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An amperometric biosensor based on laccase immobilized onto MnO_2NPs/cMWCNT/PANI modified Au electrode

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ABSTRACT

A method is described for construction of an amperometric biosensor for detection of phenolic compounds based on covalent immobilization of laccase (Lac) onto manganese dioxide nanoparticles (MnO_2NPs) decorated carboxylated multiwalled carbon nanotubes (cMWCNTs)/PANI composite electrodeposited onto a gold (Au) electrode through *N*-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS) chemistry. The modified electrode was characterized by scanning electron microscopy (SEM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The biosensor showed optimum response at pH 5.5 (0.1 M sodium acetate buffer) and 35 °C, when operated at 0.3 V vs. Ag/AgCl. Linear range, response time, detection limit were 0.1–10 μM (lower concentration range) and 10–500 μM (higher concentration range), 4 s and 0.04 μM, respectively. Biosensor measured total phenolic content in tea leaves extract. The enzyme electrode was used 150 times over a period of 5 months.

1. Introduction

Phenolic compounds as antioxidants are important constituents of natural products, such as fruits and vegetables. These are free radical scavengers, neutralizing oxygen reactive species and chelate metal ions. They cause great impact on prevention of cardiovascular diseases and even some of them prevent cancer. These have been detected in industrial effluents in the form of phenols, catechols, guaiacols and cresols. Therefore, their determination is very important [1,2]. Analytical methods available for determination of phenolic content include spectrophotometry, gas chromatography, liquid chromatography and capillary electrophoresis. However, some of these methods (e.g. spectrophotometry) are not sensitive and specific while others (e.g. gas chromatography, liquid chromatography and capillary electrophoresis) are cumbersome and require time consuming sample pre-treatment, expensive equipments and skilled persons to operate [3]. Therefore, there is an interest in developing a simple, sensitive, rapid, cost-effective and portable system such as biosensor for determination of phenolic compounds in important areas such as clinical, biomedical, environmental, industrial and pharmaceutical analysis [4]. Laccase, a glycosylated polyphenol oxidase containing four copper ions per molecule, catalyses single-electron oxidation of a wide variety of organic and inorganic substrates including mono-, di- and polyphenols, methoxyphenols, aromatic amines and ascorbate with concomitant four-electrons reduction of oxygen to water [5]. For catalysing the oxidation of non-phenolic substrates, laccase requires the presence of a mediator in the medium. A mediator is a small molecule that behaves like an 'electron shuttle' between laccase and substrate and these small molecular mass compounds are converted into stable radicals by means of enzymatic oxidation. Although the specificity for the electron donor is low, the specificity for the acceptor (oxygen) is essential. Peroxides are not produced in the reaction. A number of biosensors for detection of phenolic compounds have been reported, which were based on immobilization of laccase onto various supports such as graphite electrode [6], silane-modified platinum surface [7], titania gel matrix through adsorption [8], carbon nanotubes-chitosan composite by crosslinking [9], hydrophilic matrix by covalent coupling [10]. All these immobilization methods except covalent coupling suffer from leaking of enzyme during reuse, limited electron flow and storage stability. Recently, the biocompatible nanomaterials have opened a new area towards the development of third generation biosensors based on direct electron transfer between enzyme and electrode [11]. Multi-walled carbon nanotubes (MWCNTs) possess many unique properties such as good electrical conductivity, strong adsorptive ability and excellent biocompatibility [12] and thus have ability to promote the electron transfer of hydrogen peroxide [13]. Composite materials based on integration of CNT with some conducting polymer have been exploited for further

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improvement in the analytical performance of electrochemical biosensors. Polyaniline (PANI) is also an attractive conducting polymer, as it exhibits two redox couples facilitating efficient enzyme–polymer charge transfer [14].

Among various metal oxide nanoparticles, manganese dioxides nanoparticles (MnO_2NPs) have great surface area and exhibit remarkable electrocatalytic activity for the oxidation of H_2O_2. In recent years, several kinds of MnO_2 nanoparticles were synthesized for construction of biosensors [15].

We describe herein the construction of an amperometric biosensor based on covalently immobilized laccase onto MnO_2NPs/cMWCNT/PANI/Au electrode using *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS) chemistry and its application for measurement of total phenolic content in tea leaves extract.

2. Materials and methods

2.1. Reagents

Sephadex G-100 and DEAE-Sephacel from Sigma (Aldrich), St. Louis, USA. Guaiacol, aniline, manganese sulphate, ammonium persulphate, silver nitrate from MERCK India Pvt. Ltd., Mumbai. *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), Folin–Ciocalteu's phenol reagent from SISCO Research Laboratory Mumbai, India, carboxylated multi-walled carbon nanotubes (c-MWCNT) (functionalized MWCNT) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana), India were used. All other chemicals used were of Analytical Reagent (AR) grade. Tea leaves extract of various commercial brands were purchased from local market. Double distilled water (DW) was used throughout the experiments.

2.2. Apparatus

All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (Eco Chemie BV, The Netherlands) with GPES 4.9 software. All experiments were carried out at room temperature. Scanning electron microscopic (SEM) images were taken in a scanning electron microscope (Make: Model Joel JSM-6510, Japan) at Advanced Instrumentation Research Facility, JNU, New Delhi.

2.3. Purification of laccase

The cell free extract of *Ganoderma* sp. Rckk02 grown in malt extract broth (MEB) [16,17] was used as crude laccase. The crude enzyme was purified by combination of 0–80% ammonium sulphate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel using linear gradient of KCl (0.1–0.6 M) at 4–10 °C.

2.4. Assay of laccase

Assay of laccase was based on the oxidative polymerization of guaiacol [16]. The reaction mixture contained 0.2 μmol of guaiacol, 230 μmol 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 is defined as

$$\text{Unit activity} = \frac{\Delta A_{470}/\text{min.} \times \text{Total vol. of reaction mixture}}{\text{Extinction coefficient of guaiacol} \times \text{vol. of enzyme (mL)}}$$

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

2.5. Preparation of manganese dioxide nanoparticles (MnO_2NPs)

MnO_2NPs were synthesized according to Zhang et al. [15]. Analytical grade $MnSO_4 \cdot H_2O$ (0.3380 g, 2 mM), $(NH_4)_2S_2O_8$ (0.4564 g, 2 mM) and 2 mL concentrated sulphuric were mixed in 50 mL DW at room temperature. Then, 1 mL of 10 mL $AgNO_3$ (0.1052 g, 0.059 mM) solution was added. The homogeneous solution was allowed to stand for 1–2 days, the products were filtered off, washed with absolute ethanol followed by DW for several times, and then dried in vacuum.

2.6. Preparation of MnO_2NPs/cMWCNT/PANI/Au electrode

Prior to electrodeposition, the Au electrode was polished with alumina slurry. The c-MWCNT and aniline were electrodeposited onto Au electrode through CV as described [18]. The electrodeposition of MnO_2NPs onto the cMWCNT/PANI modified Au electrode was carried out by immersing the modified electrode (cMWCNT/PANI/Au) into a mixture of 23 mL of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) and 2 mL of MnO_2NPs solution and then applying potential between −0.2 and +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 V s^{-1}. After rinsing with DW, the MnO_2NPs/cMWCNTs/PANI/Au electrode was dried in air.

2.7. Immobilization of Lac on MnO_2NPs/cMWCNT/PANI/Au electrode

Lac was immobilized onto MnO_2NPs/cMWCNT/PANI/Au electrode by EDC–NHS chemistry [19]. Firstly, MnO_2NPs/cMWCNT/PANI/Au electrode was immersed into 0.1 M phosphate buffer (pH 7.5) containing EDC and NHS of same concentration (10 mM) for 6 h and then excess of EDC and NHS were removed by washing with 0.1 M sodium phosphate buffer (PB) (pH 7.5). Finally, EDC–NHS activated electrode was immersed into 5 mL 0.1 M PB, pH 7.5 containing 100 μL enzyme solution and kept overnight at 4 °C and thereafter taken from PB and washed with DW. The fabricated electrode was characterized by SEM and electrochemical impedance spectroscopy (EIS) techniques at different stages of its construction. The enzyme electrode was stored in refrigerator at 4 °C when not in use.

2.8. Response measurement of Lac/MnO_2NP/cMWCNT/PANI/Au electrode

All electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the Lac/MnO_2NPs/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. Cyclic voltammetric measurements were carried out in a three electrode cell containing 20 mL electrolyte [2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1)], 5 mL acetate buffer (0.1 M, pH 5.0) and 0.1 mL of guaiacol (10 μM). Current measurements were performed applying CV in the potential range, −0.2 and +0.6 V (vs. Ag/AgCl). The optimum current was obtained at 0.3 V (vs. Ag/AgCl), hence the subsequent electrochemical studies were carried out at this potential.

The principle of working of this biosensor is as follows: guaiacol (standard phenolic substrate) is oxidized to its corresponding *o*-quinone by Lac/MnO_2NPs/cMWCNT/PANI/Au electrode bound laccase and then regenerated through electrochemical reduction of o-quinone, thus forming a bioelectrocatalytic amplification cycle and thereby generating electrons, which were passed to working electrode from solution and relayed to potentiometer, in which it is read as current in mA (milliampere). The electrons then reached Ag/AgCl electrode, where Ag^+ ions were reduced to Ag metal. The

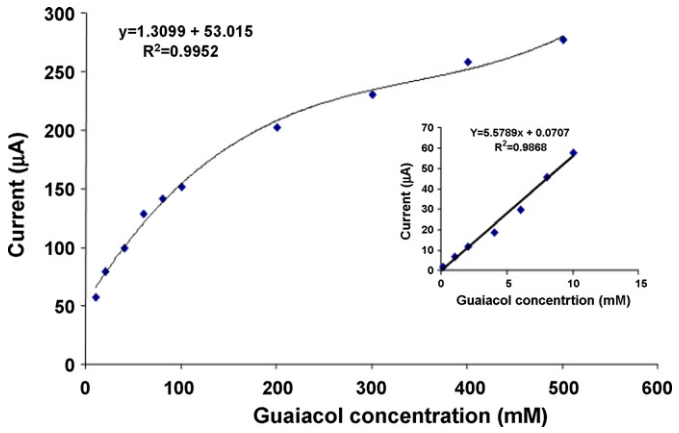


Fig. 1. Standard curve for guaiacol by biosensor based on laccase immobilized MnO_2NPs/cMWCNT/PANI/Au electrode.

current is measured, which is directly proportional to guaiacol concentration.

2.9. Optimization of Lac/MnO_2NPs/cMWCNT/PANI/Au electrode

To determine the optimum working conditions of the enzyme electrode, the pH of reaction buffer (0.1 M) was varied from pH 3.0 to 7.0 at an interval of pH 0.5 using acetate buffer in acidic pH range (pH 3.0–5.0) and phosphate buffer in pH range 6.0–7.0. Similarly, to determine optimum temperature, the reaction mixture was incubated at 15–50 °C at an interval of 5 °C. To determine the optimum response time, the current response was measured from 2 s to 8 s at an interval of 2 s. To study the effect of substrate concentration, different guaiacol concentrations, ranging from 0.1 to 500 μM were used.

2.10. Evaluation

The following criteria were studied to evaluate the analytic performance of the biosensor, e.g. linearity, analytical recovery, detection limit, precision and correlation. Analytical recovery was studied using tea leaves extract with two different standard concentrations (5.0 μM and 10.0 μM) of guaiacol. To test the reproducibility and reliability of the present biosensor, phenolic content in six tea leaves extract was measured five times on single day (within batch) and five times again after their storage at −20 °C for 1 week (between batch). To study the correlation the phenolic content was measured in 10 brands of tea leaves extract by the standard spectrophotometric method and the present method and their correlation was studied using regression equation.

To examine the long-term storage stability, the activity of enzyme electrode was tested up to 5 months at an interval of 1 week, when stored at 4 °C. To reuse the enzyme electrode, it was washed 3–4 times with reaction buffer before its use in next assay.

2.11. Amperometric measurement of total phenolic content in tea leaves extract

The biosensor was employed for determination of total phenolic content in 10 commercial brands of tea leaves extract. The tea leaves extract was diluted 10 times with acetate buffer (pH 5.5).

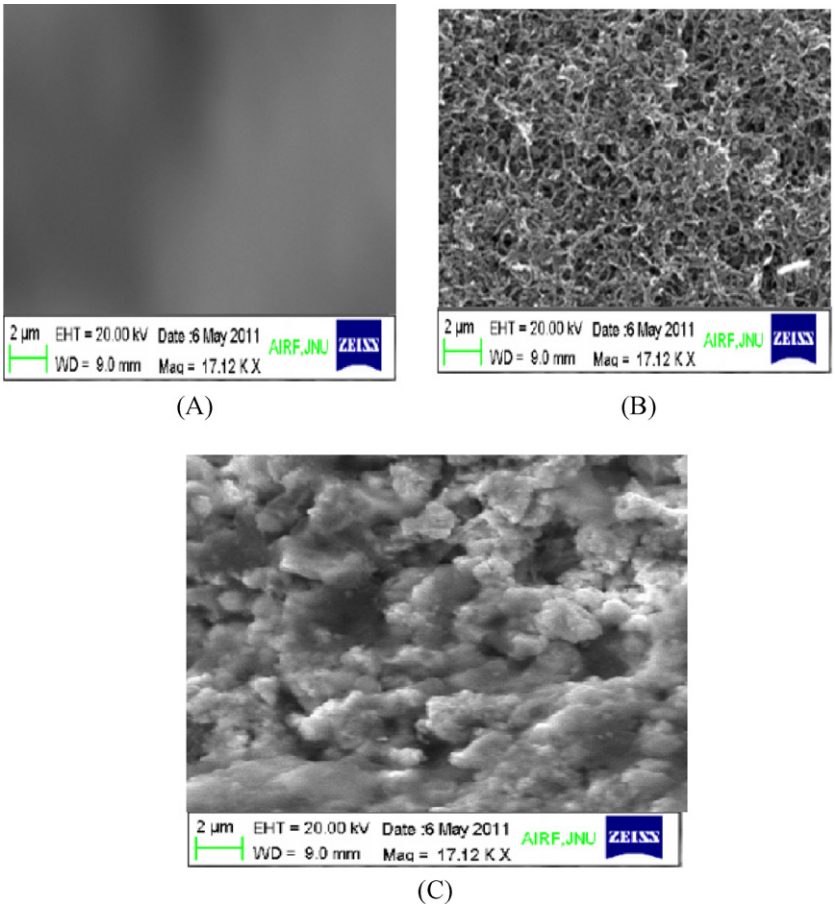


Fig. 2. Scanning electron micrographs of: (a) bare Au electrode, (b) MnO_2NPs/cMWCNT/PANI/Au electrode, and (c) Lac/MnO_2NPs/cMWCNT/PANI/Au electrode.

Total phenolic content in tea leaves extract 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 sample. The current (μA) 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. 1).

3. Results and discussion

3.1. Electrode surface characterization by SEM

Fig. 2 shows morphology of bare Au electrode, cMWCNTs/PANI/Au electrode and Lac/MnO_2NPs/cMWCNT/PANI/Au electrode as characterized by SEM studies. The SEM of the bare Au electrode (Fig. 2A) showed a homogeneous surface. The cMWCNTs/PANI composite film showed net like structure (Fig. 2B). Film was more uniform and porous and hence effective surface area was larger. The SEM images for Lac/MnO_2NPs/cMWCNT/PANI/Au showed a globular structures on this film morphology (Fig. 2C), indicating the successful immobilization of laccase to the surface of cMWCNT/PANI layer.

A method is described for construction of a Lac/MnO_2NPs/cMWCNT/PANI/Au. Scheme 1 summarizes the different chemical reactions involved in the fabrication of Lac electrode based on covalent immobilization of Lac onto MnO_2NPs/cMWCNT/PANI/Au electrode through amide bond formation between the free –COOH groups of MnO_2NPs/cMWCNT/PANI/Au electrode and free –NH_2 groups on surface of enzyme.

3.2. Response towards guaiacol at the Lac/MnO_2NPs/cMWCNT/PANI/Au electrode

To evaluate the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry technique was employed, using potassium ferricyanide–potassium ferrocyanide mixture as electrolyte. Cyclic voltamogramms were recorded in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ [(1:1)] and 0.1 M acetate buffers (pH 5.5) (Fig. 3A). No peak was observed for bare Au electrode (curve a). The cyclic voltammogram of MnO_2NPs/cMWCNT/PANI/Au identified an oxidation peak at +180 mV (vs. Ag/AgCl) (curve b) and Lac/MnO_2NPs/cMWCNT/PANI/Au modified electrode also showed an oxidation peak at +300 mV (vs. Ag/AgCl) (curve c). The electrodeposition of MnO_2NPs onto cMWCNT/PANI modified Au electrode surface leads to increase in current intensity, as nanocomposite provided a conductive path for electron transfer and promoted electron transfer reactions at a low potential. On such prepared electrode surface, pretreated with a binding agent EDC/NHS, when few drops of enzyme solution were added, it resulted into immobilization of enzyme on it.

A control experiment was also performed to study the difference between detection ability of lac/MnNPs/cMWCNT/PANI/Au and lac/cMWCNT/PANI/Au electrode (Fig. 3B). It was observed that lac/cMWCNT/PANI/Au electrode (curve a) generates much lesser current in comparison to lac/MnNPs/cMWCNT/PANI/Au electrode (curve b).

3.3. Electrochemical impedance spectroscopy

Fig. 4 shows EIS of (a) bare Au electrode, (b) MnO_2NPs/cMWCNT/PANI/Au electrode, and (c) Lac/MnO_2NPs/cMWCNT/PANI/Au electrode, respectively. EIS analysis was employed to investigate the change in charge transfer after immobilization of Lac onto MnO_2NPs/cMWCNT/PANI/Au electrode. The value of the charge transfer resistance (semicircle diameter) (R_{CT}) depends on the dielectric and insulating features at the electrode/electrolyte

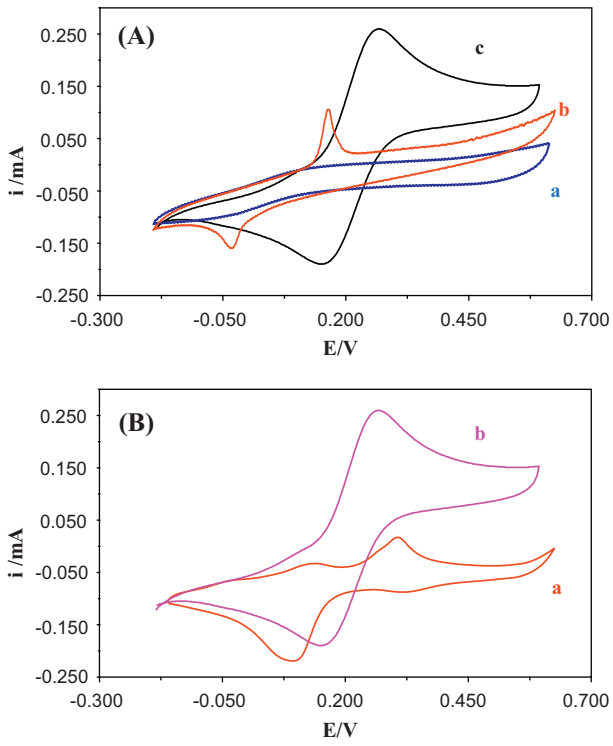


Fig. 3. Comparative cyclic voltammogram of: (A) Au electrode (a), MnO_2NPs/cMWCNT/PANI/Au electrode (b), and Lac/MnO_2NPs/cMWCNT/PANI/Au electrode (c) and (B) lac/cMWCNT/PANI/Au (a) and lac/MnO_2NPs/cMWCNT/PANI/Au electrode (b) towards phenolic compounds in a solution of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ [(1:1)] and acetate buffer (0.1 M, pH 5.0) at 20 mV s^{-1}.

interface. In EIS, the bare electrode exhibited an almost straight line (curve a), which is characteristic of a diffusional limiting step of the electrochemical process. The value of R_{CT} was 700 Ω for MnO_2NPs/cMWCNT/PANI/Au electrode (curve b) and then increases to 1200 Ω for Lac/MnO_2NPs/cMWCNT/PANI/Au electrode (curve c). 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. These results confirm the successful assembly of the enzyme electrode.

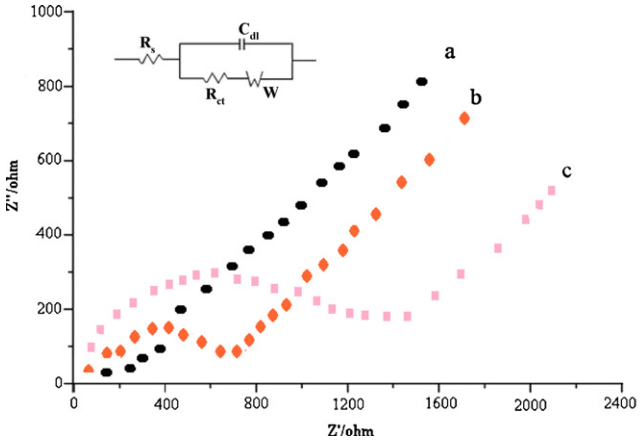
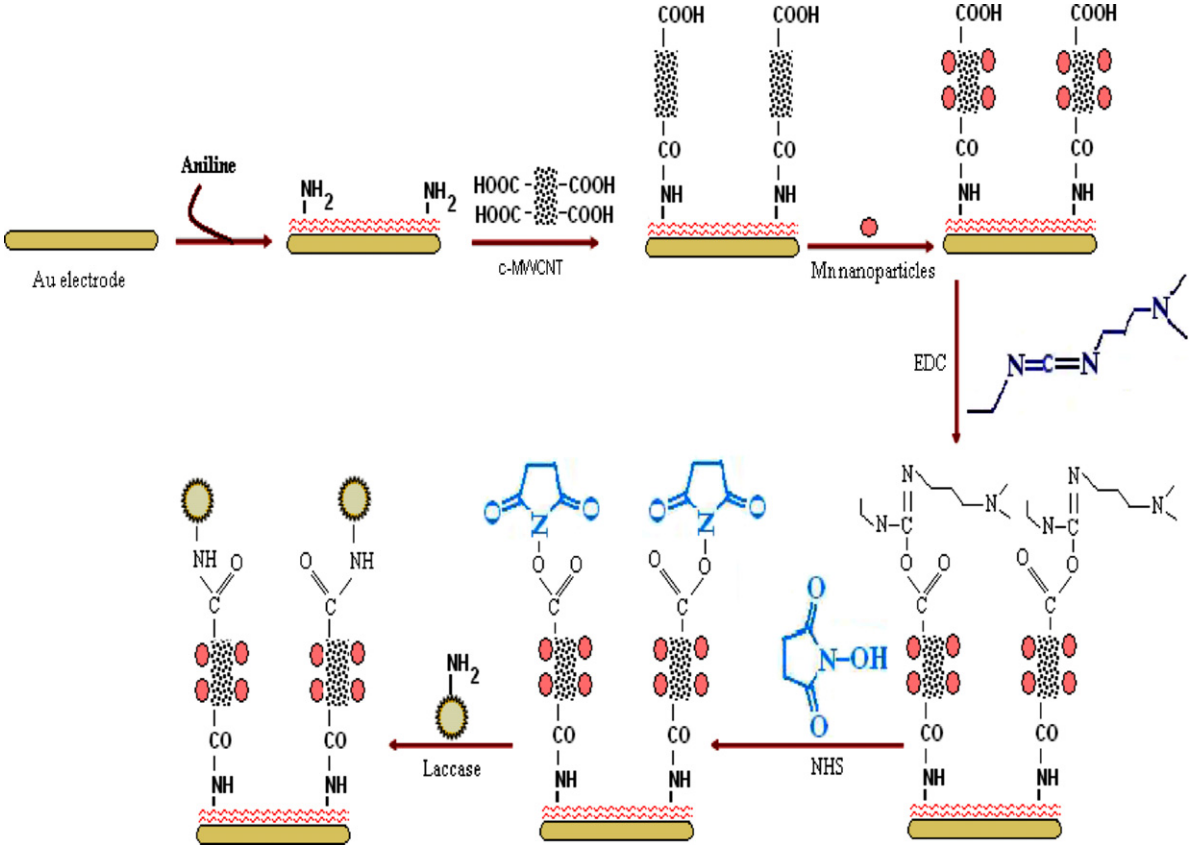


Fig. 4. Impedance spectra of: (a) bare Au electrode, (b) MnO_2NPs/cMWCNT/PANI/Au electrode, and (c) Lac/MnO_2NPs/cMWCNT/PANI/Au electrode.



Scheme 1. Schematic representation of laccase immobilized onto modified Au electrode (Lac/MnO_2NPs/cMWCNT/PANI/Au).

3.4. Optimization of experimental conditions of biosensor

The biosensor showed optimum response (current in μA) at pH 5.5, which is higher than that those based on immobilization of laccase onto different supports, e.g. *Lentinula edodes* laccase immobilized onto chitosan (pH 4.0) [20], *Pycnoporus sanguineus* laccase immobilized onto magnetic chitosan microspheres (pH 3.0) [21] but lower than that of *Trametes versicolor* laccase immobilized onto platinum nanoparticles (pH 6.5) [22]. The optimum temperature of biosensor was 35 °C, which is lower than that immobilized on magnetic chitosan microspheres (55 °C) [21] but higher than that on carbon paste modified graphite electrode [23] and nanoparticles (25 °C) [24]. Polyaniline being a good thermal insulator for enzymes might have provided the microenvironment to protect the enzyme from environmental and thermal variations, which favours the repeated use of MnO_2NPs/cMWCNT/PANI electrode [12]. The biosensor showed maximum response at 4 s after which it became constant. The effect of the scan rate from 10 to 100 mV s^{-1} on the biosensor response using 2 mM guaiacol in 0.1 M acetate buffer solution (pH 5.5) was also investigated. A potential scan rate of 20 mV s^{-1} was selected, since with these experimental conditions, the highest analytical signal and very good cyclic voltammogram profiles were obtained.

3.5. Chronoamperometric determination of guaiacol

Fig. 5 shows a typical current–time plot of the biosensor under the optimized experimental conditions with successive addition of guaiacol to the acetate buffer under stirring. With the increase in substrate concentration, the biosensor responded rapidly and 95% steady-state current was reached within 4 s (Fig. 5), which indicated a very fast diffusion process.

3.6. Evaluation of biosensor

3.6.1. Linearity

There was a linear relationship between current (mA) and guaiacol concentration ranging from 0.1 to 10 μM (lower concentration range) with linear equation being $y = 5.5789x + 0.0707$ and 10–500 μM (higher concentration range) with linear equation being $y = 1.3099x + 53.015$ (Fig. 1) in reaction mixture. The detection limit of the present sensor was 0.04 μM (S/N = 3).The *Ganoderma* sps. laccase is known to have wide substrate specificity [25]. So the present biosensor based was not selective.

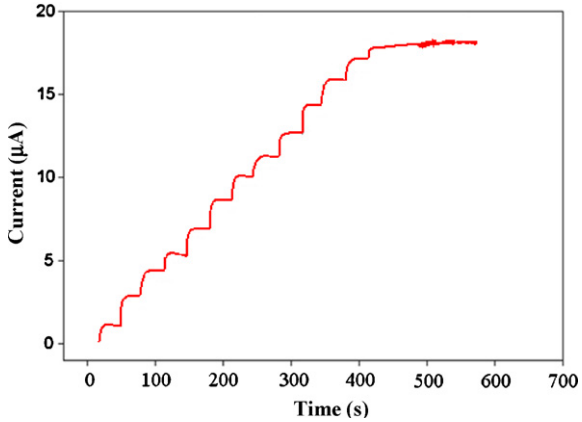


Fig. 5. Current–time response curve of the enzyme electrode on increasing the guaiacol concentration by 10 μM in each step in acetate buffer (0.1 M, pH 5.0) at an applied potential of −0.3 V vs. Ag/AgCl.

Table 1
Comparison of total phenolic content in different brands of tea leaves extract as measured by MnO_2NPs/cMWCNT/PANI/Au electrode and standard spectrophotometric method.

Samples	Total phenolic content by present method Mean ± SD[a] (n = 4) (μmol guaiacol/g)	Total phenolic content by standard spectrophotometric method Mean ± SD[a] (n = 4) (μmol guaiacol/g)
Tea leaves extract		
Brand a	0.498 ± 0.008	0.490 ± 0.008
Brand b	0.520 ± 0.012	0.510 ± 0.012
Brand c	0.772 ± 0.013	0.765 ± 0.013
Brand d	0.642 ± 0.014	0.636 ± 0.014
Brand e	0.494 ± 0.013	0.490 ± 0.013
Brand f	0.616 ± 0.008	0.606 ± 0.008
Brand g	0.580 ± 0.012	0.568 ± 0.012
Brand h	0.672 ± 0.013	0.660 ± 0.013
Brand i	0.657 ± 0.014	0.650 ± 0.014
Brand j	0.798 ± 0.013	0.790 ± 0.013

[a] sd = standard deviation.

3.6.2. Recovery and precision

The analytical recoveries of added guaiacol in tea leaves extract (5.0 μM and 10.0 μM) were 82.78% and 97.00%, respectively, demonstrating the accuracy of the present method. Within and between coefficients of variation were <2.23% and <3.54%, respectively. These high precision indicated the good reproducibility and consistency of the present method.

3.6.3. Correlation

A comparison of phenolic content in 10 tea leaves extract by the present method (y) with those obtained by standard spectrophotometric method (x) showed good correlation with r = 0.99 with a regression equation, $y = 0.9991x + 0.0078$ (Fig. 6). These results indicate the high accuracy of the method.

3.6.4. Interference study

The addition of the following interferrants such as ascorbic acid (4.8 mM), cysteine (0.24 mM), fructose (60 mM), glucose (22.2 mM), citric acid (100 mM) and ethanol (70 mM) showed a negligible interference (a decrease of only 8.56% for glucose, 9.75% for citrate, 5.68% for fructose, 9.20% for cysteine and 7.85% for ascorbic acid on the biosensor response).

3.6.5. Determination of total phenolic content in tea leaves extract

Total phenolic content in 10 different tea leaves extract of different brands as measured by the present sensor ranged from 0.494 to 0.798 μmol guaiacol/g (Table 1).

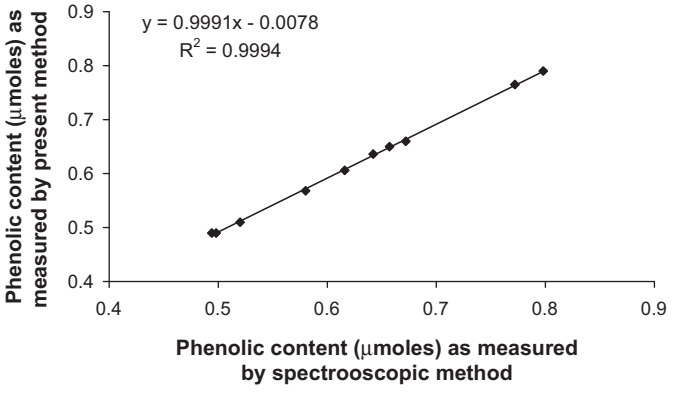


Fig. 6. Correlation between total phenolic content values determined by standard spectrophotometric method (x-axis) and present biosensor employing MnO_2NPs/cMWCNT/PANI/Au electrode method (y-axis).

Three similarly constructed enzyme electrodes were employed for determination of total phenolic content in tea leaves extracts in order to establish the reproducibility and variability of the present electrode. The results presented showed practically no significant variation (coefficient of variation less than 5% in all cases), confirming the reproducibility of present electrode.

3.6.6. Storage Stability and reusability of biosensor

The electrode lost 25% of its initial activity after its 150 uses during the span of 5 months when stored at 4 °C. The results showed practically no variation in storage stability of the three electrodes.

4. Conclusion

Lac/MnO_2NPs/cMWCNT/PANI modified Au electrode exhibited improved performance of biosensor in terms of wide linear range viz. 0.1–10 μM (lower concentration range) and 10–500 μM (higher concentration range), response time (4 s), detection limit (0.04 μM), reusability (150 times) and stability (5 months). Thus this work illustrates a simple and novel approach for the development of an improved amperometric enzyme sensor for determination of phenolic compounds, employing Lac/MnO_2NPs/cMWCNT/PANI nanocomposite.

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