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An amperometric biosensor based on multiwalled carbon nanotube-poly(pyrrole)-horseradish peroxidase nanobiocomposite film for determination of phenol derivatives

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ABSTRACT

An amperometric biosensor based on horseradish peroxidase (HRP) and carbon nanotube (CNT)/polypyrrole (PPy) nanobiocomposite film on a gold surface has been developed. The HRP was incorporated into the CNT/PPy nanocomposite matrix in one-step electropolymerization process without the aid of cross-linking agent. Amperometric response was measured as a function of concentration of phenol derivatives, at a fixed bias voltage of −50 mV. Optimization of the experimental parameters was performed with regard to pH and concentration of hydrogen peroxide. The linear range, sensitivity and detection limit of the biosensor were investigated for eighteen phenol derivatives. The sensitivity in the linear range increased in this order: 4-methoxyphenol > 2-aminophenol > guaiacol = *m*-cresol > 2-chlorophenol = 4-chlorophenol = hydroquinone = pyrocatechol > 2,6-dimethoxyphenol > 3-chlorophenol > *p*-cresol > *p*-benzoquinone = 4-acetamidophenol > catechol > phenol = pyrogallol = 2,4-dimethylphenol. CNTs was shown to enhance the electron transfer as a mediator and capable to carry higher bioactivity owing to its intensified surface area. The biosensor exhibited low detection limits with a short response time (2 s) for the tested phenolics compared to the reported working electrodes. It retained 70% of its initial activity after using for 700 measurements in 1 month.

1. Introduction

The determination of phenol and its derivative compounds is of the environmental importance, since these species are toxic and evolved in many industrial processes. They are present in many wastewater streams of the oil, paint, polymer and pharmaceutical industries [1]. Electrochemical methods have been widely used for measuring these compounds due to their advantages such as good selectivity in the presence of phenol oxidases, relatively low cost of realization and storage and the potential for miniaturization and automation [2–4]. Regarding the amperometric enzyme biosensors, tyrosinase has been the most currently used enzyme for the detection of phenolic compounds [5–9]. However, these tyrosinase biosensors are restricted to the monitoring of phenolic compounds having at least one *ortho*-position free [10]. On the other hand, laccase biosensors give response to phenolic compounds with free *para*- and *meta*-position with a complicated catalytic cycle [11]. HRP having less selectivity to phenolics is capable of giving response to a large number of phenol derivatives [12], and shows a high stability and efficiency for different biosensor designs [3,13].

The selectivity and sensitivity of the modified electrodes depends on the stability of the phenoxy radicals produced in the enzyme reaction, electrode material, immobilization method, and the magnitude of the applied potential [11]. In addition to this, the performance of biosensor is mainly affected by the electrocatalytic activity of modified electrode material and composites. CNTs have emerged as a new class nanomaterials that are receiving considerable interest owing to their ability to promote electron transfer reactions with enzymes [14,15]. The high conductivity of this carbon material leading to level of $10^2\ \Omega^{-1}\,cm^{-1}$ improves electrochemical signal transduction, while its nano-architecture imposes the electron contact between the redox centres, deeply inlaided in enzyme structure, and the smooth surface of the electrode [16]. Sotiropoulou et al. reported that CNTs have a metallic character in the range of potentials between −1.5 and +1.5 V, since there are no apparent oxidation or reduction peaks. Based on this, CNTs can donate and accept electrons in a wide range of potentials, and could therefore be used as mediators in biosensor systems [17]. A key barrier for developing CNT-based biosensors is the insolubility of CNTs in most solvents [18]. Functionalization of CNTs has been achieved by an oxidation process, which involves extensive

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ultrasonic treatment in a mixture of concentrated hydrogen peroxide and sulfuric acid [19]. The ends and sidewalls of the treated CNTs are mainly decorated with carboxyl groups. CNTs functionalized in this manner obtain good solubility in water and retain their pristine electronic and mechanical properties [20]. Recently, there has been growing interest in using CNT-based electrode configurations with tyrosinase and laccase polyphenol oxidases for detection of phenolics [21,22]. However, the use of HRP, alternative polyphenol oxidase, in modified CNT electrodes has been less reported for the measurement of phenol derivatives.

The aim of this study is to develop a CNT/PPy/HRP nanobiocomposite film for the bioanalytical applications. The working electrode was constructed in one-step by the electropolymerization process of multiwalled CNT, pyrrole and HRP. The parameters such as operating potential, pH level and concentration of hydrogen peroxide were investigated and were tested using a large set of eighteen phenol derivatives.

2. Experimental

2.1. Reagents

Horseradish peroxidase (E.C.1.11.1.7) with an activity of 10,000 U/vial (according to pyrogallol method performed by the supplier), aqueous solution of hydrogen peroxide (30%), lithium chloride, di-potassium hydrogen phosphate, citric acid, tri-sodium citrate, acetic acid (96%), sodium acetate tri-hydrate and potassium di-hydrogen phosphate were purchased from Merck. Phenol, *ρ*-benzoquinone, hydroquinone, 2,6-dimethoxyphenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2-aminophenol, 4-methoxyphenol, pyrocatechol, guaiacol, *m*-cresol, *o*-cresol, *p*-cresol, catechol, 4-acetamidophenol, pyrogallol, 2,4-dimethylphenol, pyrrole (99%), CHES buffer and sodium dodecyl sulfate (SDS) were obtained from Sigma. The phenol reagents were used as purchased without any further pre-treatment. Stock solutions of various phenols were daily prepared in 0.1 M phosphate buffer solution (pH 7.0). Multiwalled CNTs were obtained from Nanocs, Inc., NY, USA.

2.2. Apparatus

Electrochemical experiments were performed by using a CHI Model 800B electrochemical analyzer. A gold working electrode (2 mm diameter), a Platinum wire counter electrode, an Ag/AgCl (3 M NaCl) reference electrode, and a conventional three-electrode electrochemical cell were obtained from CH Instruments.

2.3. Preparation of CNT/PPy/HRP nanobiocomposite film coated gold electrode

Gold electrode was polished with slurries of fine alumina powders (0.3 and 0.05 mm) on a polishing microcloth pad. The electrode was then rinsed with distilled water. The facile routine for preparation of water-soluble CNTs was a modification of the acid oxidative method developed by Smalley's group [23]. Firstly, 14 mg of multiwalled CNTs were added into 5 mL of a 9:1 concentrated H_2SO_4/H_2O_2 (30%) aqueous solution and the mixture was stirred for 30 min for CNTs oxidation. After the reaction, 15 mL of the 9:1 concentrated H_2SO_4/H_2O_2 (30%) aqueous solution was added into the mixture. The mixture was placed in an ultrasonic bath (Elma 460-H) and sonicated for 5 min. Resulting CNTs dispersion was diluted using 1 L of distilled water, then was filtered through a 0.45 μM cellulose membrane. After, the filtrate was washed with 10 mM NaOH solution and distilled water till the pH level reaching to 7, the filtrate was separated from the membrane and dispersed in distilled water (0.03 mg/L). The resulting CNTs solution was sonicated for 2 min to obtain a homogeneous CNTs solution [19].

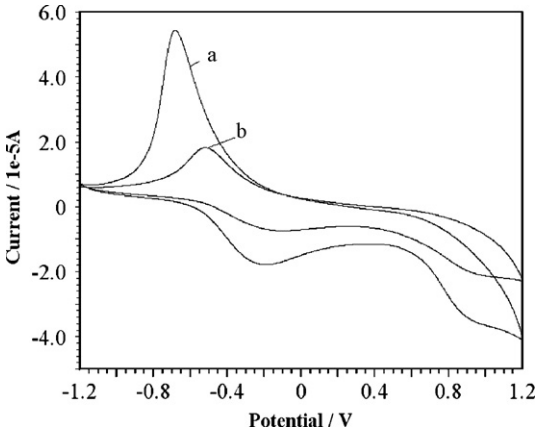

Fig. 1. Multiscan cyclic voltametric curves for CNT/PPy/HRP nanobiocomposite film (a), CNT/PPy film (b) in 0.1 M phosphate buffer (pH 7.0). Potential was scanned between −1.2 and +1.2 V at the scan rate of 100 mV s^{-1}.

CNT/PPy/HRP nanobiocomposite film was coated onto the surface of the gold working electrode by electrochemical polymerization in a three-electrode cell. The polymerization medium contained 5 mL of oxidized CNTs solution, 5 mL of 50 mM pH 6.5 citrate buffer including 0.01 M pyrrole, 0.6 mg/mL SDS and 0.3 mg/mL. HRP used in this study is a water-soluble enzyme. SDS is one of the best supporting electrolyte for electropolymerization of pyrrole in aqueous medium [24]. Since the anion of the SDS represents the dopant ions that stabilize the cationic sites in the polypyrrole, it might have a certain effect on the amount of the immobilized enzymes as well as its activity. Cyclic voltammogram of the nanobiocomposite film was scanned between 0 and 1.2 V for 4 min.

2.4. Electrochemical measurements

Electrochemical batch measurements were carried out in a 0.1 M phosphate buffer solution (pH 7.0) in the presence of 0.7 mg/mL lithium chloride with an applied working potential of −50 mV and a continuous stirring at 600 rpm in three-electrode cell mentioned in Section 2.2. Various phenol derivatives were tested to produce *i*–*t* curves of chronoamperometric measurements.

3. Results and discussion

3.1. Characterization of the CNT/PPy/HRP nanobiocomposite film

Fig. 1 shows the CV of the CNT/PPy film and CNT/PPy/HRP nanobiocomposite film in 0.1 M phosphate buffer (pH 7.0) at the scan rate of 100 mV s^{-1}. The peak current increased by the introduction of HRP into the film, indicating the synergy effect between HRP and CNT similar to the previous study [25]. In addition to this, the electrocatalytic sites placed in the active centre of HRP would join into the CNT/PPy nanobiocomposite film. There was virtually no change in the shape or peak potentials of the CV suggests that there was no hindrance for electron transfer process between electrode and HRP. The well-defined peaks indicate that films are highly homogeneous.

3.2. Effect of applied potential and pH on biosensor response

The response of the peroxidase biosensors to phenolic compounds is based on the so-called double displacement or

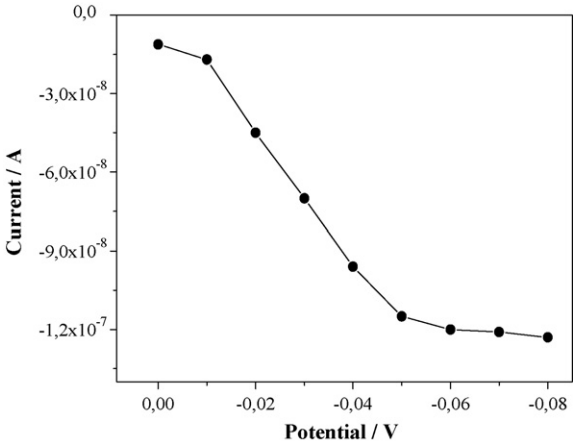

Fig. 2. Influence of applied potential on amperometric response of the biosensor to 16 μM hydroquinone in 0.1 M, pH 7.0 phosphate buffer.

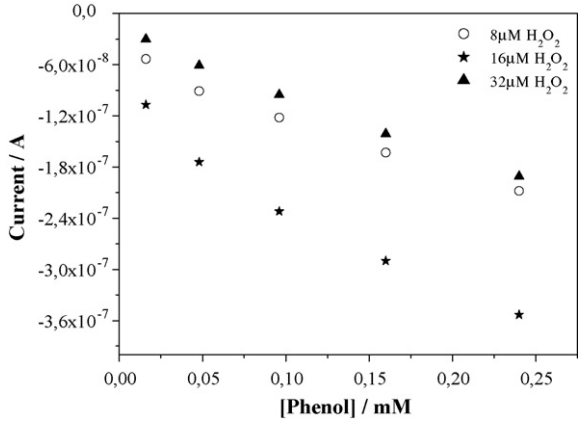

Fig. 4. The dependence of the biosensor response for phenol concentration in the presence of different hydrogen peroxide concentrations in 0.1 M, pH 7.0 potassium phosphate buffer solution. Applied potential −50 mV vs. Ag/AgCl, 3 M NaCl.

"ping-pong" mechanism in which two substrates, a peroxide and the given phenolic compound are involved [26,27]. At the surface of an electrode, where resting state and oxidized state of enzyme can be electrochemically reduced directly, the use of a second substrate such as a phenolic compound gives rise to a much faster process [12]. The monitoring of the enzymatic reaction is carried out by the electrode reduction of the phenoxy radicals. The reduction current is proportional to the concentration of phenolic compound in the medium, if adequate concentration of hydrogen peroxide is present. The dependence of CNT/PPy/HRP nanobiocomposite film electrode response on applied potential is shown in Fig. 2. The reduction peak tends to increase with the additions of hydroquinone. Hydroquinone is already reduced around 0 mV, and the reduction current increases rapidly as the applied potential moves negatively from 0 to −50 mV, which is due to the increased driving force for the fast reduction of hydroquinone at low potential. The current change approaches a plateau at −50 mV, thus this value is selected as the working potential. Same results were obtained for the other phenol deriatives tested (*not shown*).

The influence of pH on the response of CNT/PPy/HRP nanobiocomposite film electrode was investigated to optimize the reaction conditions. The change of chronoamperometric current between the pH level of 5–9 at a constant *p*-benzoquinone concentration (0.1 μM) is shown in Fig. 3. As seen in Fig. 3 the maximum response was obtained at pH 7.0 which is very similar to those observed for

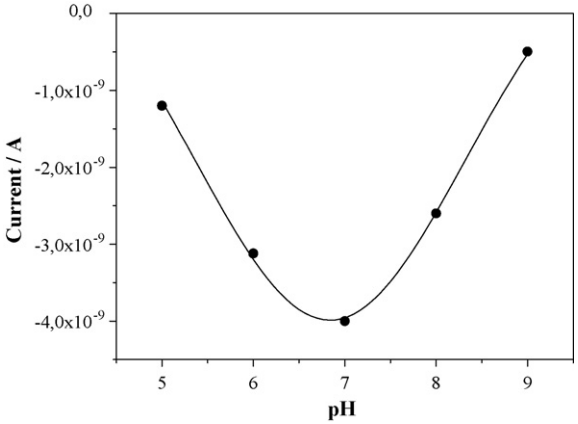

Fig. 3. Dependence of the current response of CNT/PPy/HRP nanobiocomposite film electrode to 0.1 μM *p*-benzoquinone on the pH of buffer solutions at an applied potential of −50 mV vs. Ag/AgCl, 3 M NaCl.

the soluble enzyme [28]. The optimized pH level of 7.0 was then used through further experiments.

3.3. Determination of optimum hydrogen peroxide concentration

The peroxide concentration is very important in HRP reactions to produce good sensitivity and avoid the inactivation of the enzyme due to high concentration of peroxide [29]. Fig. 4 shows dependence of the hydrogen peroxide concentration of CNT/PPy/HRP nanobiocomposite film electrode response for phenol concentrations in the range of 16–240 μM. The analytical curve obtained for phenol at various hydrogen peroxide concentrations of 8, 16, and 32 μM, showed a linear response ranging from 16 to 48 μM with a correlation coefficient (*r*) of 0.98 and a sensitivity of 0.7 nA/μM, 16 to 144 μM with a *r* value of 0.99 and a sensitivity of 1 nA/μM, and 16 to 48 μM with a *r* value of 0.98 and a sensitivity of 0.65 nA/μM, respectively. Thus, 16 μM of hydrogen peroxide was used for further experiments due to its maximum sensitivity and the widest linear range with an excellent correlation coefficient.

3.4. Response of the CNT/PPy/HRP nanobiocomposite film electrode to eighteen phenol derivatives

Eighteen phenolic compounds (phenol, catechol, *p*-benzoquinone, *m*-cresol, *o*-cresol, *p*-cresol, guaiacol, 2,4-dimethylphenol, 2,6-dimethoxyphenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, hydroquinone, 4-acetamidophenol, pyrogallol, 4-methoxyphenol, pyrocatechol, 2-aminophenol) were detected by CNT/PPy/HRP nanobiocomposite film electrode in 0.1 M phosphate buffer solution (pH 7.0) at a working potential of −50 mV (vs. Ag/AgCl). Fig. 5 (*not included all the phenolics tested*) illustrates typical amperometric responses for the CNT/PPy/HRP working electrode after the addition of successive aliquots of phenolic compounds under continuous stirring. Table 1 summarizes the characteristics of the calibration plots obtained for the phenol derivatives, as well as the corresponding limits of detection calculated according to the $3s_b/m$ criteria in Ref. [30], where m is the slope of the linear range of the respective calibration plot, and s_b is estimated as the standard deviation of the signals from different solutions of the phenolics at the concentration level corresponding to the lowest concentration of the calibration plot. The lowest detection limit was found to be 0.027 μM ($S/N = 3$) for *p*-benzoquinone and the highest detection limit was found to be 27.9 μM ($S/N = 3$) for 2,4-dimethylphenol among the tested derivatives. Detection limit ranges between 0.00003 and 346 μM

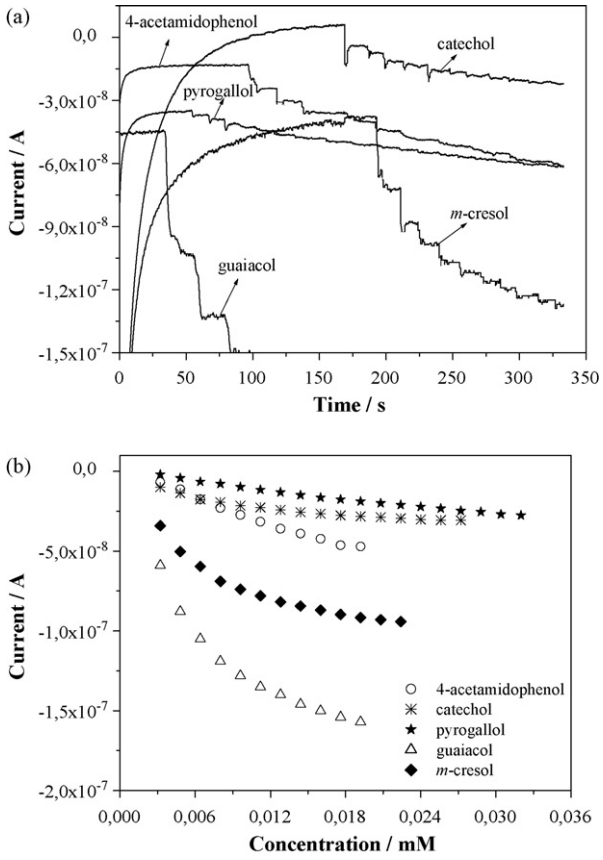

Fig. 5. (a) Current–time recordings and (b) calibration curve of the CNT/PPy/HRP nanobiocomposite film electrode to increasing 4-acetamidophenol, catechol, pyrogallol, guaiacol and *m*-cresol concentrations (initial phenolic concentration is 1.6 μM). Applied potential: −50 mV vs. Ag/AgCl, 3 M NaCl.

(S/*N* = 3) for various phenol derivatives with different phenol oxidases in recently reported biosensors [31–44].

The sensitivity of HRP-based biosensor depended on the stability of the phenoxy radicals produced in the enzyme reaction, electrode material, HRP immobilization method and the magnitude of the applied potential [11]. For the detection of different phenolic compounds, the trend of the sensitivity was consistent with the ability of the substituents for forming electron-donor conjugation. Usually, stronger ability of electron-donor conjugation resulted in higher sensitivity for the detection. According to Wilkolazka et al. [31], the enzymatic oxidation products of the *ortho*-substituted phenols are more rapidly produced showing an increase in the amplification reaction cycle. In this study, 2-aminophenol and guaiacol showed the higher sensitivity among the *ortho*-substituted phenols tested. For 2-aminophenol, the corresponding conjugation structure could be easily formed because of strong ability of electron-donor conjugation of the substituent. Kane et al. [45] reported that the phenol compounds with electron-donor substituents in an *ortho*-position gave no response. CNT/PPy/HRP nanobiocomposite film electrode did not give any response to *o*-cresol. The sensitivity was calculated from the slope of the calibration curves. The sensitivity in the linear range increase in this order: 4-methoxyphenol > 2-aminophenol > guaiacol = *m*-cresol > 2-chlorophenol = 4-chlorophenol = hydroquinone = pyrocatechol > 2,6-dimethoxyphenol > 3-chlorophenol > *p*-cresol > *p*-benzoquinone = 4-acetamidophenol > catechol > phenol = pyrogallol = 2,4-dimethylphenol (Table 1). It can be deduced that there is obvious difference among the phenol derivatives with regard to sensitivity. The different sensitivities observed can be attributed to the formation of *o*-quinones during the enzymatic reaction for each phenolic compounds [32]. The highest sensitivity was obtained from the calibration of 4-methoxyphenol. The presence of $-OCH_3$ group of 4-methoxyphenol allows HRP to oxidize more efficiently. A lower sensitivity was observed for 2,4-dimethylphenol, as expected, for the one having the *ortho*-position occupied by a methyl group. The sensitivity ranges between 1 and 50 nA/μM for the phenolics tested (Table 1). It is reported in the range between 0.011 and 746 nA/μM for various phenol derivatives at recent studies of phenoloxidases [31–44].

According to Koile and Johnson, a lose in linearity at higher concentration of phenolic compounds is attributed to slow surface fouling by the reaction products [46]. CNTs-based electrode was reported to increase electrocatalytic activity and to minimize surface fouling [32]. In this study, wide linear range with high *r*-value (0.99) was obtained for each phenolic tested. This result can be attributed to the nanobiocomposite structure based on CNTs.

Fig. 6 shows the comparison of the produced responses to the successive additions of 16 μM hydroquinone for CNT/PPy/HRP and PPy/HRP working electrodes in 0.1 M phosphate buffer (pH 7.0) at the potential of −50 mV (vs. Ag/AgCl). PPy/HRP biocomposite film electrode showed no reproducible signals to the hydroquinone

Table 1
Calibration plot parameters and analytical characteristics for various phenolic compounds at CNT/PPy/HRP nanobiocomposite film electrode

Compound	*r*	Sensitivity (nA/μM)	Lineer range (μM)	LOD (μM)	Response time (*s*)	%RSD
Phenol	0.99	1	16–44	3.52	2	2.89
p-Benzoquinone	0.99	3	0.02–0.16	0.027	2	4.43
Hydroquinone	0.99	8	16–240	6.42	2	6.5
2,6-Dimethoxyphenol	0.99	7	1.6–9.2	0.29	2	1.8
2-Chlorophenol	0.99	8	1.6–8	0.26	2	1.7
3-Chlorophenol	0.99	6	1.6–12.8	0.2	2	1.1
4-Chlorophenol	0.99	8	1.6–14.4	0.3	2	1.87
2-Aminophenol	0.99	40	8–60.8	1.53	2	5.4
4-Methoxyphenol	0.99	50	1.6–81.6	1.06	2	2.8
Pyrocatechol	0.99	8	1.6–446.4	6.27	2	6.7
Guaiacol	0.98	9	1.6–9.6	0.3	2	1.92
m-Cresol	0.99	9	8–20.8	1.5	2	2.84
o-Cresol			no response			
p-Cresol	0.98	5	128–832	24	2	2.5
Catechol	0.98	2	1.6–8	0.93	2	3.8
4-Acetamidophenol	0.99	3	1.6–16	1.11	2	2.57
Pyrogallol	0.98	1	1.6–22.4	1.24	2	1.2
2,4-Dimethylphenol	0.98	1	64–240	27.9	2	2.2

Applied potential; −50 mV, 0.1 M phosphate buffer (pH 7.0) containing 16 μM H_2O_2.

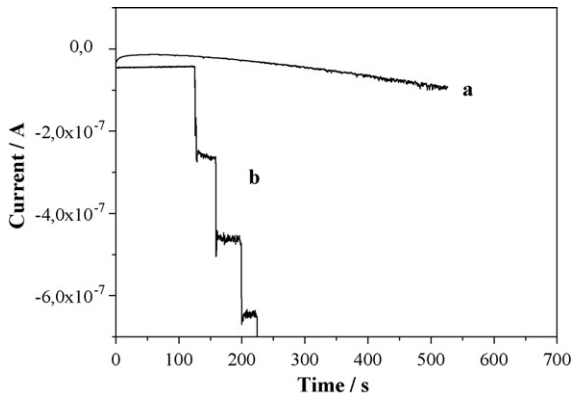

Fig. 6. Steady-state current–time responses to the successive additions of hydroquinone (16 μM) at PPy/HRP working electrode (a), at CNT/PPy/HRP working electrode (b). Applied potential: −50 mV vs. Ag/AgCl, 3 M NaCl.

additions. Nevertheless, CNT/PPy/HRP nanobiocomposite film electrode achieved to produce measurable responses by the regular growth of reduction currents. CNTs were thought to impose the electron transfer of the mediated reaction. It was previously reported that peroxidases were able to do direct electron transfer between enzyme molecules and electrode thus they did not need electron mediators for electron transfer [47]. However, in this study, the available responses could only be obtained by CNTs-based electrode. Furthermore, the amount of active immobilized enzyme in CNT/PPy/HRP nanobiocomposite film and PPy/HRP biocomposite film was found to be 6.1 and 2.7 μg, respectively. The immobilized enzyme quantity was measured by using the enzyme activity assay according to the procedure performed by Vojinovic et al. [48]. Nanobiocomposite film, involving CNTs, attached higher amount of enzyme than the composite film without CNTs due to their unique structure having activated large surface area. The nanostructure of the biocomposite could intensify the surface for higher biocatalytic activity. The sensitivity value of CNT/PPy/HRP nanobiocomposite film electrode was calculated as 8 nA/μM in the concentration range of 16–240 μM hydroquinone.

Time to allow the system to come to equilibrium is defined as "response time". The response of CNT/PPy/HRP nanobiocomposite film electrode was rapidly reached to steady-state current in about 2 s for all phenolics tested. This value is obviously shorter than most of the previously reported biosensors where the response time is ranged between 5 and 35 s for various phenolic compounds [32,38,41,44].

3.5. Stability of the CNT/PPy/HRP nanobiocomposite film electrode

The stability of the CNT/PPy/HRP nanobiocomposite film electrode was monitored by the measurement of the response for a series of 50 successive additions of a 1.6 μM phenolic to 0.1 M phosphate buffer (pH 7.0) at the potential of −50 mV (vs. Ag/AgCl). Well-defined reduction responses were obtained for all phenolics with relative standard deviations (RSD) range between 1.1% and 6.7% (Table 1). The biosensor response lost 30% of its initial value at the end of 1 month after 700 measurements. Relatively high stability can be attributed to the mild enzyme immobilization condition. Since, the acid treatment of CNTs attaches various oxygen groups (mainly carbonyl groups) to the sidewalls and ends of multiwalled CNT [49], large amount of HRP can be immobilized on the activated surfaces of CNTs. Therefore, nanostructure of CNT can provide favorable architecture for retaining the bioactivity of the enzyme and contribute to the stability of the working electrode.

4. Conclusions

The relative response of the CNT/PPy/HRP nanobiocomposite film electrode was investigated for eighteen phenol derivatives. Due to the relatively low oxidation potential of the pyrrole monomer, the immobilization of enzyme in a CNT/PPy nanocomposite film through electropolymerization enables films to be grown in aqueous solution that are compatible with most of the biological elements. The properties of good compatibility and film-forming ability make CNT/PPy nanocomposite film electrode suitable and robust matrix to incorporate enzyme in a one-step without using any cross-linking reagents. CNTs was shown to enhance the electron transfer as a mediator and capable to carry higher bioactivity owing to its intensified surface area. In conclusion, the fabricated biosensor can easily detect various phenolics in a wide range of linearity. The developed biosensor exhibited available sensitivity and low detection limit for the most of phenolics comparable to other polyphenol oxidase based electrodes. The response time of the biosensor for all phenol derivatives was very short, reaching 99% of its maximum response in about 2 s. The biosensor subjected to at least 700 measurements showed 70% of its initial activity after 1-month storage at 4 °C in the dark.

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