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An amperometric biosensor based on laccase immobilized onto Fe_3O_4NPs/cMWCNT/PANI/Au electrode for determination of phenolic content in tea leaves extract

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ABSTRACT

A method is described for the construction of an amperometric biosensor for detection of phenolic compounds based on covalent immobilization of laccase onto iron oxide nanoparticles (Fe_3O_4NPs) decorated carboxylated multiwalled carbon nanotubes (cMWCNTs)/polyaniline (PANI) composite electrodeposited onto a gold (Au) electrode. The modified electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The biosensor showed optimum response within 3 s at pH 6.0 (0.1 M sodium acetate buffer) and 35 °C, when operated at 0.3 V vs. Ag/AgCl. Linear range, detection limit were 0.1–10 μM (lower concentration range) and 10–500 μM (higher concentration range), and 0.03 μM respectively. The sensor measured total phenolic content in tea leaves extract. The enzyme electrode lost 25% of its initial activity after its 150 uses over a period of 4 months, when stored at 4 °C.

1. Introduction

Polyphenolic compounds that have been identified in industrial effluents include phenols, catechols, guaiacols and cresols. Therefore, their determination is very important due to their toxicity and persistency in the environment [1,2]. These compounds are important constituents of natural products, such as fruit and vegetables. Their role as antioxidants has caused great impact on prevention of cardiovascular diseases and some of them have been claimed to prevent cancer. They are free radical scavengers, neutralizing oxygen reactive species and chelating metal ions. However some polyphenols are pro-oxidants [3,4]. They are responsible for the organoleptic properties of wine and olive oil [5]. The biological effect of polyphenols and derivatives were tested in order to assess their environmental impact and their use as byproducts for agriculture and industry was evaluated after a detoxification treatment with laccase [6]. The determination of environmental pollutants using immunoassays is a continuously growing area and, within this frame, laccase proved to be an excellent alternative to peroxidase as a bioanalytical tool for monitoring polar pollutants [7]. A number of commonly used analytical techniques are available for determination of phenolic content such as spectrophotometry, gas chromatography, liquid chromatography and capillary electrophoresis. However, some of these techniques (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 [8]. To overcome these drawbacks, enzyme based-amperometric biosensors, useful for polyphenols determination have been developed.

Nanotechnology has recently become one of the most exciting forefront fields in analytical chemistry. A wide variety of nanoscale functional materials such as carbon nanotubes and iron nanoparticles have been employed to modify the enzyme electrodes and enhance the analytical performance of the enzyme-based biosensors extensively for their unique chemical and physical properties. Carbon nanotubes (CNTs) possess many unique properties such as good electrical conductivity, strong adsorptive ability and excellent bioconsistency [9] and thus have ability to promote the electron transfer of hydrogen peroxide [10]. Composite materials based on integration of CNT and some other materials possess properties of the individual components with a synergistic effect have gained growing interest also. For its facile preparation, high

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conductivity and good environmental stability, polyaniline (PANI), a conducting polymer, has become the most attractive one in the formation of CNTs based composites. PANI/CNT composites synthesized chemically or electrochemically have improved the electrical conductivity, electrochemical capacitance and electrocatalytic properties of polymers [11–14].

As one kind of novel nanomaterial, magnetic nanoparticles have gained huge interest for their excellent properties in recent years. They have been widely studied and applied in various fields of biology, medicine and biochemical analysis including enzyme catalytic biosensing. The application of the magnetic Fe_3O_4 nanoparticles in the enhancement of the electron transfer and electrochemical biosensing of heme proteins was studied by Cao and Hu, 2006 [15].

In the present study, we immobilized laccase (purified from *Ganoderma sps.*) onto Fe_3O_4NPs/cMWCNTs/PANI/Au electrode through covalent coupling to construct an enzyme electrode for determination of total phenolic content, which is likely to overcome the problem of leakage of enzyme beside promoting electrical conductivity and elecrocatalytic property of enzyme. The resulting phenolic biosensor has also been employed for amperometric determination 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, ferrous chloride, ferric chloride from MERCK India Pvt. Ltd. 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 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. Fourier transform infrared (FTIR) spectroscopy was performed on FTIR spectrometer (Model iS10, Thermoelectron, USA).

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 M–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

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

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

2.5. Preparation of iron oxide nanoparticles (Fe_3O_4NPs)

Iron oxide nanoparticles were prepared by method as described by, Kuang et al., 2002 [18]. The synthesis of Fe_3O_4 was carried out following a typical procedure. Before mixing, 0.4 M hydrochloric acid and 0.7 M ammonia solution were bubbled by N_2 for 10 min. 8.5 g $FeCl_3 \cdot 6H_2O$ and 3 g $FeCl_2 \cdot 4H_2O$ were dissolved in 38 mL of 0.4 M hydrochloric acid, then added quickly to 375 mL ammonia solution under vigorous stirring (non-magnetic) at room temperature. After half-an-hour stirring, the precipitates were isolated by magnetic force. The precipitates were washed three times with distilled water then diluted with 150 mL DW.

2.6. Preparation of Fe_3O_4NP/cMWCNT/PANI/Au electrode

Prior to electrodeposition, the Au electrode was polished with alumina slurry. c-MWCNT and aniline were electrodeposited onto Au electrode through CV as described [19]. The electrodeposition of Fe_3O_4NPs 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 Fe_3O_4NPs solution and then applying potential between −0.2 + 0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 Vs^{-1}. After rinsing with DW, the Fe_3O_4NPs/cMWCNTs/PANI/Au electrode was dried in air.

2.7. Immobilization of laccase on Fe_3O_4NPs/cMWCNT/PANI/Au

The purified laccase was immobilized onto the surface of Fe_3O_4NPs/cMWCNTs/PANI/Au electrode through covalent coupling by layering 10 μl of enzyme solution (40 mg ml^{-1} protein) in 0.1 M acetate buffer (pH 5.0) and keeping it undisturbed for about 12 h at 4 °C. The electrode was finally washed with DW to remove unbound enzyme.

2.8. Response measurement of Lac/Fe_3O_4NP/cMWCNT/PANI/Au

All electrochemical characterizations and measurements were carried out using a conventional three-electrode system with the Lac/Fe_3O_4NP/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 follow: Guaiacol (standard phenolic substrate) is oxidized to its corresponding o-quinone by Fe_3O_4NPs/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 (miliAmpere). The electrons then reached Ag/AgCl electrode, where Ag^+ ions were reduced to Ag metal. The current is measured, which is directly proportional to guaiacol concentration.

2.9. Optimization of Lac/Fe_3O_4NP/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 0 s to 12 s at an interval of 3 s. To study the effect of substrate concentration, different guaiacol concentration, ranging from 0.1 to 500 μM were used.

2.10. Evaluation

The following criteria was 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 one 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 by using regression equation.

To examine the long-term storage stability, the activity of enzyme electrode was tested upto 4 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 ten commercial brands of tea leaves extract. The tea leaves extract were diluted 10 times with acetate buffer (pH 5.5). 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).

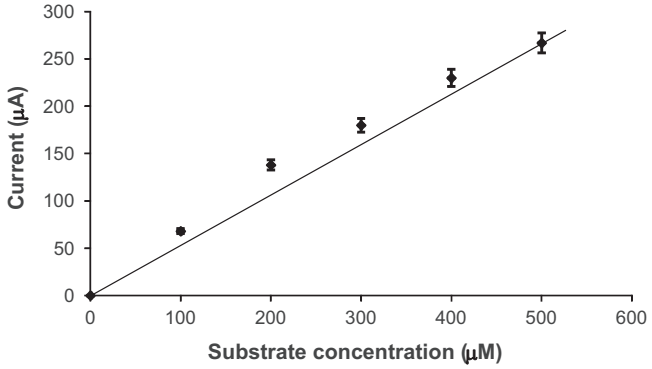


Fig. 1. Standard curve for guaiacol by phenolic biosensor based on laccase immobilized Fe_3O_4NPs/cMWCNT/PANI/Au electrode.

3. Results and discussion

3.1. Electrode surface characterization by SEM and FTIR

Fig. 1 shows morphology of bare Au electrode, cMWCNTs/PANI/Au electrode and Lac/Fe_3O_4NP/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). The films were more uniform and porous and hence effective surface area was larger. The SEM images for Lac/Fe_3O_4NPs/cMWCNT/PANI/Au showed a regular globular structural morphology (Fig. 2C), indicating the successful immobilization of laccase to the surface of cMWCNT/PANI layer.

Fig. 3 displays the FTIR spectra for cMWCNT/PANI/Au (curve a) and Lac/Fe_3O_4NPs/cMWCNT/PANI Au (curve b). The FTIR spectrum of electrodeposited cMWCNT/PANI composite (curve a) showed benzenoid and quinoid ring stretching bands (C=C) present at 1447 and 1595 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 1710 cm^{-1} is attributed to –C=O stretching vibrations indicating 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/Fe_3O_4NPs/cMWCNT/PANI/Au electrode (curve b) showed characteristic peaks at 1550 and 1600 cm^{-1} which could be attributed to the primary and secondary amide linkages between $-NH_2$ groups on the surface group on the surface of enzyme and free –COOH groups of MWCNT. Also, absorption bands in the low frequency region 800 cm^{-1} were obtained, due to the iron nanoparticle skeleton. Other prominent bands at 617 and 582 cm^{-1} belong to the stretching vibration mode and the torsional vibration mode of Fe–O bonds in the tetrahedral sites and in the octahedral sites of Fe_3O_4 nanoparticles. Also the peaks for free –COOH groups were not found in curve b, which confirms that the free –COOH groups have been occupied by $-NH_2$ group of enzyme, thus conforming the formation of a strong covalent bond.

A method is described for construction of a Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode. Scheme 1 summarizes the different chemical reactions involved in the fabrication of biosensor based on covalent immobilization of lac onto Fe_3O_4NPs/cMWCNT/PANI/Au electrode through amide bond formation between the free –COOH groups of cMWCNT of Fe_3O_4NPs/cMWCNT/PANI/Au electrode and free $-NH_2$ groups on surface of enzyme. Our results showed that Fe_3O_4NPs and

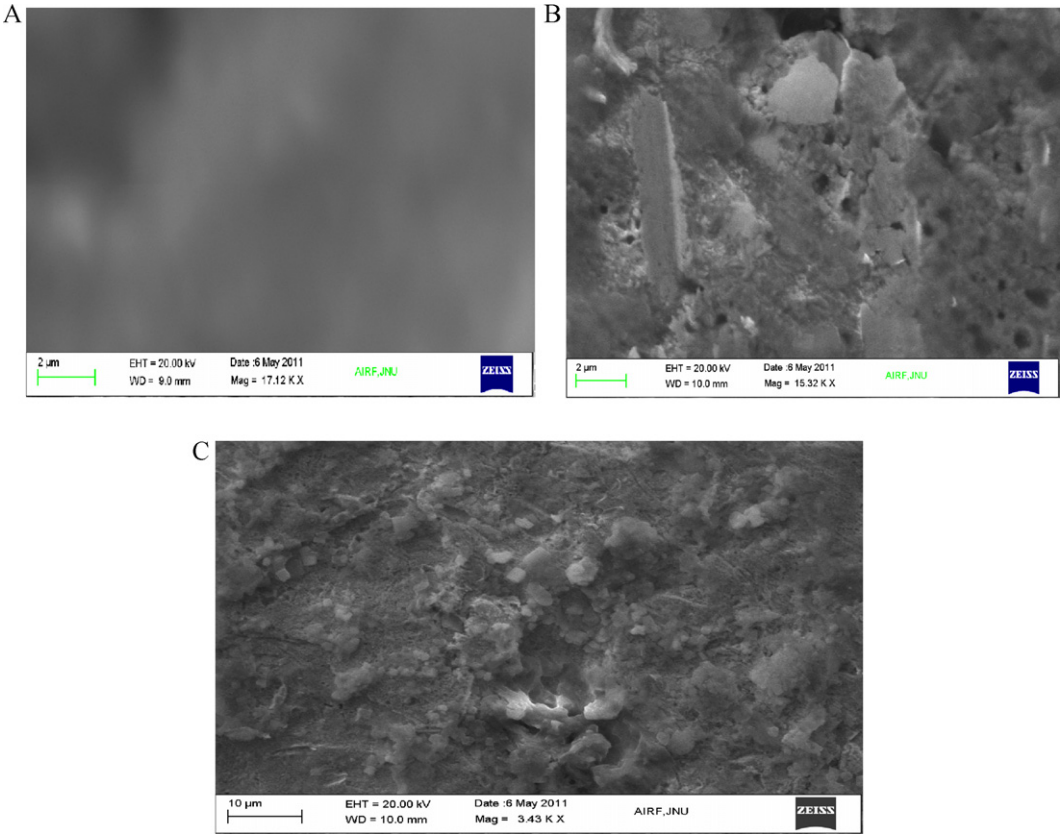


Fig. 2. Scanning electron micrographs of (A) bare gold electrode (B) cMWCNT/PANI/Au electrode (C) Laccase/Fe_3O_4NPs/cMWCNT/PANI/Au electrode.

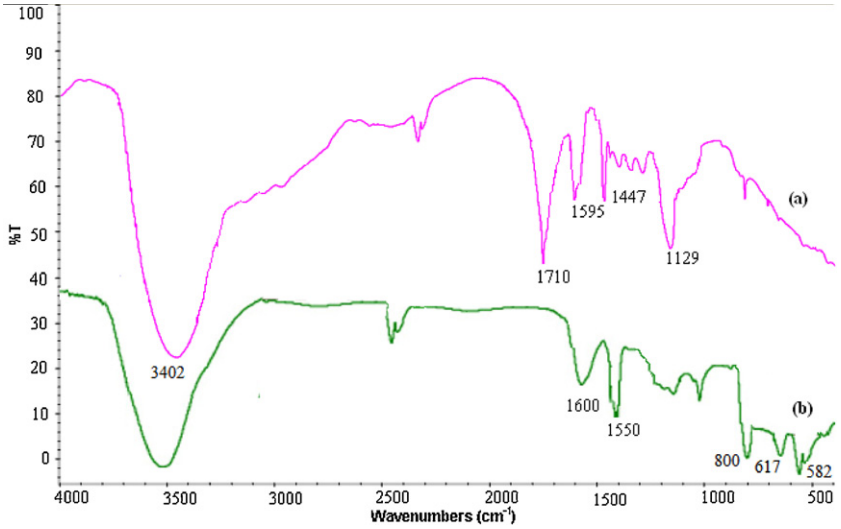
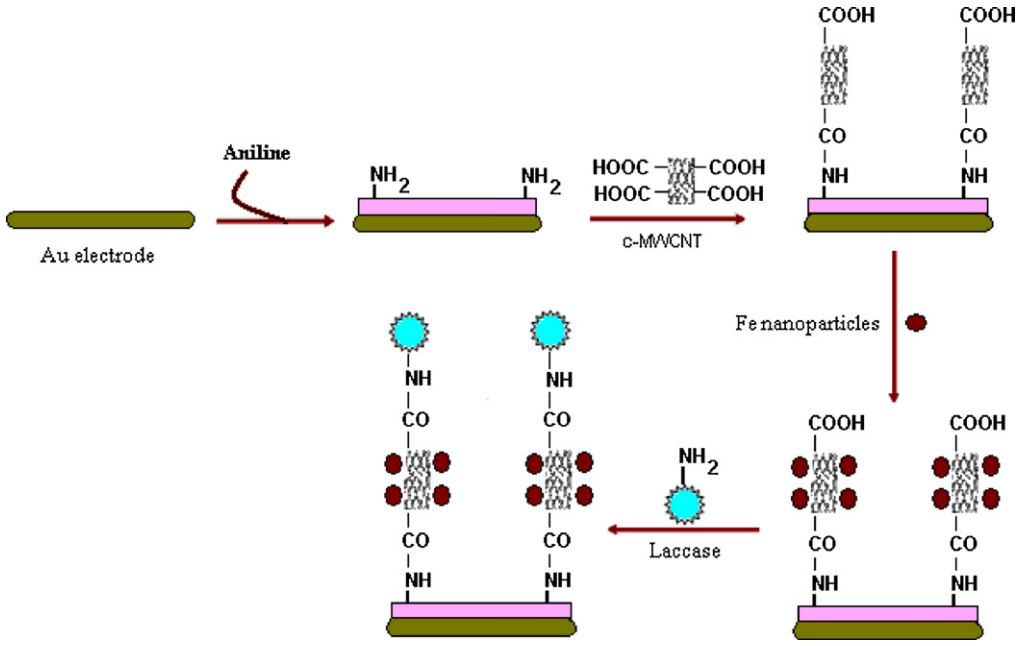


Fig. 3. The FTIR spectra of (a) c-MWCNT/PANI/Au electrode and (b) Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode.



Scheme 1. Schematic representation of Laccase immobilized onto modified Au electrode (Lac/Fe_3O_4NPs/cMWCNT/PANI/Au).

cMWCNTs provided a remarkable synergistic effect toward the oxidation of guaiacol.

3.2. Response towards guaiacol at the Lac/Fe_3O_4NP/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. 4). No peak was observed for bare Au electrode (curve a). The cyclic voltammogram of Fe_3O_4NPs/cMWCNT/PANI/Au identified an oxidation peak at +180 mV (vs. Ag/AgCl) (curve b) and Lac/Fe_3O_4NPs/cMWCNT/PANI/Au modified electrode also showed an oxidation peak at +325 mV (vs. Ag/AgCl) (curve c). The electrodeposition of Fe_3O_4NPs onto cMWCNT/PANI modified Au electrode surface leads to an increase in current intensity, as a result of

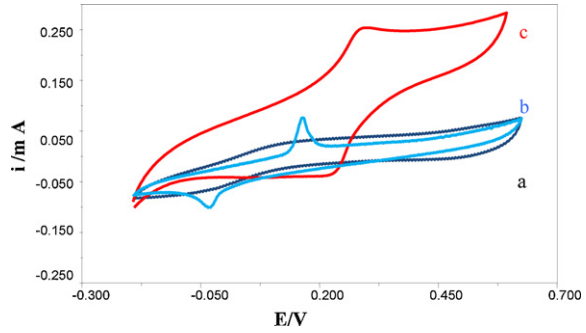


Fig. 4. Comparative cyclic voltammogram of (a) Au electrode (b) Fe_3O_4NPs/cMWCNT/PANI/Au electrode (c) Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode in a solution of 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ [(1:1)] and acetate buffer (0.1 M, pH 5.0) at 20 mVs^{-1}.

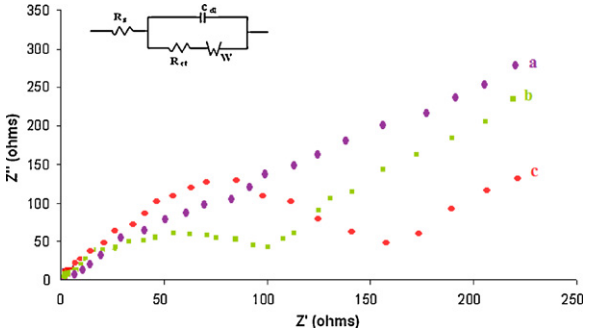


Fig. 5. Impedance spectra of (a) bare Au electrode (b) Fe_3O_4NPs/cMWCNT/PANI/Au electrode (c) Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode.

increase in electroactive area. The preservation of a quasi reversible electrode process and the large increase in peak currents for the nanocomposite film modified electrode proved that Fe_3O_4NPs exerted an obvious improvement effect on cMWCNT/PANI/Au electrode.

3.3. Electrochemical impedance spectroscopy

Fig. 5 shows EIS of (a) bare Au electrode (b) Fe_3O_4NP/cMWCNT/PANI/Au electrode (c) Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode respectively. EIS analysis was employed to investigate the change in charge transfer after immobilization of laccase onto Fe_3O_4NPs/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 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 100 Ω for Fe_3O_4NPs/cMWCNT/PANI/Au electrode (curve b) which then increases to 165 Ω for Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode (curve c). The increased R_{CT} value of Lac/Fe_3O_4NPs/cMWCNT/PANI/Au electrode is attributed to the fact that most biological molecules, including enzymes are poor electrical conductors at low frequencies and cause hindrance to the electron transfer. These results confirm the successful assembly of the enzyme electrode.

3.4. Optimization of experimental conditions of biosensor

The biosensor showed optimum response (current in μA) at pH 6.0, which is higher than that immobilized 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]. Therefore, a pH of 6.0 was used in further experiments. 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 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 Fe_3O_4NPs/cMWCNT/PANI electrode [11]. The biosensor showed maximum response at 3 s. The effect of the scan rate from 10 to 100 mVs^{-1} on the biosensor response using 2 mM guaiacol in 0.1 M acetate buffer solution (pH 6.0) was also investigated. A potential scan rate of 20 mVs^{-1} was selected, since with these experimental conditions, the highest analytical signal and very good cyclic voltammogram profiles were obtained.

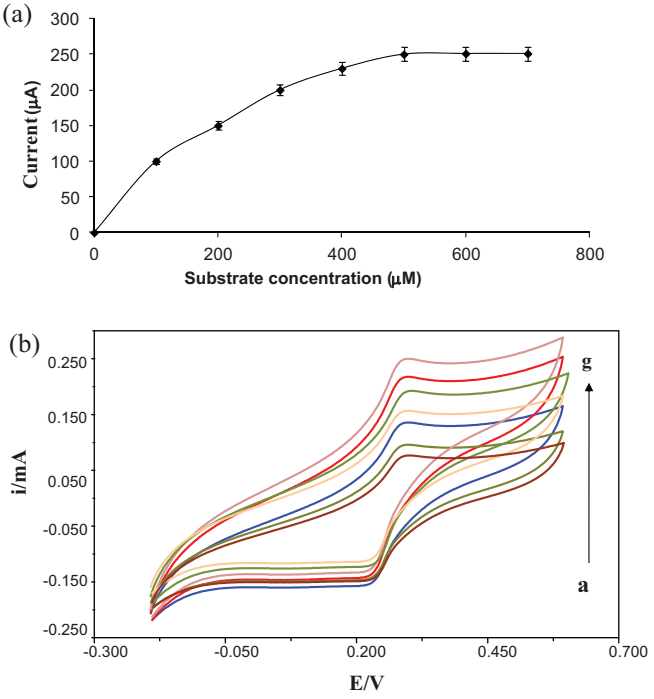


Fig. 6. (a) Effect of substrate concentration on the biosensor response (b) cyclic voltammograms recorded at different concentration of guaiacol from (a) 25 (b) 50 (c) 100 (d) 200 (e) 300 (f) 400 (g) 500 scan rate: 20 mVs^{-1}.

3.5. Evaluation of biosensor

3.5.1. Linearity

There was a linear relationship between current (μA) and guaiacol concentration ranging from 0.1–500 μM (Fig. 6) in reaction mixture. The detection limit of the present sensor was 0.03 μM (S/N = 3).

The *Ganoderma sps.* laccase is known to have wide substrate specificity [25]. So the present biosensor based on this enzyme is not selective.

3.5.2. Recovery and precision

The analytical recoveries of added guaiacol in tea leaves extract (5.0 μM and 10.0 μM) were 90.00% and 98.00% respectively, demonstrating the satisfactory accuracy of the present method. Within and between coefficients of variation were <2.17% and

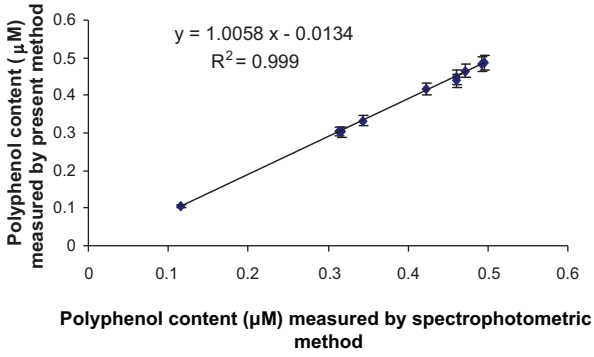


Fig. 7. Correlation between phenolic content as determined by standard spectrophotometric method (*x*-axis) and present biosensor method employing Laccase/Fe_3O_4NPs/cMWCNT/PANI/Au electrode (*y*-axis).

Table 1
Comparison of total phenolic content in different brands of tea leaves extract as measured by Fe_3O_4NPs/cMWCNT/PANI/Au electrode and standard spectrophotometric method.

Samples	Total phenolic content by present method mean ± SD[a] $n = 4$)	Total phenolic content by standard spectrophotometric method mean ± SD[a] ($n = 4$)
Tea leaves extract	(μmol guaiacol/g)	(μmol guaiacol/g)
Brand a	0.116 ± 0.008	0.104 ± 0.008
Brand b	0.560 ± 0.012	0.548 ± 0.012
Brand c	0.872 ± 0.013	0.860 ± 0.013
Brand d	0.792 ± 0.014	0.784 ± 0.014
Brand e	0.844 ± 0.013	0.832 ± 0.013
Brand f	0.616 ± 0.008	0.602 ± 0.008
Brand g	0.520 ± 0.012	0.510 ± 0.012
Brand h	0.772 ± 0.013	0.765 ± 0.013
Brand i	0.602 ± 0.014	0.594 ± 0.014
Brand j	0.814 ± 0.013	0.804 ± 0.013

[a] SD = standard deviation.

<3.87% respectively. The high precision indicated the good reproducibility and consistency of the present method.

3.5.3. 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, $y = 1.0058x - 0.0134$ (Fig. 7). These results indicate the high accuracy of the method.

3.5.4. 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) evoked a negligible interference (a decrease of only 7.14% for glucose, 8.73% for citrate, 4.69% for fructose, 8.19% for cysteine and 6.66% for ascorbic acid on the biosensor response).

3.5.5. Determination of total phenolic content in tea leaves extract

Total phenolic content as measured by the present sensor ranged from 0.116–0.872 μmol guaiacol/g in ten different tea leaves extract of different brands (Table 1).

Three similarly constructed enzyme electrodes were employed for determination of total phenolic content in tea leaves extract 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.

3.5.6. Storage Stability and reusability of biosensor

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

4. Conclusion

Lac/Fe_3O_4NPs/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 (3 s), detection limit (0.03 μM), reusability (150 times) and stability (4 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 Fe_3O_4NPs/cMWCNT/PANI.

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