Nyquist diameter of cMWCNT/PANI/Au electrode (curve a, R_{CT} = 2200 Ω) is much larger than that of AgNPs/cMWCNT/PANI/Au electrode (curve b, R_{CT} = 1700 Ω), suggesting that cMWCNT/PANI modified electrode increases the resistance of the electrodes [35], whereas AgNPs act as a nanoscale electrode and promote electron transfer. However, after immobilization of laccase onto AgNPs/cMWCNT/PANI/Au electrode, the R_{CT} was found to increase to 2700 Ω (curve c). This suggests that immobilized molecules strongly bind with hybrid nanobiocomposite and block charge carriers in the nanobiocomposite matrix. These results clearly indicate the immobilization of laccase onto AgNPs/cMWCNT/PANI/Au electrode.

The change in the R_{CT} value reflecting the increase or decrease in the diameter of the semicircle at high frequencies in the impedance spectra is associated with the blocking behaviour of the electrode surface for the charge transfer to the redox probe.

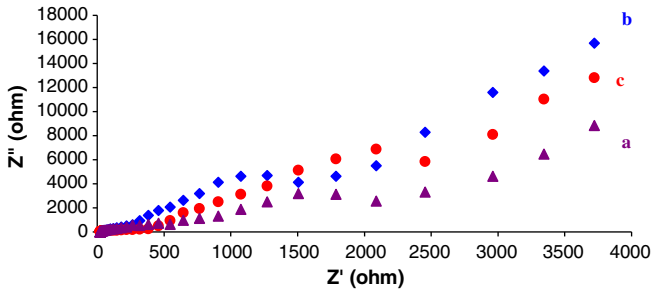


Fig.6. Nyquist plots of EIS comparison of cMWCNT/PANI/Au electrode (curve a), AgNPs/cMWCNT/PANI/Au electrode (curve b), and laccase/AgNPs/cMWCNT/PANI/Au electrode (curve c) in 0.1 M KCl.

Optimization of experimental conditions of biosensor

To optimize the biosensor (laccase/AgNPs/cMWCNT/PANI/Au electrode), the effects of pH, incubation temperature, and scan rate were studied. The current response resulting from the AgNPs/cMWCNT/PANI/Au-bound enzyme-catalyzed reaction achieved a maximum value at pH 5.5 (Fig. 7A). Therefore, a pH of 5.5 was used in further experiments. The optimal temperature of the biosensor was 35 °C (Fig. 7B). Polyaniline being a good thermal insulator for enzymes might have provided the microenvironment to protect the enzyme from environmental and thermal variations, favoring the repeated use of AgNPs/cMWCNT/PANI electrode [36]. The biosensor showed a maximum response at 6 s (Fig. 8A) after which it became constant. The effect of the scan rate from 25 to 200 mV s^{-1} on the biosensor response using 2 μM guaiacol in 0.1 M acetate

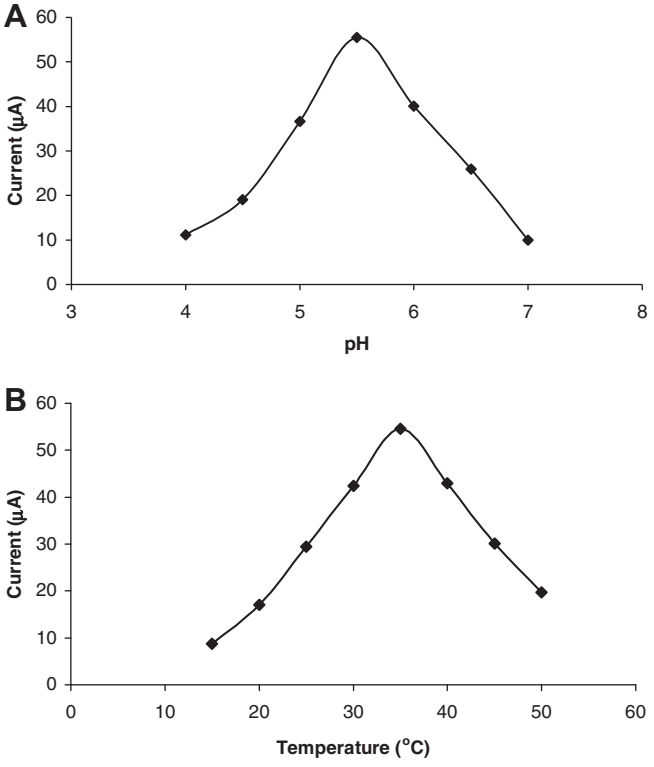


Fig.7. Effects of pH (A) and temperature (B) on the current response of laccase/AgNPs/cMWCNT/PANI/Au electrode.

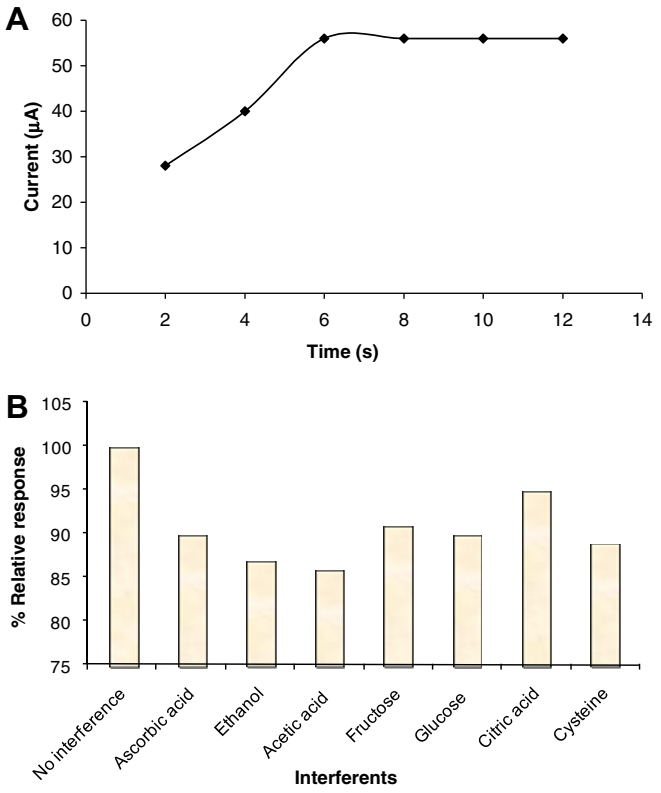


Fig.8. (A) Time course study of laccase/AgNPs/cMWCNT/PANI/Au electrode. (B) Effect of potential interferents on biosensor response.

buffer solution (pH 6.0) was also investigated. A potential scan rate of 50 mV s^{-1} was selected because with these experimental conditions the highest analytical signal and very good cyclic voltammogram profiles were obtained.

The effect of several possible interfering substances, such as ascorbic acid, acetic acid, ethanol, fructose, glucose, citric acid, and cysteine at a final concentration of 0.1 mM, on the current biosensor was investigated versus Ag/AgCl in acetate buffer (pH 5.5). The decrease in the current was calculated as guaiacol concentration equivalence (i.e., the concentration of guaiacol that can produce the same decrease as the interferents). Negligible interference was observed with ascorbic acid, glucose, fructose, and citric acid (Fig. 8B).

Evaluation of biosensor

Linearity

There was a linear relationship between current (μA) and guaiacol concentration, ranging from 0.1 to 10 μM (lower concentration range) with the linear equation being $y = 0.2709x + 9.8704$ and ranging from 10 to 500 μM (higher concentration range) with the linear equation being $y = 0.6938x + 2.4635$ (Fig. 2) in reaction mixture.

The detection limit of the present sensor was 0.05 μM (S/N = 3). K_m for guaiacol was 307.69 μM and Imax was 153.84 μA (Fig. 9). The *Ganoderma* sp. laccase is known to have wide substrate specificity [37]. So, the current biosensor based on this enzyme is not selective.

The detection limit of the current sensor was 0.05 μM (signal/noise ratio = 3), which is lower than that of CNT modified glassy carbon electrode (0.29 μM) [38] and Cu–OMC/chitosan matrix-based electrode (0.67 μM) [39].

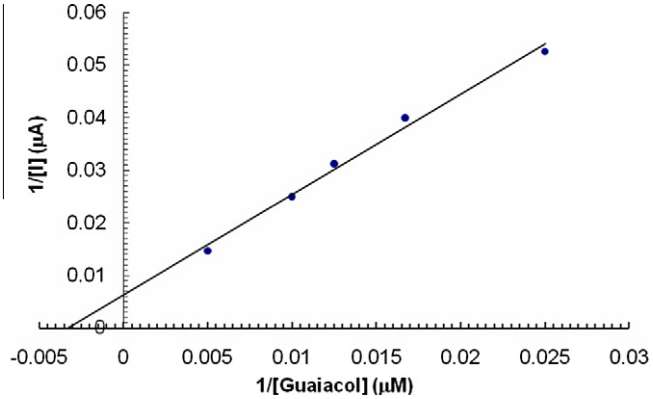


Fig.9. Lineweaver–Burk plot for the effect of L-guaiacol concentration on response Laccase/AgNP's/cMWCNT/PANI/Au electrode.

Stability and reusability of biosensor

The electrode lost 20% of its initial activity after 200 uses during the span of 4 months when stored at 4 °C, which is much better than earlier biosensors [38,39].

To test the reproducibility and reliability of the current biosensor, the polyphenol level in six tea samples was determined on a single day (within batch) and five times again after storage at −20 °C for 1 week (between batch). The results showed that determinations were consistent, and within- and between-batch coefficients of variation (CVs) were 2.3% and 4.4%, respectively, which is better than earlier reports [39,40]. The high precision indicated the reproducibility and consistency of the current method. The good stability, repeatability, and reproducibility observed for the proposed biosensor could be attributed to the excellent immobilization of laccase onto AgNPs/cMWCNT/PANI/Au electrode.

Recovery

A recovery study was performed using tea leaf extract by adding 0.1 mM guaiacol. The results showed that average recovery of added guaiacol was 96.03%, demonstrating the high accuracy of the biosensor.

Correlation

Comparing the values of polyphenols in 15 pharmaceutical samples by the current method (y) with those obtained by the

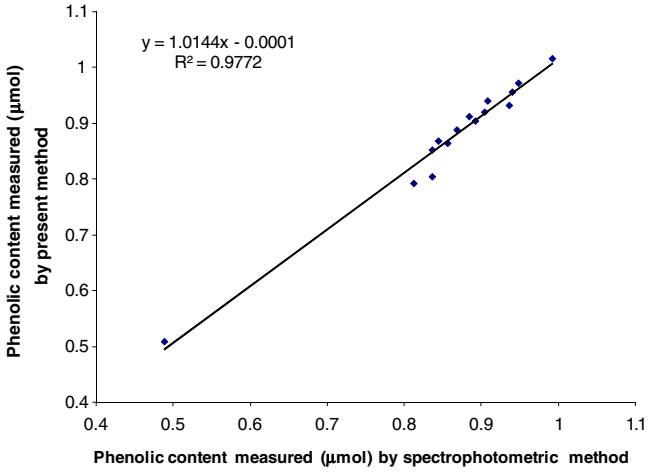


Fig.10. Correlation between total phenolic content values determined by standard spectrophotometric method (x axis) and current biosensor employing laccase/AgNPs/cMWCNT/PANI/Au electrode method (y axis).

Table 1
Comparison of total phenolic content in different brands of tea, alcoholic beverages, and pharmaceutical formulations as measured by lac/AgNPs/cMWCNT/PANI/Au electrode and standard spectrophotometric method.

Samples	Total phenolic content by present method (means ± SD, $n = 4$)	Total phenolic content by standard spectrophotometric method (means ± SD, $n = 4$)
Tea	*(μmol guaiacol/g)*	
Red label	0.116 ± 0.032	0.104 ± 0.024
Tata	0.560 ± 0.048	0.548 ± 0.042
Vijay	0.872 ± 0.052	0.860 ± 0.049
RCM	0.792 ± 0.056	0.784 ± 0.051
Nescafe	0.844 ± 0.052	0.832 ± 0.043
Alcoholic beverages	*(μM guaiacol)*	
Officer's choice	204 ± 0.048	200 ± 0.040
Royal challenge	235 ± 0.056	233 ± 0.042
Aristocrat	202 ± 0.064	199 ± 0.046
Bagpiper	237 ± 0.076	235 ± 0.053
Bonnie	232 ± 0.056	230 ± 0.041
Pharmaceutical formulations	*(μmol guaiacol/g)*	
Syndopa-110	0.852 ± 0.068	0.840 ± 0.034
Syndopa-275	0.888 ± 0.048	0.872 ± 0.029
α-Dopa	0.956 ± 0.044	0.948 ± 0.030
Dopamine	0.804 ± 0.080	0.800 ± 0.043
Isoprenaline	0.508 ± 0.036	0.496 ± 0.028
Adrenaline	0.904 ± 0.052	0.892 ± 0.027

Note: SD, standard deviations.

standard spectrophotometric method (x) showed good correlation with $r = 0.99$ and a regression equation of $y = 1.0144x - 0.0001$ (Fig. 10). These results indicate a very good analytical performance of the current biosensor in biological samples.

Determination in tea, alcoholic beverages, and pharmaceutical formulations

Total phenolic content as measured by the current sensor ranged from 0.116 to 0.872 μmol guaiacol/g in tea, from 202 to 237 μM in alcoholic beverages, and from 0.804 to 0.956 μmol guaiacol/g in pharmaceutical formulations (Table 1).

Conclusions

The current study showed that AgNPs/cMWCNT/PANI hybrid film is a very good candidate for the construction of a highly sensitive polyphenol biosensor. The fabricated enzyme electrode showed good characteristics such as a larger determination range, faster response time, and higher stability.

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