Journal of Biotechnology 156 (2011) 39–45

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Fabrication of polyphenol biosensor based on laccase immobilized on copper nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline-modified gold electrode

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

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

ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form 30 July 2011
Accepted 8 August 2011
Available online 16 August 2011

Keywords:
Polyphenol
Laccase
Amperometric biosensor
CuNP's
cMWCNT
CHIT

ABSTRACT

A high-performance amperometric polyphenol biosensor was developed, based on covalent immobilization of *Ganoderma* sp. laccase onto copper nanoparticles (CuNP's)/chitosan (CHIT)/carboxylated multiwalled carbon nanotube (cMWCNT)/polyaniline (PANI)-modified gold (Au) electrode. The CuNP's and cMWCNT had a synergistic electrocatalytic effect in the matrix of CHIT. The biosensor showed optimum response at pH 6.0 (0.1 M acetate buffer) and 35 °C, when operated at 50 mV s^{-1}. The biosensor exhibited excellent sensitivity (the detection limit was down to 0.156 μM for guaiacol), fast response time (less than 4 s) and wide linear range (from 1 to 500 μM). Analytical recovery of added guaiacol was 96.40–98.46%. Within batch and between batch coefficients of variation were <2.6% and <5.3%, respectively. The enzyme electrode was used 300 times over a period of 7 months, when stored at 4 °C.

1. Introduction

Polyphenols, known for their antioxidant properties are commonly found in legumes, cereals, fruits and medicinal plants. These compounds provide a protective role against many chronic human diseases associated with oxidative stress, like cancer or cardiovascular diseases (Lu and Foo, 2002) and also employed as markers in taxonomic studies and food quality control (Friedman, 1997). Therefore, it is very important to quantify the polyphenolic content in natural foodstuffs and pharmaceuticals to evaluate their antioxidant activity. A number of techniques for measurement of total polyphenolic content in plant extracts have been reported in literature such as liquid chromatography (LC) with photodiode array detector (DAD) (Zgorka and Kawka, 2001), mass spectrometry (MS) (Almela et al., 2006) and gas chromatography (GC) coupled to flame ionization or mass spectrometer detectors (Aleksovski and Sovova, 2007). Though some of these methods were sensitive, yet are relatively cumbersome and often need derivatisation and pre-concentration steps. Enzyme based-amperometric polyphenol biosensors have overcome these problems (Rajesh and Kaneto, 2005; Roy et al., 2005; Odaci et al., 2007). Two types of phenoloxidases, namely tyrosinase and laccase have been used in these biosensors. Tyrosinase is known to catalyze the oxidation of monophenols, o-diphenols to o-quinones, while laccase (Lac) catalyzes the oxidation of o-, m-, and p-benzenediols and phenol to o-, m- and p-quinones or radical species respectively and does not require H_2O_2 as a co-substrate or any cofactors for the catalytic reaction (Gamella et al., 2006). Lac can reduce O_2 directly to water in a four-electron transfer step without forming H_2O_2, at the expense of oxidation of a variety of mediators, e.g., 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), guaiacol and catechol, etc. A number of polyphenol biosensors based on immobilized laccase have been reported (Yaropolov et al., 1995; Fernandes et al., 2008) but most of them suffered from leakage of enzyme due to non covalent immobilization of enzyme.

Carbon nanotubes (CNT) have been extensively used for improvement of amperometric biosensors, as they possess many unique properties such as good electrical conductivity, strong adsorptive ability, excellent bioconsistency, high electrocatalytic effect, fast electron-transfer rate and thus possess great promise for amperometric biosensors (Zhang et al., 2004; Lin et al., 2005). Due to excellent film-forming ability, good adhesion, biocompatibility and high mechanical strength, chitosan (CHIT) has been used as a support for immobilization of biomolecules (Chen and Gorski, 2001; Okuma and Watanabe, 2002; Luo et al., 2005; Jiang et al., 2006). Copper nanoparticles (CuNP's) are known to increase the response current, relatively inexpensive and also have good biocompatibility. Polyaniline (PANI), a conducting polymer, has

* Corresponding author. Tel.: +91 9416492413.
E-mail address: pundircs@rediffmail.com (C.S. Pundir).

0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.08.008

become the most attractive one for formation of multi-walled carbon nanotubes (MWCNTs/PANI) nanocomposite because of facile preparation, superior transducing ability and good environmental stability. We describe herein the construction of a new nanocomposite of CuNP's and MWCNTs/PANI in the matrix of CHIT and its application for fabrication of an improved polyphenol biosensor. Further, the covalent immobilization of enzyme onto hybrid electrode is likely to overcome the problem of enzyme leakage.

2. Experimental

2.1. Chemicals

Sephadex G-100 and DEAE-Sephacel were from Sigma–Aldrich, St. Louis, USA. Guaiacol, aniline and chitosan (MW $\sim 1 \times 10^6$; 75–85% deacetylation) were from SRL, Mumbai and glutaraldehyde from Merck India Pvt. Ltd., Mumbai. Carboxylated multi-walled carbon nanotubes (c-MWCNT) (Functionalized MWCNT) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana) India were used. All other chemicals were of Analytical Reagent (AR) grade. Fruit juices, alcoholic beverages of various commercial brands and pharmaceutical formulations (tablets + Intravenous injections) were purchased from local market.

2.2. Apparatus

All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (Eco Chemie BV, The Netherlands) with the GPES 4.9 software. The modified gold electrode of 1.0 mm diameter as working electrode, a KCl-saturated Ag/AgCl electrode as reference electrode and Pt electrode as counter electrode were used in all electrochemical experiments. All potentials were measured with respect to the reference electrode. All experiments were carried out at room temperature ($30 \pm 5\,^{\circ}C$). Scanning electron microscopic (SEM) images were taken at Department of Chemistry, M.D. University, Rohtak. Fourier transform infrared (FTIR) spectroscopy was performed on FTIR spectrometer (Model iS10, Thermoelectron, USA) in our laboratory.

2.3. Purification of laccase

The cell free extract of *Ganoderma* sp. Rckk02 grown in malt extract broth (MEB) (Dhawan et al., 2003) was used as crude laccase. The crude laccase was purified at 4 °C using 0–80% ammonium sulfate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel using linear gradient of KCl (0.1–0.6 M). The activity and protein content of various enzyme preparations were measured.

2.3.1. Enzyme assay

Assay of laccase was based on the oxidative polymerization of guaiacol (Vasdev and Kuhad, 1994). The reaction mixture contained 0.2 μmoles of guaiacol, 230 μmoles of acetate buffer (pH 5.0) and enzyme in a total volume of 2.5 ml. Change in absorbance of 0.01 min^{-1} at 470 nm was studied. The unit activity of enzyme was calculated as follow:

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

2.3.2. Protein determination

Protein content in various enzyme preparations was determined by method of Lowry et al. (1951).

2.4. Preparation of CuNP's/CHIT/cMWCNT/PANI/Au electrode

Prior to electrodeposition, the gold electrode was polished with alumina slurry. One mg of c-MWCNT was suspended in 1 ml mixture of concentrated H_2SO_4 and HNO_3 in 3:1 ratio and ultrasonicated for 2 h to get a finely dispersed black colored solution of c-MWCNTs. Aniline (50 μl) was added to 10.0 ml of 1 M HCl and mixed with 1.0 ml finely dispersed solution of c-MWCNT in a glass cell. c-MWCNT and aniline were electrodeposited onto Au electrode by immersing it in 25 ml of 1 M KCl solution through cyclic voltammetry (CV) by applying 30 polymerization cycles at 0.0–1.5 V as described (Ye et al., 2006). The resulting cMWCNT/PANI/Au modified electrode was washed thoroughly with distilled water to remove unbound matter and kept in dry petri plate at 4 °C.

CuNP's were then electrochemically deposited on this electrode in a 0.1% of chitosan solution containing 20.0 mM $CuCl_2$ (Cu/CHIT) by cycling in the potential range from −0.4 to +0.4 V (Wang et al., 2008) at a scan rate of 50 mV s^{-1} for different cycles in a 25 ml solution containing 20 ml 1 M KCl and 5 ml CuNP's/CHIT solution (CHIT solution was prepared by dissolving 0.1 g of CHIT flakes into 100 ml of 2.0% acetic acid solution and stirred for 3 h at room temperature until complete dissolution. The CHIT solution was stored at 4 °C when not in use).

2.5. Preparation of enzyme electrode (Lac/CuNP's/CHIT/cMWCNT/PANI/Au electrode)

Purified laccase was immobilized onto the CuNP's/CHIT/cMWCNT/PANI modified gold electrode surface by glutaraldehyde coupling. The modified electrode was activated by glutaraldehyde by dipping it in 10 ml of 2.5% glutaraldehyde for 30 min and then washing it thoroughly with distilled water (DW). Laccase (100 μl containing 5.16 U) was mounted on the activated electrode and kept at 4 °C overnight to dry up. The resulting enzyme electrode was washed in DW followed by reaction buffer and then stored in the refrigerator at 4 °C. The washing discard was tested for enzyme activity and protein. No enzyme activity and protein were detected in washing discard, which indicated 100% retention of the enzyme on the electrode. The principle of working of this biosensor was as follow: Guaiacol is oxidized into its corresponding o-quinone by CuNP's/CHIT/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 are passed to working electrode from solution (Fig. 1).

2.6. Optimization of Lac/CuNP's/CHIT/cMWCNT/PANI modified gold 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 intervals 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 °C to 55 °C at an interval of 5 °C. To determine the optimum response time, the current response was studied from 5 s to 25 s. To study the effect of substrate concentration, different guaiacol concentration were prepared, ranging from 1 μM to 500 μM were used. K_m for guaiacol was calculated from Line weaver Burk plot whose equation is given below

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

where I = current, K_m = Michaelis–Menten constant, I_{max} = maximum current and $[S]$ = guaiacol concentration.

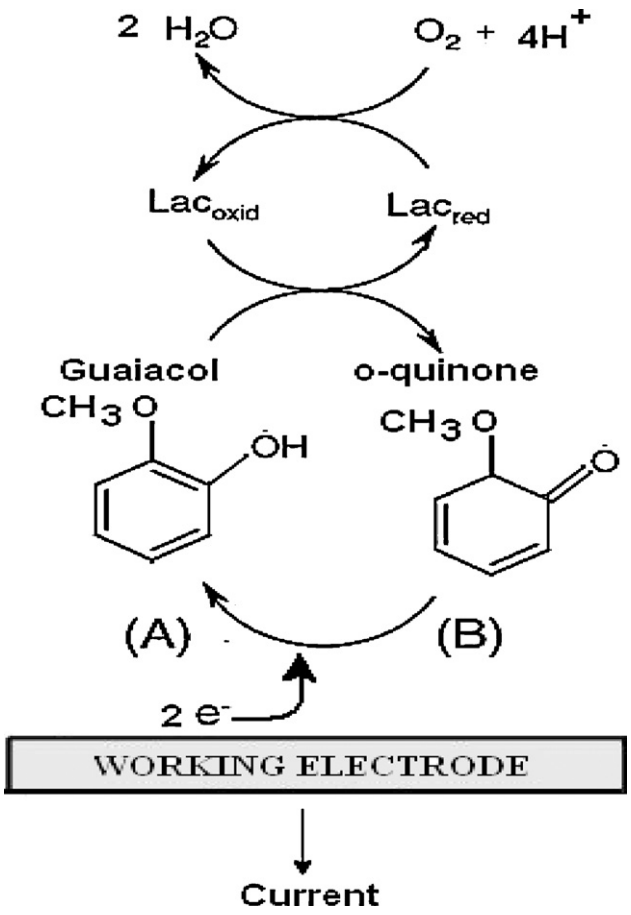


Fig. 1. Chemical reaction catalyzed by laccase.

2.7. Amperometric measurement of polyphenols in dried tea leaves, alcoholic beverages and pharmaceutical formulations

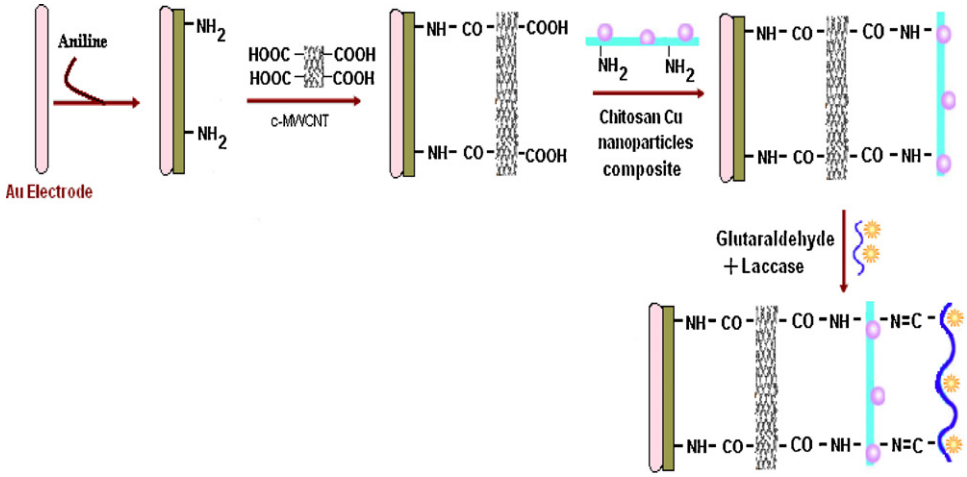
The biosensor was employed for determination of polyphenols in five commercial brands of tea leaves, alcoholic beverages and pharmaceutical samples. Dried tea leaves (5 g) were boiled in 20 ml of distilled water and filtered through a sieve. The pharmaceutical tablets were ground to powder and dissolved in 5 ml phosphate buffer (pH 6.0). Polyphenol level in tea leaf extract, alcoholic beverages and pharmaceutical samples were determined using the present biosensor in the same manner as described above for its response measurement under its optimal working conditions except that guaiacol was replaced by the sample. The current (mA) was recorded and the amount of polyphenols was interpolated from standard curve between guaiacol concentrations and current (mA) prepared under optimal working conditions.

3. Results and discussion

3.1. Electrode surface characterization by SEM and FTIR

The morphology of bare Au electrode, cMWCNTs/PANI/Au electrode, CuNP's/CHIT/cMWCNT/PANI/Au electrode and Lac/CuNP's/CHIT/cMWCNT//PANI/Au electrode was characterized by SEM studies. SEM of the bare Au electrode (Fig. 2A) showed a smooth and featureless morphology, whereas nanocomposites (Fig. 2B–D) displayed a stepwise immobilization of PANI/cMWCNT, CuNP's and laccase onto the gold electrode. Fig. 3 shows FTIR spectra obtained for cMWCNT/PANI/Au electrode (curve a), and lac/CuNP's/CHIT/cMWCNT/PANI/Au electrode (curve b). The FTIR spectrum of electrochemically deposited cMWCNT/PANI composite (curve a) showed 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} is attributed to B–N^{+}=Q stretching and –N–H stretching vibrations of PANI in the composite. The peak at 1704 cm^{-1} is attributed to –C=O stretching vibrations, which indicates the presence of carboxyl group (–COOH). The presences of these peaks reveal the existence of both cMWCNT and PANI on the Au electrode. In the FTIR spectrum of lac/CuNP's/CHIT/cMWCNT/PANI/Au (curve b), appearance of additional absorption bands at 1550 cm^{-1} is attributed to the primary amide linkage of PANI/cMWCNT and cMWCNT/CHIT and secondary amide linkage (C=N bond) of enzyme molecule with CHIT through glutaraldehyde showed peak at 1615–1700 cm^{-1} confirmed the covalent immobilization of enzyme. The characteristic absorption bands of amino saccharide in chitosan were also observed at 3415 cm^{-1}, which were due to overlapping of –OH and –NH_2 stretching.

The fabrication of the polyphenol biosensor based on CuNP's onto c-MWCNT/PANI modified Au electrode is summarized in Scheme 1. Firstly PANI was electrodeposited onto Au electrode with free –NH_2 group at their ends which get linked to –COOH groups



Scheme 1. Scheme of chemical sequence of electropolymerization of CuNP's/CHIT/cMWCNT/PANI on gold electrode and chemical reaction of immobilization of enzyme on layer on Au electrode.

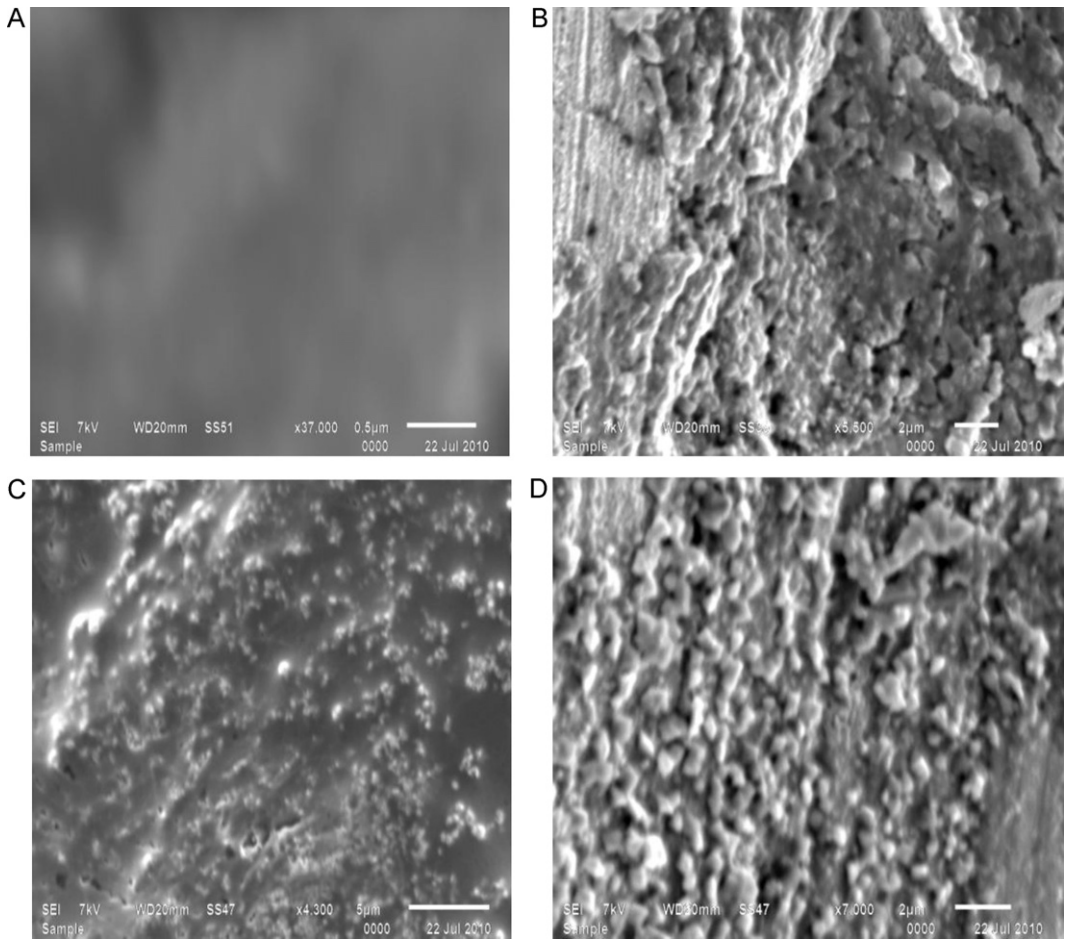


Fig. 2. Scanning electron micrographs of (A) bare gold electrode; (B) cMWCNT/PANI/Au electrode; (C) CuNP's/CHIT/cMWCNT/PANI/Au electrode; (D) laccase/CuNP's/CHIT/cMWCNT/PANI/Au electrode.

of c-MWCNT through amide bond (–CO–NH). Then, CuNPs in chitosan solution were electrochemically deposited on the surface of PANI/cMWCNT. The electrodeposition method was selected to produce CuNP's onto electrode surface, because this method is easy to be carried out and the layer thickness can be controlled. The experiment results showed that CuNP's and cMWCNTs provided a remarkable synergistic effect towards the oxidation of guaiacol. When the modified electrode (CuNP's/CHIT/cMWCNT/PANI/Au electrode) was treated with glutaraldehyde, its –CHO group at one end get attached to $-NH_2$ group of CHIT, while the other –CHO group get linked to free $-NH_2$ group of enzyme through C=N bond and thus the enzyme is linked covalently.

To evaluate the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry was employed, using KCl as electrolyte. Cyclic voltammograms recorded in 1 M KCl solution are shown in Fig. 4. Fig. 4A and B displays the cyclic voltammograms of cMWCNT/PANI/Au modified electrode and CuNP's/CHIT/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 (Feldheim and Foss, 2002). The electrodeposition of CuNP's/CHIT film on cMWCNT/PANI modified gold electrode surface leads to increase in current intensity as a result of increase in electroactive area (Fig. 4B). The preservation of a quasi reversible electrode process and the large increase in peak currents for the nanocomposite film modified electrode proved that CuNP's/CHIT exerted an obvious improvement effect on cMWCNT/PANI/Au electrode properties.

3.2. Response towards guaiacol at the CuNPs/CHIT/cMWCNT/PANI/Au electrode

To evaluate the catalytic activity of laccase at the CuNP's/CHIT/cMWCNT/PANI/Au electrode, the modified electrode was characterized by a cyclic voltammogram in the potential range, −0.4 V to +0.4 V using guaiacol as substrate. Fig. 4C shows cyclic voltammograms of the CuNP's/CHIT/cMWCNT/PANI/Au electrode in an unstirred 0.1 M KCl solution (20 ml) and 0.1 M acetate buffer, pH 6.0 (5 ml) without (curve a) and with (curve b) guaiacol solution at scan rate 50 mV s^{-1}. The addition of 2 μM guaiacol led to increased oxidation peak current but suppressed reduction current, which showed the catalytic properties of modified electrode towards the oxidation of guaiacol.

3.3. Optimization of experimental conditions of biosensor

The biosensor showed maximum response at pH 6.0, which is higher than laccase bound to polyvinylpropylidone (PVP) gel in a cellophane membrane (pH 5.5) (Roy et al., 2005), poly(ethyleneimine) microcapsules (pH 5.75) (Rochefort et al., 2008), CNT modified glassy carbon electrode (pH 5.6) (Liu et al., 2008), copper-containing ordered mesoporous carbon (Cu-OMC)/chitosan matrix (pH 5.0) (Xu et al., 2010) but lower than that of hydrophilic matrix (pH 6.5) (Vianello et al., 2006). Therefore, a pH of 6.0 was used in subsequent experiments. The optimum working temperature of biosensor was 35 °C, which is

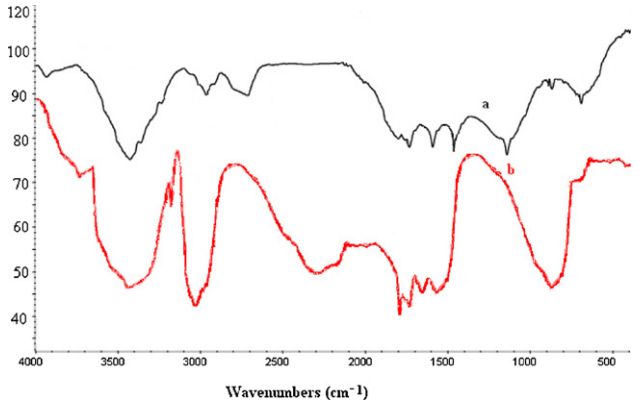


Fig. 3. FTIR spectra of (a) cMWCNT/PANI/Au electrode and (b) laccase/CuNP's/CHIT/cMWCNT/PANI/Au electrode.

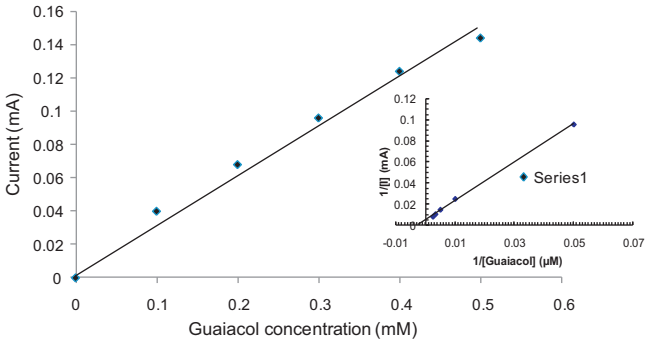


Fig. 5. The calibration curve for polyphenol biosensor Inset: Lineweaver Burk plot for effect of guaiacol concentration on response of polyphenol biosensor based on CuNP's/CHIT/cMWCNT/PANI/Au electrode bound laccase.

similar to that of copper-containing ordered mesoporous carbon (Cu-OMC)/chitosan matrix (Xu et al., 2010), but lower than silane-modified platinum surface (Quan et al., 2004). PANI, being a good thermal insulator for enzymes provided the microenvironment to protect the enzyme from environmental and thermal variations, which favours the repeated use of CuNP's/CHIT/cMWCNT/PANI electrode (Iijima, 1991). 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 buffer solution (pH 6.0) was also investigated. A potential scan rate of 50 mV s^{-1} was selected to obtain the highest analytical signal and very good cyclic voltammogram profiles.

3.4. Evaluation of biosensor

3.4.1. Linearity

There was a linear relationship between current (mA) and guaiacol concentration ranging from 1.0 to 500 μM in the reaction mixture (Fig. 5), which is better than biosensor based on laccase immobilized onto matrix of carbon nanotubes–chitosan composite (1.2–30 μM and 3.3–40 μM) (Liu et al., 2006; Diaconu et al., 2010), Cu-OMC/chitosan matrix (0.67 μM–15.75 μM) (Xu et al., 2010), Pt nanoparticles (0.999–213 μM) (Brondani et al., 2009) but similar to that based on enzyme immobilized onto hydrophilic matrix (0–500 μM) (Vianello et al., 2006). K_m for guaiacol was 333.33 μM, which is comparable to that of laccase bound to hydrophilic membrane (339 μM) (Vianello et al., 2006). A linear relationship was

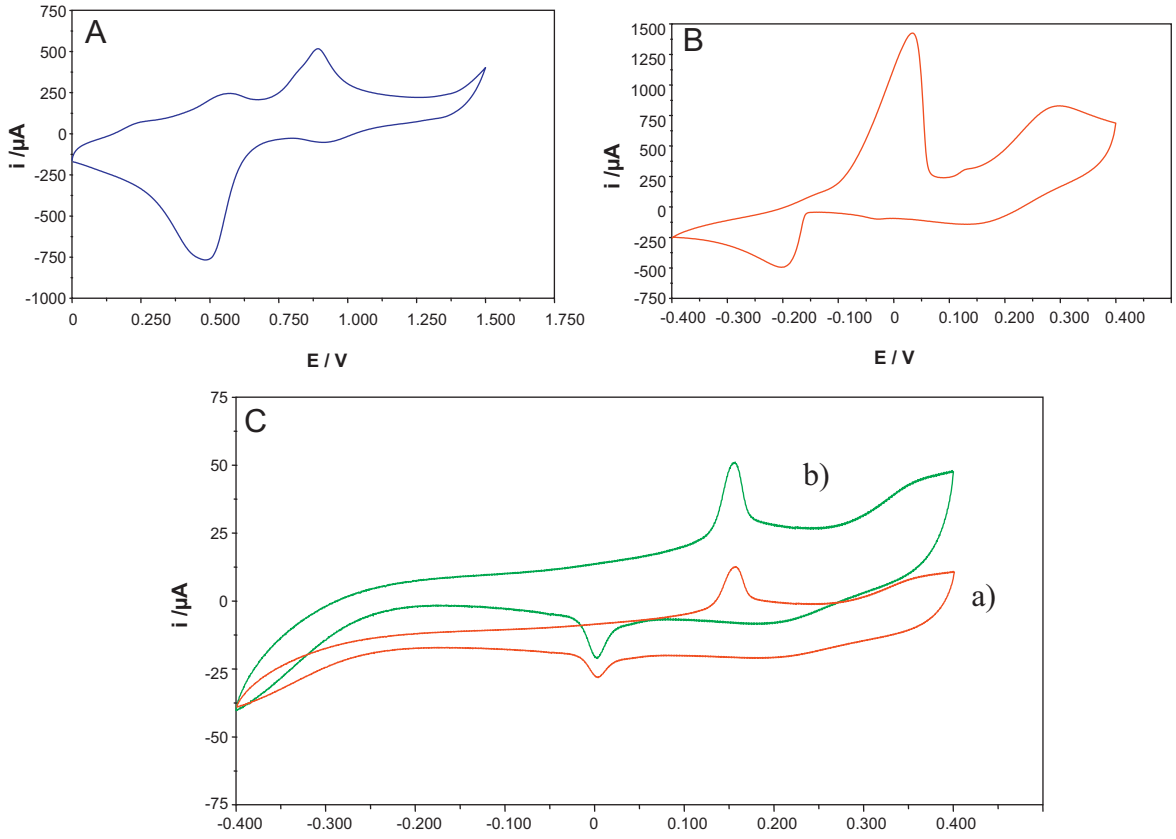


Fig. 4. Cyclic voltammogram of electrochemical deposition of (A) PANI/cMWCNT onto gold electrode and (B) Cu nanoparticles in the matrix of chitosan onto cMWCNT/PANI coated gold electrode. (C) Cyclic voltammetric (CV) curves of CuNP's/CHIT/cMWCNT/PANI/Au electrode in 0.1 M acetate buffer (pH 6.0) (a) without and (b) with 2 μM guaiacol solution. Supporting electrolyte: 1 M KCl solution; scan rate: 50 mV s^{-1}.

also observed for standard adrenaline in the range 1–500 μM by the present biosensor (Fig. not shown).

3.4.2. Detection limit

The detection limit of the present sensor was 0.156 μM, which is lower than that of enzyme electrode based on carbon nanotubes–chitosan composite (0.66 μM and 0.275 μM) (Liu et al., 2006; Diaconu et al., 2010) and Cu-OMC/chitosan matrix (0.67 μM) (Xu et al., 2010).

3.4.3. Stability and reusability of biosensor

The electrode lost 20% of its initial activity after its 300 uses over a period of 7 months, when stored at 4 °C which is much better than earlier biosensors (Vianello et al., 2006; Brondani et al., 2009; Xu et al., 2010). The enzyme electrode was constructed thrice and effect of storage at 4 °C was studied on the response of these three similarly constructed electrodes. The results given in Fig. 6 show practically no variation in storage stability of the three electrodes.

To test the reproducibility and reliability of the present biosensor, polyphenol level in six tea samples was determined on single day (within batch) and five times again after storage at −20 °C for one week (between batch). The results showed that determinations were consistent and within and between coefficients of variation (CVs) were 2.6% and 5.3% respectively, which is better than earlier reports (Montereali et al., 2010; Brondani et al., 2009). These results showing high precision indicated the good reproducibility and consistency of the present method, which could be attributed to the excellent immobilization of laccase onto CuNP's/CHIT/cMWCNT/PANI/Au electrode.

3.4.4. Recovery

The analytic recovery of a known amount of added adrenaline into a pharmaceutical formulation (adrenaline injection) was determined by the present biosensor. The analytic recoveries of added 30 and 60 μM of adrenaline (final concentration in reaction mixture) were 96.40% and 98.46% respectively, which is comparable to the earlier reports (Montereali et al., 2010; Brondani et al., 2009).

3.4.5. Accuracy

To determine the accuracy of the method, the level of polyphenol in 15 alcoholic beverages was determined by standard spectrophotometric method (x) and the present method (y). The polyphenol values obtained by both the methods were in agreement with each other with a good correlation ($r = 0.97$) (Fig. 7). These evaluation studies showed that the method was fairly reliable with high recovery and in agreement with the standard method.

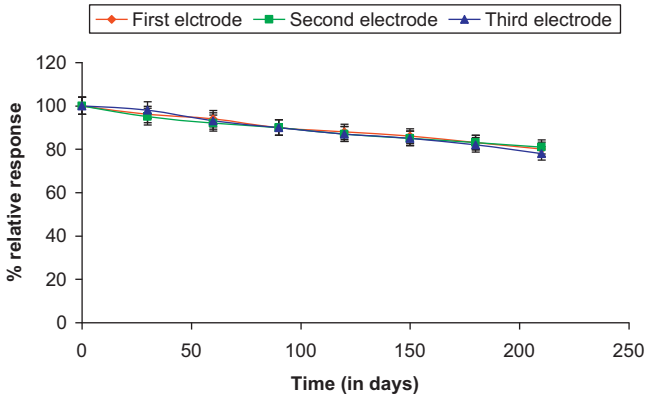


Fig. 6. Effect of storage at 4 °C on the response of three similar CuNP's/CHIT/cMWCNT/PANI/Au electrodes.

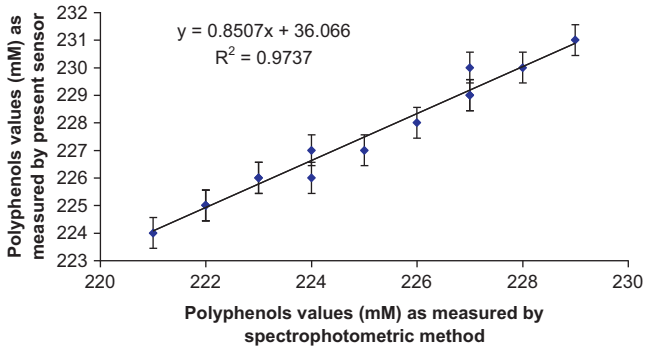


Fig. 7. Correlation between polyphenol values in alcoholic beverages determined by standard spectrophotometric method and present method.

3.5. Determination of polyphenolic level in tea, alcoholic beverages and pharmaceutical formulations

Polyphenolic level, as measured by the present biosensor, ranged from 0.025–0.225 mM in dried tea leaves, 0.216–0.232 mM in alcoholic beverages and 0.138–0.242 mM in pharmaceutical formulations (Table 1).

Three similarly constructed enzyme electrodes (laccase/CuNP's/CHIT/cMWCNT/PANI/Au electrode) were employed for determination of polyphenolic level in dried tea leaves in order to establish the reproducibility and variability of the present electrode (Table 2). The results presented in Table 2 show no significant variation (CV less than 6% in all cases), confirming the reproducibility of present electrode.

Table 1
Polyphenol level in different brands of dried tea leaves, alcoholic beverages and pharmaceutical formulations as measured by laccase/CuNP's/CHIT/cMWCNT/PANI/Au electrode.

Samples	Polyphenol level (mM) Mean ± SD[a] ($n = 4$)
1. Tea	
(a) Red label	0.025 ± 0.003
(b) Tata	0.143 ± 0.002
(c) Vijay	0.224 ± 0.005
(d) RCM	0.205 ± 0.004
(e) Nescafe powder	0.225 ± 0.003
2. Alcoholic beverages	
(a) Officer's choice	0.216 ± 0.002
(b) Royal challenge	0.228 ± 0.004
(c) Aristocrat	0.232 ± 0.002
(d) Bagpiper	0.230 ± 0.003
(e) Bonnie	0.223 ± 0.004
3. Pharmaceutical formulations	
(a) Syndopa-110	0.218 ± 0.003
(b) Syndopa-275	0.213 ± 0.002
(c) Alpha-dopa	0.225 ± 0.005
(d) Dopamine	0.185 ± 0.004
(e) Isoprenaline	0.138 ± 0.003
(f) Adrenaline	0.242 ± 0.003

[a] SD = standard deviation.

Table 2
Determination of polyphenol level (mM) in different dried tea leaves by laccase/CuNP's/CHIT/cMWCNT/PANI/Au electrode, prepared thrice.

Sample no.	First electrode Mean ± SD[a] ($n = 4$)	Second electrode Mean ± SD[a] ($n = 4$)	Third electrode Mean ± SD[a] ($n = 4$)
1	0.025 ± 0.003	0.023 ± 0.005	0.022 ± 0.004
2	0.143 ± 0.005	0.140 ± 0.002	0.141 ± 0.007
3	0.224 ± 0.005	0.226 ± 0.008	0.223 ± 0.003
4	0.202 ± 0.004	0.205 ± 0.009	0.207 ± 0.006
5	0.226 ± 0.003	0.225 ± 0.005	0.228 ± 0.008

[a] SD = standard deviation.

4. Conclusion

A novel amperometric polyphenol biosensor was fabricated, based on covalent immobilization of laccase onto CuNP's/CHIT/cMWCNT/PANI/Au electrode. The resulting working electrode showed better analytic characteristics such as a wider determination range, faster response time and higher stability compared to earlier biosensors.

Acknowledgments

Authors are grateful to Prof. R.C. Kuhad, Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi-110021, India for providing crude laccase (cell free extract of *Ganoderma* sp. Rckk02 grown in malt extract broth) used in present study.

References

Aleksovski, S.A., Sovova, H., 2007. Supercritical CO_2 extraction of *Salvia officinalis* L. J. Supercrit. Fluids 40, 239–245.

Almela, L., Sanchez-Muñoz, B., Fernandez-Lopez, J.A., Roca, M.J., Rabe, V., 2006. Liquid chromatographic–mass spectrometric analysis of phenolics and free radical scavenging activity of rosemary extract from different raw material. J. Chromatogr. A 1120, 221–229.

Brondani, D., Scheeren, C.W., Dupont, J., Vieira, I.C., 2009. Biosensor based on platinum nanoparticles dispersed in ionic liquid and laccase for determination of adrenaline. Sens. Actuators B 140, 252–259.

Chen, L., Gorski, W., 2001. Bioinorganic composites for enzyme electrodes. Anal. Chem. 73, 2862–2868.

Dhawan, S., Lal, R., Kuhad, R.C., 2003. Ethidium bromide stimulated hyper laccase production from birds nest fungus *Cyathus bulleri*. Lett. Appl. Microbiol. 36, 64–67.

Diaconu, M., Litescu, S.C., Radu, G.L., 2010. Laccase–MWCNT–chitosan biosensor—a new tool for total polyphenolic content evaluation from in vitro cultivated plants. Sens. Actuators B 145, 800–806.

Feldheim, D.L., Foss, C.A. 2002. In: 'Metal nanoparticles: synthesis, characterization and applications', Jr., eds. Marcel Dekker, New York.

Fernandes, S.C., Rosane, I., de Oliveira, W.Z., Fatibello-Filho, O., Spinelli, A., Vieira, I.C., 2008. Biosensor based on laccase immobilized on microspheres of chitosan crosslinked with tripolyphosphate. Sens. Actuators B: Chem. 133, 202–207.

Friedman, M., 1997. Chemistry, biochemistry, and dietary role of potato polyphenols. A review. J. Agric. Food Chem. 45, 1523–1540.

Gamella, M., Campuzano, S., Reviejo, A.J., Pingarró, J.M., 2006. Electrochemical estimation of the polyphenol index in wines using a laccase biosensor. J. Agric. Food Chem. 54, 7960–7967.

Iijima, S., 1991. Helical microtubules of graphitic carbon. Nature 1354, 56–58.

Jiang, Z.Y., Yu, Y.X., Wu, H.J., 2006. Preparation of CS/GPTMS hybrid molecularly imprinted membrane for efficient chiral resolution of phenylalanine isomers. J. Membr. Sci. 280, 876–882.

Lin, Y., Wang, R.X., Li, X.M., 2005. Electrochemical oxidation behavior of nitrite on a chitosan-carboxylated multiwall carbon nanotube modified electrode. Electrochem. Commun. 7, 597–601.

Liu, J.X., Zhou, W.J., Gong, J.L., Tang, L., Zhang, Y., Yu, H.Y., Wang, B., Xu, X.M., Zeng, G.M., 2008. An electrochemical sensor for detection of laccase activities from Penicillium simplicissimum in compost based on carbon nanotubes modified glassy carbon electrode. Biores. Technol. 99, 8748–8751.

Liu, Y., Qu, X., Guo, H., Chen, H., Liu, B., Dong, S., 2006. Facile preparation of amperometric laccase biosensor with multifunction based on the matrix of carbon nanotubes–chitosan composite. Biosens. Bioelectron. 21, 2195–2201.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lu, Y., Foo, L.Y., 2002. Polyphenolics of Salvia—a review. Phytochemistry 59, 117–140.

Luo, X.L., Xu, J.J., Wang, J.L., Chen, H.Y., 2005. Electrochemically deposited nanocomposite of chitosan and carbon nanotubes for biosensor application. Chem. Commun. 16, 2169–2171.

Montereali, M.R., Della Seta, L., Vastarella, W., Pilloton, R., 2010. A disposable Laccase-Tyrosinase based biosensor for amperometric detection of phenolic compounds in must and wine. J. Mol. Catal. B: Enz. 64, 189–194.

Odaci, D., Timur, S., Pazarlioglu, N., Montereali, M.R., Vastarella, W., Pilloton, R., Telefoncu, A., 2007. Determination of phenolic acids using Trametes versicolor laccase. Talanta 71, 312–317.

Okuma, H., Watanabe, E., 2002. Flow system for fish freshness determination based on double multi-enzyme reactor electrodes. Biosens. Bioelectron. 17, 367–372.

Quan, D., Kim, Y., Shin, W., 2004. Characterization of an amperometric laccase electrode covalently immobilized on platinum surface. J. Electroanal. Chem. 561, 181–189.

Rajesh, Kaneto, K., 2005. A new tyrosinase biosensor based on covalent immobilization of enzyme on N-(3-aminopropyl) pyrrole polymer film. Curr. Appl. Phys. 5, 178–183.

Rochefort, D., Kouisni, L., Gendron, K., 2008. Physical immobilization of laccase on an electrode by means of poly(ethyleneimine) microcapsules. J. Electroanal. Chem. 617, 53–63.

Roy, J.J., Abraham, T.E., Abhijith, K.S., Sujith Kumar, P.V., Thakur, M.S., 2005. Biosensor for the determination of phenols based on Cross-Linked Enzyme Crystals (CLEC) of laccase. Biosens. Bioelectron. 21, 206–211.

Vasdev, K., Kuhad, R.C., 1994. Induction of laccase production in Cyathus Bulleri under shaking and static culture conditions. Folia Microbiol. 39, 326–330.

Vianello, F., Ragusa, S., Cambria, M.T., Rigo., A., 2006. A high sensitivity amperometric biosensor using laccase as biorecognition element. Biosens. Bioelectron. 21, 2155–2160.

Wang, Y., Wei, W., Zeng, J., Liu, X., Zeng, X., 2008. Fabrication of a copper nanoparticle/chitosan/carbon nanotube-modified glassy carbon electrode for electrochemical sensing of hydrogen peroxide and glucose. Microchim. Acta 160, 253–260.

Xu, Guo, M., Lu, P., Wang, R., 2010. Development of amperometric laccase biosensor through immobilizing enzyme in copper-containing ordered mesoporous carbon (Cu-OMC)/chitosan matrix. Mater. Sci. Eng. 30, 722–729.

Yaropolov, A.I., Kharybin, A.N., Emnéus, J., Marko-Varga, G., Gorton, L., 1995. Flow-injection analysis of phenols at a graphite electrode modified with co-immobilised laccase and tyrosinase. Anal. Chim. Acta 308, 137–144.

Ye, J.S., Cui, H.F., Wen, Y., Zhang, W.D., Xu, G.Q., Sheu, F.S., 2006. Electrodeposition of platinum nanoparticles on multi-walled carbon nanotubes for electrocatalytic oxidation of methanol. Microchim. Acta 152, 267–275.

Zgorka, G., Kawka, S., 2001. Application of conventional UV, photodiode array (PDA) and fluorescence (FL) detection to analysis of phenolic acids in plant material and pharmaceutical preparations. J. Pharm. Biomed. Anal. 24, 1065–1072.

Zhang, M.G., Smith, A., Gorski, W., 2004. Carbon nanotube-chitosan system for electrochemical sensing based on dehydrogenase enzymes. Anal. Chem. 76, 5045–5051.