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ORIGINAL PAPER

Electrochemical detection of phenolic compounds using composite film of multiwall carbon nanotube/surfactant/tyrosinase on a carbon paste electrode

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Abstract A new tyrosinase-based biosensor was developed for detection of phenolic compounds using composite film of multiwall carbon nanotube (MWCNT)/dimethylditetradecylammonium bromide (DTDAB)/tyrosinase (Tyr) on a Nafion-incorporated carbon paste electrode. The biosensor showed a sensitive electrochemical response to the reduction of the oxidation products of different phenolic compounds (phenol, catechol, *p*-cresol, and *p*-chlorophenol) by dissolved O_2 in the presence of the immobilized enzyme. The effects of pH, operating potential, MWCNT concentration, and the DTDAB/Tyr ratio on electrochemical response were explored for optimum analytical performance. The biosensor exhibited a linear response range of 1.5–25.0, 2.0–15.0, 2.0–15.0, and 2.5–25.0 μM and sensitivity of 2,900, 3,100, 3,100, and 1,500 μA/mM for phenol, catechol, *p*-cresol, *p*-chlorophenol, respectively. In addition, the response of the enzyme electrode showed Michaelis–Menten behavior at concentrations of the phenolic compounds higher than 5.0 μM. The stability and the application of the biosensor were also evaluated.

Keywords Carbon paste electrode · Tyrosinase · Phenolic compounds · Multiwall carbon nanotube · Surfactant

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Introduction

Phenolic compounds are important contaminants in food and environmental matrices [1]. Many of them are very toxic, showing harmful effects on plants, animals, and human health. Therefore, the identification and quantification of these compounds are important for environmental monitoring [2, 3]. The commonly used techniques for determination of phenolic compounds are spectrophotometry, chromatography, and capillary electrophoresis [4–7]. However, these methods are time-consuming and the equipments are expensive. Therefore, there is an interest in developing simple, sensitive, and effective analytical techniques for their determination. Among them, electrochemical biosensors based on tyrosinase have been shown to be very simple and sensitive tools for phenolic compounds assay [8–16].

Tyrosinase (Tyr, monophenol, dihdrophenylalanine (DOPA):dioxygen oxidoreductase, EC 1.14.18.1) contains a coupled binuclear copper active site (the T3 site) [17]. This enzyme catalyzes both the *ortho*-hydroxylation of monophenols (cresolase activity), and two-electron oxidation of *o*-diphenols to *o*-quinones (catecholase activity) using molecular oxygen [18, 19]. *o*-quinones can be electrochemically reduced to *o*-diphenols with a low overpotential. However, quinones may spontaneously react with each other to form oligomers or may be attacked by nucleophiles [20, 21]. Indeed, the surface of the biosensor may be blocked by some products or by insulating polymer film which cause low stability and significant inhibition of the enzyme activity. Therefore, the search for reliable methods to improve enzyme stabilization and to retain the catalytic activity of the enzyme and eventually to enhance performance of the biosensor is still interesting.

Recently, designing of electrochemical biosensors based on the immobilization of Tyr in carbon paste electrodes have been widely reported in the literature [10, 22–25]. In all of these works, many attempts have been made to improve the sensitivity and operational stability of the biosensors using the modification of carbon paste electrode with various sillicon and parafin oils [22], short-chain hydrocarbon binders [24, 25], and well-known nanomaterials [10, 11]. Generally, among the various nanomaterials, carbon nanotubes (CNTs) with excellent electrical conductivity, high mechanical strength, and good stability have been extensively used in the development of modified electrodes [26]. An impediment for the potential applications of CNTs is their insolubility in most solvents [27]. It is, however, reported that the solubility of CNTs can be improved using polymeric chain and surfactant molecules [27–33]. Surfactants are a type of amphiphilic molecules with a polar head at one end and a long hydrophobic tail at the other. They can spontaneously be adsorbed on the interfaces of two phases with different polarities and formed stable films. Surfactants usually used in these films have two long hydrocarbon chains. These films allow detailed studies of protein electron transfer and catalysis in biomembrane-like environments [34].

Given the ability of surfactants to disperse CNTs as individual tubes in media and to create a biocompatible environment for enzyme immobilization onto the surface of CNT, we used DTDAB as a two chain surfactant to prepare a composite film assembled by the DTDAB surfactant, MWCNT and Tyr on a Nafion-incorporated carbon paste electrode (NCPE) to determine phenolic compounds in water samples.To the best of our knowledge, no study on electrochemical detection of phenolic compounds using MWCNT/DTDAB/Tyr film on a carbon paste electrode has been reported.

Materials and methods

Reagents

We purchased Tyr (EC 1.14.18.1, 5,370 U mg^{-1}, from mushroom) and DTDAB from Sigma, hydrogen peroxide (30%), phenol, catechol, *p*-cresol, and *p*-chlorophenol from Merck, MWCNTs (with diameter of 110–170 nm, length 5–9 μm) and perfluorinated ion-exchange resin (Nafion, 5% in ethanol) from Aldrich. The phosphate buffer solution (PBS) consisted of a sodium phosphate solution (NaH_2PO_4 and Na_2HPO_4 from Merck; 0.05 M total phosphate) at pH 6.5.

Tyrosinase activity was assayed by the measurement of L-DOPA formed from tyrosine in phosphate buffer solution (0.05 M, pH 6.5 and 25 °C) using a continuous spectrophotometric rate determination as described in Sigma-Aldrich protocols. Under these conditions, the enzyme activity was about 150.0 units/mg of solid protein.

Apparatus

We used a Metrohm electroanalyzer Model 797 VA for voltammetric measurements. A conventional three-electrode cell was used with an Ag/AgCl electrode, a platinum rod and a modified carbon paste with a 3.0 mm diameter as reference, counter, and working electrode respectively. All experiments were performed at 25±°C.

Fabrication of the enzyme electrode

The NCPE was prepared as follows: Nafion solution, nujol (mineral oil), and graphite powder (10:15:75, *w*/*w*%) were mixed by hand to produce a homogeneous carbon paste. The carbon paste was packed into the end of a Teflon tube (*ca.* 3.0 mm diameter) and smoothed on a weighing paper.

MWCNT (1 mg) was dispersed in 1 mL aqueous solution of DTDAB (10 mM) with the aid of ultrasonication to give a 1-mg/mL stable black MWCNT suspension. MWCNT suspension (11 μL) was mixed with 40 μL Tyr solution (9.4 μM in PBS, pH 6.5) thoroughly. Then, 10 μL of the mixture (MWCNT/DTDAB/Tyr) was cast onto the surface of the NCPE (MWCNT/DTDAB/Tyr-NCPE). Finally, it was allowed to dry at room temperature for 3 h. For comparative studies, DTDAB and Tyr mixtures of different ratios were prepared. All the above prepared solutions and carbon paste-modified electrodes were stored at 4 °C when not in use. Nafion is a perfluorosulfonate ionomer that contains less than 15% ionizable sulfonate groups per monomer unit and has hydrophobic perfluoro-poly-ether chains which can bind to hydrophobic moiety of surfactant–protein complex. Incorporating the macromolecules into Nafion is revealed to improve their permeability and stability [35–37].

Results and discussion

Cyclic voltammetric characterization

Figure 1 shows the cyclic voltammograms of air saturated 0.05 M PBS (pH 6.5) in the presence of phenol at NCPE, Tyr-NCPE, DTDAB/Tyr-NCPE, and MWCNT/DTDAB/Tyr-NCPE. Upon addition of 50 μM phenol to the electrochemical cell, the cyclic voltammograms change dramatically with a significant increase in the reduction current at Tyr-NCPE, DTDAB/Tyr-NCPE, and MWCNT/DTDAB/Tyr-NCPE (Fig. 1 curves b, c, and d), while no change observed at NCPE (Fig. 1 curve a). Obviously, the enhanced reduction current at NCPEs containing Try can be

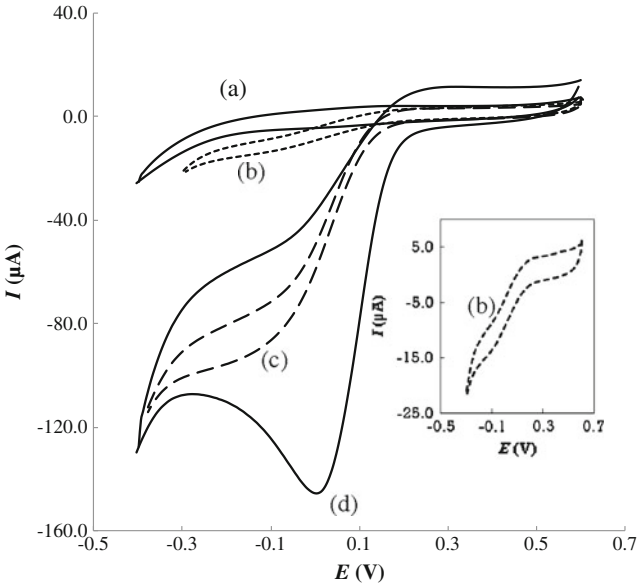


Fig. 1 Cyclic voltammograms of NCPE (**a**), Tyr-NCPE (**b**), DTDAB/Tyr-NCPE (**c**), and MWCNT/DTDAB/Tyr-NCPE (**d**) after addition of 50 μM of phenol to air-saturated 0.05 M PBS (pH 6.5) at 0.05 V s^{-1}. The inset shows curve (**b**) on an expanded scale

attributed to the reduction of *o*-quinone species liberated from the enzyme reaction catalyzed by the Tyr on the electrode surface. The steps of the enzymatic reaction on the modified electrode surface are shown as follows (Scheme 1) [18, 19].

$$\text{Phenol} + \text{tyrosinase}\,(0_2) \rightarrow \text{catechol} \quad (1)$$

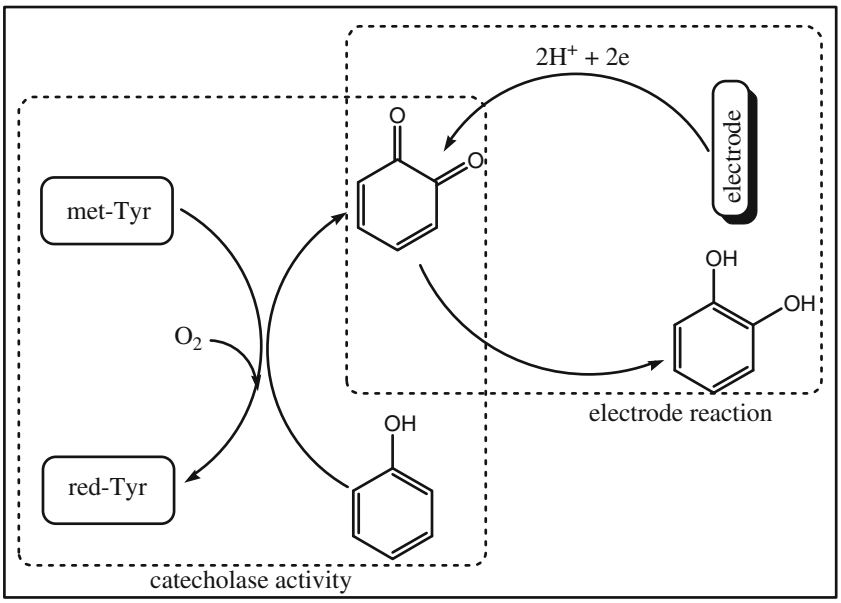
$$\text{Catechol} + \text{tyrosinase}\,(0_2) \rightarrow o - \text{quinone} + H_20 \quad (2)$$

$$o - \text{Quinone} + 2H^+ + 2e \rightarrow \text{catechol}\,(\text{at electrode surface}) \quad (3)$$

Although, the cyclic voltammogram at Tyr-NCPE shows the catalytic reactions to form *o*-quinone on the electrode surface (Fig. 1 curve b), its response is about 6.2 and 7.9 times smaller than those at DTDAB/Tyr-NCPE, and MWCNT/DTDAB/Tyr-NCPE, respectively (Fig. 1 curve c and d). Also, the reduction peak current of *o*-quinone at MWCNT/DTDAB/Tyr-NCPE is about 1.3 times higher than DTDAB/Tyr-NCPE.

It is well known that ionic surfactants can interact very strongly with oppositely charged globular proteins [38, 39]. At pH 6.5, DTDAB and Tyr possess positive and negative surface charges, respectively. Thus, the localized electrostatic attraction between the negatively charged groups of the protein and positively charged DTDAB molecules could play an important role in DTDAB/Tyr interaction and enhance the enzyme catalytic sites accessible to substrate molecule. Furthermore, the solubility of CNTs can be improved by surfactant molecules [27–33]. When carbon nanotubes are dispersed by sonication in a buffer solution containing DTDAB, the DTDAB molecules are adsorbed onto their surfaces. The adsorption of DTDAB molecules on the surfaces of carbon nanotubes can create a distribution of positive charges preventing the carbon nanotubes aggregation. Such an improvement in the solubility of carbon nanotubes in surfactant solution can be clearly seen with the naked eye.

That the MWCNTs can enhance the reduction current of *o*-quinone on the electrode surface may be due to their novel properties such as high surface area, electrical conductivity, good chemical stability, and extremely high mechanical strength [26, 40, 41].



Scheme 1 The steps of the enzymatic reaction on the modified electrode surface

Optimization of experimental variables

Influence of DTDAB/Tyr ratio for preparation of MWCNT/DTDAB/Tyr-NCPE

Figure 2 shows the cyclic voltammograms of MWCNT/DTDAB/Tyr-NCPE, prepared with different ratios of DTDAB/Tyr, in air-saturated 0.05 M PBS (pH 6.5) containing 50 μM phenol. As seen in the inset of Fig. 2, the maximum reduction current attained with DTDAB/Tyr (100:1) solution. This optimized ratio was used in all the experiments. Furthermore, to obtain a well-defined reduction peak current of *o*-quinone on the electrode surface, MWCNT concentration was optimized to 1 mg/mL (data not shown).

Effect of solution pH on the electrode response

The pH value is one of the parameters that can affect the response of the modified electrode to phenolic compounds. Figure 3 shows the cyclic voltammograms of MWCNT/DTDAB/Tyr-NCPE, obtained in air-saturated buffer solutions with different pHs, containing 50 μM phenol. As shown in the inset of Fig. 3, the best response current was achieved in the pH range between 6.0 and 7.0. Therefore, in order to obtain maximum sensitivity, pH 6.5 was chosen in subsequent experiments. This pH value is in agreement with the optimum pH reported for free Tyr [42]. However, the enzyme would lose activity irreversibly at lower or higher pH values [10, 11]. Furthermore, the decrease in the response current at pH values greater than 7.0 may be due to the involvement of protons in the reduction reaction of *o*-quinone [10].

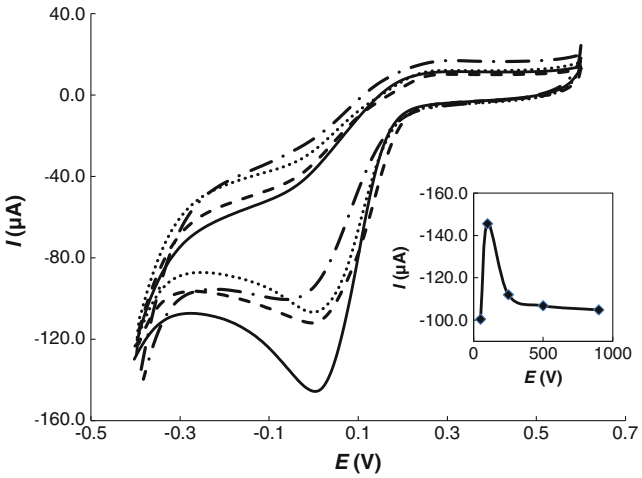


Fig. 2 Cyclic voltammograms of MWCNT/DTDAB/Tyr-NCPE, prepared with different ratios of DTDAB/Tyr (50/1 (*dashed dotted line*), 100/1 (*solid line*), 250/1 (*dashed line*), 500/1 (*dotted line*)) in air-saturated 0.05 M PBS (pH 6.5) containing 50 μM phenol. The *inset* shows the influence of DTDAB/Tyr ratio on maximum reduction current

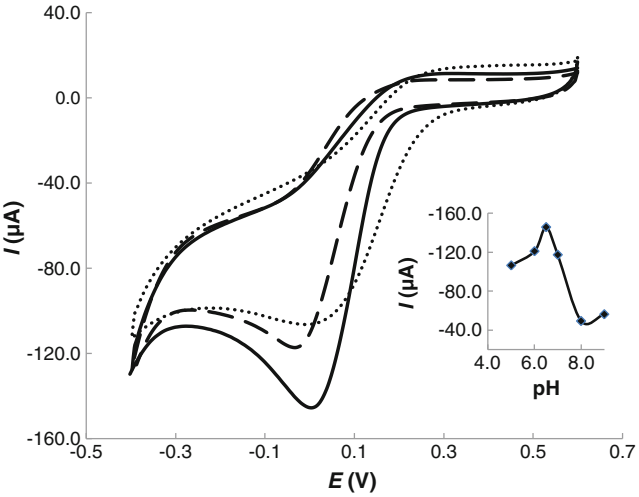


Fig. 3 Cyclic voltammograms of MWCNT/DTDAB/Tyr-NCPE, obtained in air-saturated buffer solutions with different pHs (5.0 (*dotted line*), 6.5 (*solid line*), 7.0 (*dashed line*)) containing 50 μM phenol. The *inset* shows the influence of pH on maximum reduction current

Quantitative detection of the phenolic compounds

Analytical determination of phenol using the modified electrode was studied by cyclic voltammetry. Voltammograms were obtained under the optimized experimental conditions after the addition of successive aliquots of phenol to air saturated 0.05 M PBS (pH 6.5). Upon addition of successive aliquots of phenol to PBS, a clearly defined reduction peak current proportional to the phenol concentration was observed (Fig. 4). In order to select the best applied potential for plotting the calibration curves, the effect of the applied potential on the signal and background current of the biosensor was investigated over the potential range −0.05 to −0.2 V, being the potential range near the maximum reduction peak current. As seen in Fig. 5, the highest difference

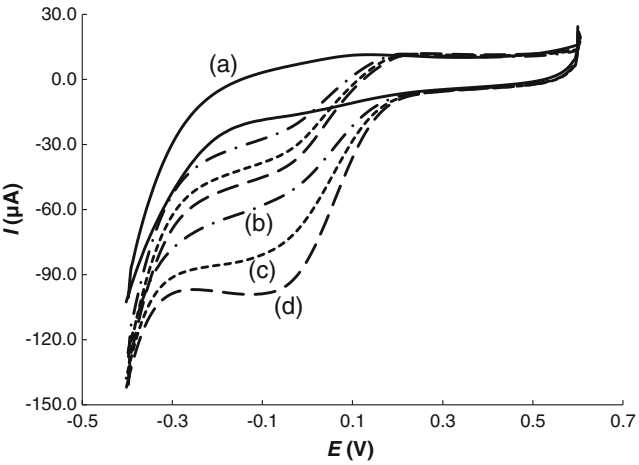


Fig. 4 Cyclic voltammograms of MWCNT/DTDAB/Tyr-NCPE before (**a**) and after addition of 10 μM (**b**), 20 μM (**c**), 30 μM (**d**) μM phenol to air-saturated 0.05 M PBS (pH 6.5) at 0.05 V s^{-1}

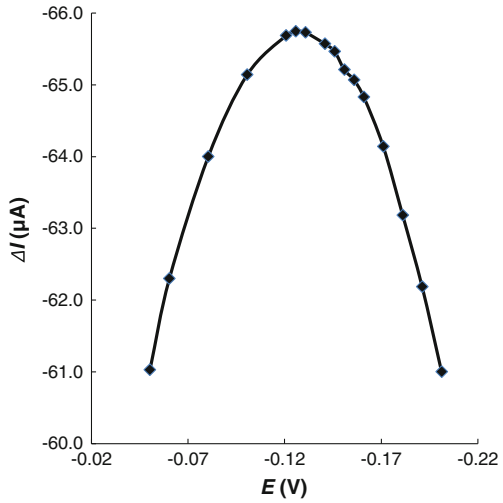


Fig. 5 Influence of applied potential on difference between signal and background current of the biosensor over the potential range −0.05 to −0.2 V, which is the potential range near the maximum reduction peak current. The highest difference between signal and background current was obtained at about −125 mV vs. Ag/AgCl

between signal and background current was obtained at about −125 mV vs. Ag/AgCl. As a result, the applied potential of −125 mV was selected for showing the dependence of the reduction current on the concentration of phenol.

The calibration curve of the enzyme electrode obtained from cyclic voltammetric data in the phenol concentration range from 0.2 to 90.0 μM under the optimal conditions is shown in Fig. 6. The response to phenol is linear (r=0.992) in the concentration range from 1.5 to 25.0 μM (inset of Fig. 6). The curvature observed for higher concentration can be attributed to slow surface fouling by the reaction

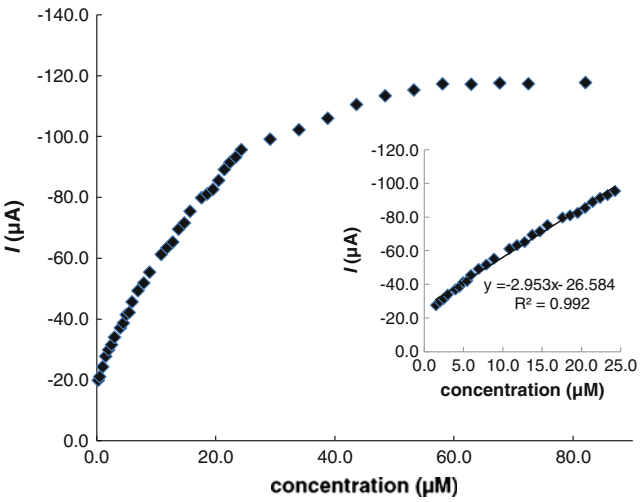


Fig. 6 Calibration curve of the enzyme electrode obtained from cyclic voltammetric data in the phenol concentration range from 0.2 to 90.0 μM under the optimal conditions. The *inset* shows the linear range of the calibration curve

products. The resulting biosensor exhibited sensitivity (slope) of 2.9 μA/μM (2,900 μA/mM; Table 1), which was higher than those reported for phenol on other modified electrodes (Table 2) [8, 10, 13, 43–45]. As mentioned above, the improvement in the sensitivity of the biosensor could be attributed to the favorable microenvironment of the proposed film, allowing the enzyme bioactivity to be retained to a large extent after the immobilization procedure and enhancing the electrochemical reduction kinetics of the corresponding *o*-quinone. The detection limit, C_m, was obtained by using the equation $C_m=3S_{bl}/m$, where S_{bl} is the standard deviation of the background current (μA) and m is the slope of the calibration plot (2.9 μA/μM) [11, 46, 47]. In the present study, 10 replicate measurements were performed on the blank solution and the resulting data were then treated statistically to obtain S_{bl}=1.1 μA. From the analysis of these data, we estimate that the detection limit of phenol is of the order of 1.1 μM.

The response of the enzyme electrode to other phenolic compounds was also investigated using cyclic voltammetry. Figure 7 displays the linear response range of the modified electrode for catechol, *p*-cresol, and *p*-chlorophenol under optimal experimental conditions. Analytical performances derived from Fig. 7 are given in Table 1. With this enzyme electrode, the range of linearity of 2.0–15.0 μM (r=0.991), 2.0–15.0 μM (r=0.997), 2.5–25.0 μM (r=0.998), for catechol, *p*-cresol, and *p*-chlorophenol, respectively, could be reached. Furthermore, the sensitivity of the modified electrode obtained towards catechol, *p*-cresol, and *p*-chlorophenol were 3,100, 3,100, 1,500 μA/mM, respectively, which are higher than those reported for phenolic compounds on other modified electrodes [8, 13]. The difference in sensitivity between each phenolic compound (the increasing order: *p*-cresol > phenol > *p*-chlorophenol) might depend on the Tyr catalytic selectivity for different compounds. The phenolic compounds containing electron-donating groups are good substrates for Tyr as compared to the electron-withdrawing groups [9, 48, 49]. However, some investigations differently reported that the solubility of phenolic compounds in an immobilized film on an electrode surface and hence the hydrophobic characteristics of the >film can also affect the sensitivity sequence of the phenolic compounds in an order of p-chlorophenol >

Table 1 Analytical performances of biosensor

Compound	Sensitivity (μA/mM)	Detection limit (μM)	Linear range (μM)	Correlation coefficient
Phenol	2,900	1.1	1.5–25.0	0.992
Catechol	3,100	0.9	2.0–15.0	0.991
p-Cresol	3,100	2.0	2.0–15.0	0.997
p-Chlorophenol	1,500	2.2	2.5–25.0	0.998

Table 2 Comparison of sensitivity of proposed modified electrode for phenol with others

Immobilization method	Sensitivity (μA/μM)	reference
MWCNT/DTDAB modified carbon paste electrode	2,900	The current study
Nano-ZnO/chitosan	182	[8]
Gold nanoparticles modified glassy carbon electrode	82	[43]
Colloidal gold-modified carbon paste electrode	23	[10]
Hybrid titania sol-gel matrix	1,605	[44]
Electropolymerized PTS-doped polypyrrole film	17	[45]
Colloidal gold/graphite/teflon	540	[13]

p-cresol > phenol [50]. In order to understand the observed difference in sensitivity order in the present study, one may notice that the presence of DTDAB as an ionic surfactant leads to a decrease in hydrophobic characteristics of the immobilized film, and therefore the effect of substitution in para position of the phenolic compounds is more important than hydrophobic characteristics of the film in determining the sensitivity order of the phenolic compounds.

In order to make certain whether the modified electrode exhibits an increase in the efficiency of the catalytic reaction to form *o*-quinone, we calculated the apparent Michaelis–Menten constant (K_M^{app}) for the phenolic compounds from the data in Figs. 6 and 7 at concentrations higher than 5 μM. According to the Lineweaver–Burk equation [51], the (K_M^{app}) values were 8.0, 8.1, 12.3, and 24.5 μM for *p*-cresol, catechol, phenol, and *p*-chlorophenol, respectively. The (K_M^{app}) value is markedly lower than that observed for the free enzyme in solution, estimated to be 700 using phenol as substrate [10]. More interestingly, in the presence of phenol as substrate, the (K_M^{app}) value is also lower than the values 53.5, 133.0, 168.0, and 245 reported in the literature for Tyr immobilized on carbon paste electrode with different methods [24]. This configuration of the modified electrode causes a higher affinity of the enzyme for phenol and, consequently, allows a higher sensitivity.

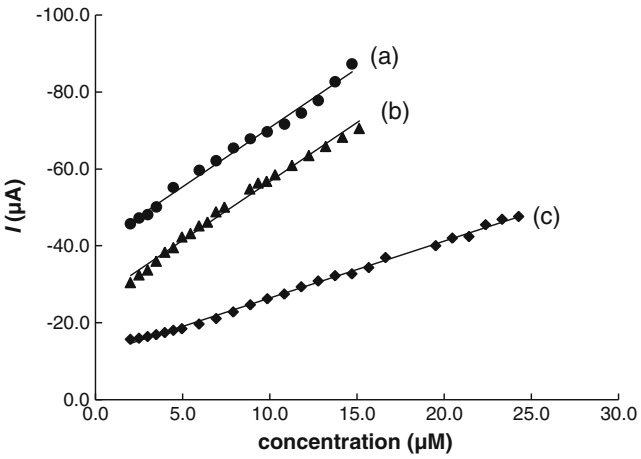


Fig. 7 Linear response range of the enzyme electrode for catechol (*a*), *p*-cresol (*b*), and *p*-chlorophenol (*c*) in air-saturated 0.05 M PBS (pH 6.5) at 0.05 V s^{-1}

Reproducibility, repeatability, and stability

The fabrication reproducibility of the modified electrode was examined from the response to 25.0 μM of phenol at six different enzyme electrodes. An acceptable reproducibility was obtained with a relative standard deviation (RSD) of 4.3%. The repeatability of the current response of one enzyme electrode to 25.0 μM of phenol was also examined. The RSD was 4.8% for six successive assays.

The long-term stability of the modified electrode was studied over a certain period of time by monitoring its current to the 25.0 μM of phenol with intermittent usage (every day). The response current of the electrode decreased to about 86% after 10 days.

Analytical application

To evaluate the accuracy of the proposed biosensor, some assays were made on standard phenol samples. Phenol concentration was determined by the standard addition method. The results presented in Table 3 seem to be satisfactory. Therefore, the biosensor provides a possible and simple method for determining the phenol with good precision and accuracy.

Table 3 Recovery of the tyrosinase biosensor

Phenol concentration (μM)		Recovery (%)
Added	Found	
2.0	2.1	105.0
3.0	2.9	96.7
4.0	3.9	97.5
4.9	5.0	102.0
5.9	6.1	103.4
9.9	10.5	106.1
13.7	14.4	105.1
17.6	16.9	96.0

Conclusion

In this work, it is demonstrated that a composite film of MWCNT/DTDAB/Tyr on a Nafion incorporated carbon paste electrode can be used for detection of different phenolic compounds (phenol, catechol, *p*-cresol, and *p*-chlorophenol). The proposed biosensor exhibited simplicity in manipulation, low cost, fast response, high sensitivity, stability, and fabrication simplicity. In addition, the response of the enzyme electrode exhibited Michaelis–Menten behavior at concentrations of the phenolic compounds higher than 5.0 μM.

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