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Enzyme Based Phenol Biosensors

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1. Introduction

Phenol and its derivatives is one of the most important parameters which should be monitored in environmental engineering. They are present in many wastewater streams of the oil, paint, paper, polymer and pharmaceutical industries. Phenolic compounds reach into the food chain by wastewaters then lead to dangerous and toxic effect on aquatic organisms. Principal standard methods for quantitative phenol measurement are high performance liquid chromatography (HPLC), electrochemical capillary electrophoresis (CE), gas chromatography (GC) and colorimetric spectrophotometry. Although, these methods are analytically capable, generally they require pretreatment processes such as extraction, cleaning, dilution of the samples as well as additional chemicals. Owing to those disadvantages, researchers have focused on enzyme based amperometric biosensors for measuring phenolic compounds due to their advantages such as good selectivity, working possibility in aqueous medium, fast responding, relatively low cost of realization and storage and the potential for miniaturization and automation. Amperometric biosensors, have been developing for phenol and its derivatives, are usually prepared with working electrodes which include polyphenol oxidases (PPO) (tyrosinase and laccase) and enzyme horseradish peroxidase (HRP). HRP reaction with phenols is faster than PPO enzyme reactions, and HRP-based working electrodes show higher sensitivity in comparison to PPO-based electrodes. Thus, the usage of HRP on working electrodes can be advised for fast and effective phenol measurements.

The design of a support matrix that binds the enzyme and bare electrode can be target specific providing efficient electron transport via added functional groups or nanoparticles into the composite structure of the electrode. Conducting polymers as supporting matrix are usually used as copolymers or composite films in biosensor systems since mechanical and processing properties of their homopolymers are weak (Tsai & Chui, 2007; Heras et al., 2005; Carvalho et al., 2007; Serra et al., 2001; Mailley et al., 2003). Copolymerization does not require rigorous experimental conditions, and can be employed for the polymerization of a large variety of monomers leading to the formation of new advantageous materials (Böyükbayram et al., 2006; Kuwahara et al., 2005; Yilmaz et al., 2004; Yilmaz et al., 2005). Nanomaterials have also been used to improve the operational characteristics of biosensors (Yang et al., 2006; Zhou et al., 2007; Rajesh et al, 2005; Shan et al., 2007). This improvement

results from both increased surface area and increased catalytic activity. Carbon nanotubes (CNTs) have emerged as a new class nanomaterials that are receiving considerable interest owing to their ability to promote electron transportation (Zhao et al., 2006; Chen et al, 2007; Zeng et al., 2007; Liu et al., 2006; Vega et al., 2007; Santos et al., 2007). The high conductivity of this carbon material leading to a level of 10^2 $\Omega^{-1}cm^{-1}$ improves electrochemical signal transduction, while its nano-architecture imposes the electron contact between redox centers, deeply inlaid in enzyme structure, and the smooth surface of the electrode.

In this chapter, we reported HRP-based amperometric phenol biosensors, which were comprised of working electrodes prepared in various designs, developed in order to get reliable, selective, sensitive and fast detection of phenol and its derivatives. Various compositions of polymeric/composite films were synthesized onto the surface of the electrodes. Various supporting matrix, designed target specific, were used for the fabrication of some of these polymeric/composite films. We are planning to cover a detailed investigation and discussion of the enzyme based working electrodes with regard to the response dependences as well as their amperometric characteristics including sensitivity, linear range, detection limit, relative standard deviation and reproducibility of the composite film electrodes.

2. General principle of enzyme-based amperometric biosensors

Amperometric biosensors are analytical devices in which a biological material is used as a biological catalyst in combination with an electrical transducer. A biosensor responds to an analyte in a sample and interprets its concentration as an electrical signal via a biological recognition system and the electrochemical transducer. Amperometric biosensors possess linear concentration dependence, compared to a logarithmic relationship in potentiometric systems and measure change in the current on the working electrode due to the direct oxidation of the products of a biochemical reaction. Electrochemical biosensors have been under development for 40 years, and over this time a wide variety of sensors has been developed. The overriding theme of biosensors is the ability to perform selective biological recognition of the target analyte in a complex sample matrix and couple this to sensitivity of electrochemical detection. The magnitude of the response of amperometric biosensors depends on a number of factors, including the kinetics of the enzymatic reaction, the construction, and the operation mode of the enzyme electrode. The response from the electrode can either be diffusionaly or kinetically controlled. With kinetically controlled enzyme electrodes, the enzyme loading is sufficiently low that the response depends on the enzyme concentration and the kinetics of the enzymatic reaction. Such behaviour has limited analytical utility as response saturation occurs at low substrate concentration. The diffusionaly controlled electrode possesses very high enzyme loadings such that the current is independent of small changes in enzyme concentration; consequently, the current response is a function of analyte concentration and diffusion. Enzyme electrodes can be operated in several measurement modes: dynamic steady-state, potential step, and flow-injection mode. The steady-state mode allows reaction equilibrium to be reached before the analytical signal is obtained, whereas in dynamic measurement the signal is obtained quickly as a predetermined timepoint after introduction of the sample. Both potential step and flow-injection measurements are transient responses due to the transient nature of the techniques (Diamond, 1998).

2.1 HRP-based amperometric phenol biosensors

Amperometric biosensors for the detection of phenolic compounds have been introduced as a mono-enzyme system using tyrosinase, laccase or HRP. Tyrosinase biosensors are restricted to the monitoring of phenolic compounds having at least one *ortho*-position free. On the other hand, laccase biosensors give response to phenolic compounds with free *para*- and *meta*-position with a complicated catalytic cycle. HRP having less selectivity to phenolics is capable of giving response to a large number of phenol derivatives, and shows a high stability and efficiency for different biosensor designs. HRP was oxidized by hydrogen peroxide and re-reduced by phenols. Phenoxy radicals, formed during the enzymatic oxidation of phenolic compounds in the presence of hydrogen peroxide, were reduced electrochemically on the electrode surface; the reduction current is proportional to concentration of phenolic compound (Korkut el al., 2008; Korkut Ozoner et al., 2011).

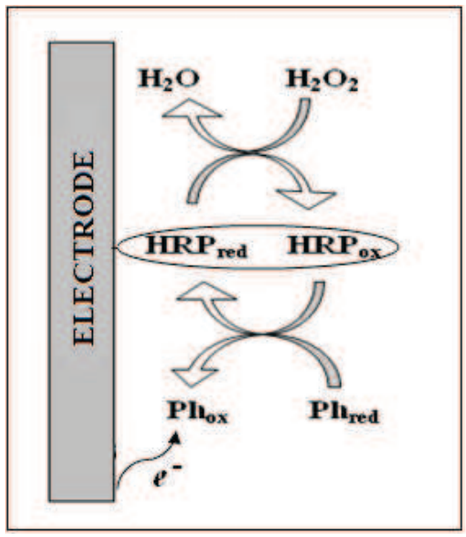

Fig. 1. The electrochemical reaction between HRP and phenol on electrode surface.

2.2 Polymers for working electrodes

To achieve high biosensor performance, it is very necessary to fabricate excellent electrode support materials for both effective immobilization of enzyme and fast electron transport between enzyme and metallic electrode. Electropolymerizable conducting polymers are generally used as supporting matrix for working electrodes. Among the conducting polymers, polythiophene has a special place due to their electrical properties, rich synthetic flexibility, and environmental stability in doped and undoped states, non-linear optical properties, and highly reversible redox switching. Synthesis of a thiophene-functionalized methacrylate monomer [3-methylthienylmethacrylate (MTM)] via the esterification of 3-thiophene methanol with methacryloyl chloride can be prepared. Thus, the MTM monomer obtained has two polymerizable groups: the vinyl group is useful for radical polymerization while the thiophene ring, with substitution at the 3-position, can be employed in both oxidative polymerization and electropolymerization. It is also possible to prepare block and random copolymers of MTM with other acrylic or vinyl monomers at different compositions. Subsequently, constant-potential electrolyses can be employed for the synthesis of the graft copolymers of the side chain thiophene (Depoli et al., 1985).

Copolymerization is the most effective and successful way among the existing polymerization techniques for incorporation of systematic changes in polymer properties. It does not require rigorous experimental conditions, and can be employed for the polymerization of a large variety of monomers leading to the formation of new materials. Reactive functional polymers can be prepared by incorporation of acrylates and methacrylates monomers containing side chain reactive functional groups into polymers. Various architectures of epoxy group possessing polymers have been developed in the literature. Copolymers of glycidyl methacrylate (GMA), an epoxy group containing methacrylate monomer, have received great interest. Epoxide is a three-membered cyclic ether and very reactive due to the large strain energy (about 25 kcal mol^{-1}) associated with the three-membered ring. Therefore, it can be employed into a large number of chemical reactions by ring opening. Various applications of chemically modified pendant copolymers, such as immobilization of enzymes, DNA, catalysts, and biomolecules, were reported (Hradil & Svec, 1985; Lukas & Kalal, 1978).

2.3 Working electrode fabrications

Polymeric coatings can be applied to a wide range of electrode support materials. Electrodes covered with polymeric coatings have thicker layers which, besides increasing the flexibility for choosing the coating material, permit to obtain a higher surface coverage and therefore to increase the amount of electroactive material attached to the surface. Polymeric coatings can be formed by electropolymerization of monomers or by solution casting of preformed polymers. Electropolymerization, a recent focus among immobilization strategies, is an electrochemical route to form polymeric coatings by entrapment of biomolecules and involves the application of an appropriate potential to a working electrode immersed in an aqueous solution containing the electropolymerizable monomer and enzyme, which is homogeneously incorporated in the growing polymer. The enzyme/polymer interaction is of paramount importance to improve the fundamental knowledge about the biological interface of the biosensor. So far, difficulties to understand the exact mechanism of entrapment and the dynamic effects on biosensors partially result from a scarcity of reports comparing different polymer matrix for immobilization of the same enzyme. In the entrapment technique which is easy and rapid one-step procedure, enzyme does not link onto the polymeric structure, and can act as it is in its free form in the pores during/after electropolimerization process. However, biological activity of the entrapped enzyme decreases probably due to the hydrophobic character of polymers and the steric hindrances caused by the surrounding polymer, which drastically reduces the accessibility to the immobilized biomolecules. Enzymes can also be chemically immobilized to a polymer matrix basically in a two-step process: a polymer film containing functional groups for enzyme immobilization is formed on electrode surface, then the electrode is dipped in enzyme solution or the enzyme solution is dropped onto the surface of the electrode. Covalent bindings are stronger and therefore less prone to biomolecule detachment, thus increasing the stability of the linkage. As an alternative to electropolymerization, polymer coatings can also be formed by casting of films from solution using preformed polymers. In this way, the amount of material on the electrode surface can be controlled by the concentration and the amount of polymer solution applied. The actual layer thickness is, however, less well defined than in electropolymerization, an important issue as often the analytical signal will depend on the thickness of the modifying layer.

3. Experimental procedures

3.1 Chemicals

Horseradish peroxidase (E.C.1.11.1.7) with an activity of 10 000U $vial^{-1}$ (according to pyrogallol method performed by the supplier), aqueous solution of hydrogen peroxide (30%), glutaraldehyde (25%), lithium chloride, dichloromethane (DCM), N,N-dimethyl formamide (DMF), α,α'-Azobisisobutyronitrile (AIBN), 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. Tetrahydrofuran (THF) was obtained from Riedel. 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 monomer (99%), sodium dodecyl sulfate (SDS) and 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-tolueno-sulfonate were obtained from Sigma. Stock solutions of various phenols were daily prepared in 0.1 M, pH 7 phosphate buffer solution. Multiwalled carbon nanotubes (MWCNTs) were obtained from Nanocs. Inc., Newyork, USA.

3.2 Synthesis of Poly(glycidyl methacrylate-*co*-3-thienylmethyl methacrylate) {Poly(GMA-*co*-MTM)}

Side chain thiophene containing monomer, 3-thienylmethylmethacrylate (MTM) was synthesized according to the previously reported papers (Yilmaz et al., 2004; 2005).

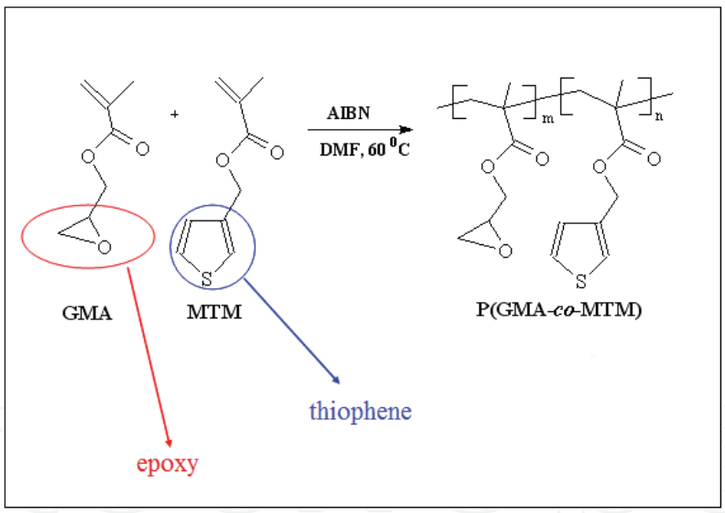

Fig. 2. Synthesis of Poly(GMA-*co*-MTM).

We have previously reported the copolymers of MTM with glycidyl methacrylate (GMA) and monomer reactivity ratios were determined for low conversion using Fineman Ross (FR) (r_{GMA}= 0:9795; r_{MTM}= 0:5641) and Kelen Tüdős (KT) (r_{GMA} = 0:9796; r_{MTM} =0:5771) graphical methods (Gunaydın & Yilmaz, 2007). Poly(GMA-*co*-MTM) was synthesized via radical polymerization of appropriate GMA/MTM feed mixture in the presence of AIBN as an initiator. Predetermined quantities of MTM, GMA and AIBN (1% of total weight of monomers) in DMF with a volume of 1.5 mL were placed in a Pyrex tube. The mixture was deoxygenated by flushing with oxygen-free argon for at least 15 min. The tube was tightly sealed and immersed in a thermostated oil bath at 60 ± 1°C. The conversion was determined

by gravimetric measurements. After the reaction, copolymer was precipitated in methanol, filtered off, and purified by reprecipitation from DCM solution into methanol and finally dried in vacuo for 24 h. The solution of Poly(GMA-*co*-MTM) was prepared in THF solvent.

3.3 Amperometric measurements

Amperometric measurements were performed by using a CHI Model 840B electrochemical analyzer. A gold working electrode (2 mm diameter), a glassy carbon working electrode with a diameter of 3 mm for batch measurements, 2 mm for flow injection analyses (FIA), a Platinum wire counter electrode, a Ag/AgCl (3M NaCl) reference electrode, and a conventional three-electrode electrochemical cell were used in the experiments. Measurements of phenolic compounds were carried out in 0.1 M, pH 7 phosphate buffer in the presence of 0.7 mg mL^{-1} lithium chloride with an applied working potential of -50 mV.

3.4 Experimental setup

3.4.1 FIA system

FIA system was set up with an HPLC pump (GBC LC1120), an injection valve (Shimadzu) and a flow cell including three-electrode system. The HPLC pump was adjusted to deliver a carrier solution at a constant flow rate. Potassium phosphate buffer was used as carrier solution. Samples were injected into the carrier solution, passing from the flow cell, by an injection valve with a volume of 1 mL. A sharp current peak was formed for each phenolic injection at a working potential of -50 mV.

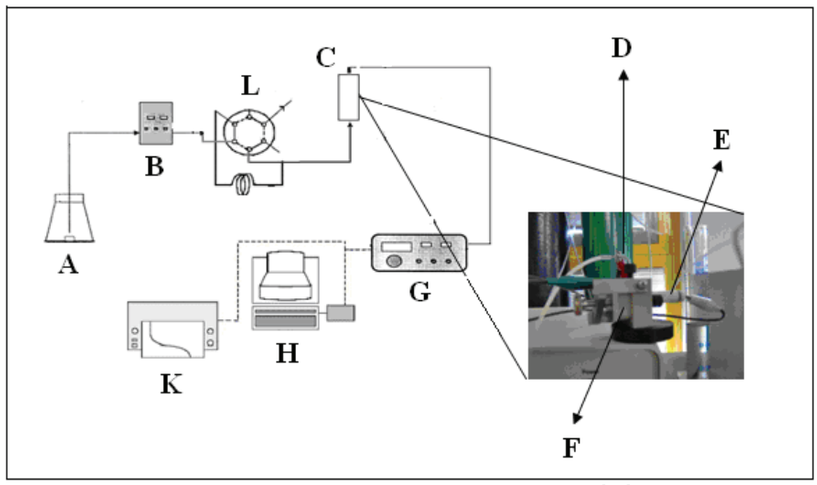

Fig. 3. Schematic diagram of the FIA system: carrier solution (A), HPLC pump (B), injection valve (L), flow cell (C), Pt counter electrode (D), Ag/AgCl reference electrode (E), glassy carbon working electrode (F), potentiostat (G), computer (H) and data recorder (K).

3.4.2 Batch system

Electrochemical batch measurements were carried out in 10 mL of potassium phosphate buffer with a continuous stirring at 600 rpm in three-electrode cell. Three-electrode system was immersed into the electrochemical cell, a working potential of -50 mV was applied and current was allowed to reach a steady-state value then, various concentrations of phenolic compounds were added into the cell to produce i-t curves of amperometric measurements.

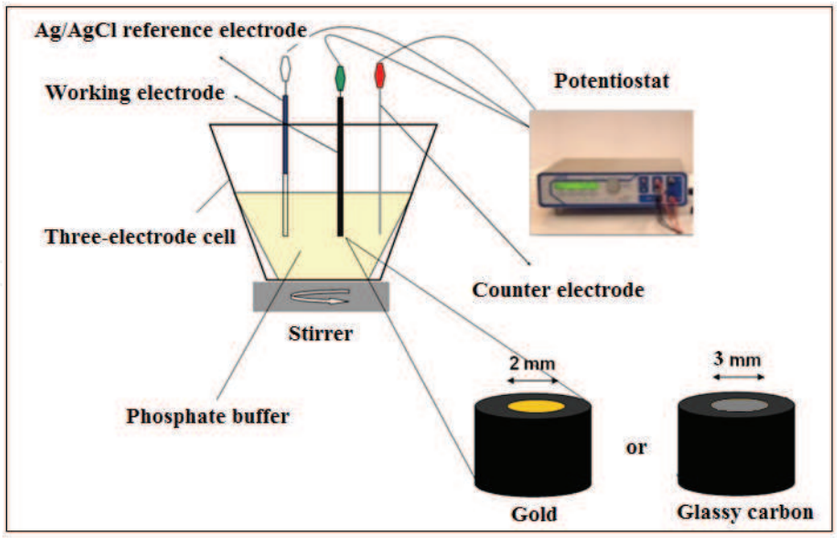

Fig. 4. Schematic diagram of the batch system.

3.5 Fabrication of the working electrodes

3.5.1 Poly(glutaraldehyde-*co*-pyrrole)/HRP {Poly(GA-*co*-Py)/HRP} composite film electrode

The composite film electrode was used in FIA system. Poly(glutaraldehyde) solution was prepared by adding 2 mL of 0.1 M NaOH and 2 mL of 25% glutaraldehyde to 10 mL of distilled water. The solution was stirred at 600 rpm for 30 minutes in order to polymerize glutaraldehyde. The final pH must be 9-10. The copolymerization medium was comprised of 0.01 M pyrrole and 0.6 mg mL^{-1} SDS in 10 mL of prepared PGA solution. This medium was circulated through the flow cell using the HPLC pump under a potential scan between 0 and +1.2 V with the scan rate of 100 mV s^{-1}. Then the copolymerized film coated electrode was immersed into 25% glutaraldehyde solution to increase the number of aldehyde groups in the composite film of Poly(glutaraldehyde-*co*-pyrrole) {Poly(GA-*co*-Py)}, and stored at +4°C overnight. The electrode was washed potassium phosphate buffer, and immersed in 0.3 mg mL^{-1} HRP solution for 20 hours. Finally, the electrode was washed again with buffer to remove excess HRP.

3.5.2 Carbon nanotube/Polypyrrole/HRP (CNT/PPy/HRP) nanocomposite film electrode

A modified acid oxidative method was used for preparation of water-soluble CNTs (Zhao et al., 2002). 14 mg of MWCNTs were added into 5 mL of a 9:1 concentrated H_2SO_4/H_20_2 (30%) solution and stirred for 30 min for CNTs oxidation. After the reaction, 15 mL of the 9:1 concentrated H_2SO_4/H_20_2 solution was added into the mixture. The mixture was placed in an ultrasonic bath and sonicated for 5 min. Resulting CNTs dispersion was diluted using 1 L of distilled water and filtered through a 0.45 μM cellulose membrane. The filtrate was washed with 0.01 M NaOH solution and distilled water till the pH level reaching to 7 and dispersed in distilled water (0.03 mg L^{-1}). The resulting CNTs solution was sonicated for 2 min to obtain a homogeneous CNTs solution. Nanocomposite film was formed onto the surface of the gold electrode by immersing the electrode to an electropolymerization medium contained 5 mL of oxidized CNTs solution, 5 mL of 0.05 M pH 6.5 citrate buffer,

0.01 M pyrrole, 0.6 mg L^{-1} SDS and 0.3 mg L^{-1} HRP under a potential scan between 0 and +1.2 V for 4 minutes at a scan rate of 100 mVs^{-1}.

3.5.3 Poly(glycidyl methacrylate-*co*-3-thienylmethyl methacrylate)-Polypyrrole-Carbon nanotube-HRP {Poly(GMA-*co*-MTM)/PPy/CNT/HRP} composite film electrode

6 mg of Poly(GMA-*co*-MTM) was dissolved in 10 mL THF. The polymer solution with a volume of 20 µL was directly spread onto the surface of a gold electrode. The electrode was then allowed to dry for solvent evaporation at room temperature. Poly(GMA-*co*-MTM) coated electrode was dipped into the electropolymerization medium contained 5 mL of oxidized CNTs solution, 5 mL of 0.05 M pH 6.5 citrate buffer, 0.01 M pyrrole, and 0.6 mg L^{-1} SDS under a potential scan between (-1.2) – (+1.2) V for 4 minutes with a scan rate of 100 mVs^{-1}. Poly(GMA-*co*-MTM)/PPy/CNT electrode was pre-treated at a potential of +2 V vs Ag/AgCl for 5 minutes in 0.1 M, pH 7 phosphate buffer. The electrode was then allowed to react for 3.5 hours within the solution of 5 mg L^{-1} 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-tolueno-sulfonate with a continuous stirring at 200 rpm. The electrode was dipped into a solution of 0.3 mg L^{-1} HRP, dissolved in 0.1 M, pH 7 phosphate buffer, and stored at +4°C overnight.

3.5.4 Poly(glycidyl methacrylate-*co*-3-thienylmethyl methacrylate)-Polypyrrole-HRP {Poly(GMA-*co*-MTM)/PPy/HRP} composite film electrode

20 µL of 0.6 mg L^{-1} of Poly(GMA-*co*-MTM) was directly spread onto the surface of the polished glassy carbon electrode. After the solvent evaporation polymer coated electrode electropolymerized with polypyrrole in a polymerization medium contained 10 mL of 50 mM pH 6.5 citrate buffer including 0.01 M pyrrole, 0.6 mg mL^{-1} SDS and 0.6 mg mL^{-1} of HRP at a potential scan between (-1.2) – (+1.2) V for 4 minutes at a scan rate of 100 mVs^{-1}.

4. Results and discussions

4.1 FIA of phenols by using Poly(GA-*co*-Py)/HRP composite film electrode

Various concentrations of *p*-benzoquinone, catechol and phenol ranging between 75 µM and 750 µM with 1.5 mM hydrogen peroxide were injected to the carrier solution at a flow rate of 1 mL min^{-1} (Fig. 5). No reproducible response was obtained for phenol from Poly(GA-*co*-Py)/HRP composite film electrode, and the best response was observed for catechol as model phenolic. The difference in response among the phenolic compounds depends on the different affinity of HRP towards its substrates, and the formation of *o*-quinones during the enzymatic reaction for each phenolic (Tsai & Cheng-Chui, 2007).

Effect of flow rate was investigated on the FIA system response to a series of *p*-benzoquinone injections at different flow rates ranged between 0.25-6 mL min^{-1} (Fig. 6). It was observed that the obtained peak currents decreased as a consequence of the short retention time of the substrate with the enzyme depending on the increase of the flow rate. In addition, unstable signals were shaped with the increasing flow rate due to the unsteady flow conditions through the composite film. The flow rate in the FIA system affected the sample throughput, detection limit and accuracy. The disadvantages of lower flow rates were low sample throughput and an increase in dispersion. By using a higher flow rate, a greater number of samples could be analyzed, and the peaks became narrower, but the detection limit increased and unstable responses were observed.

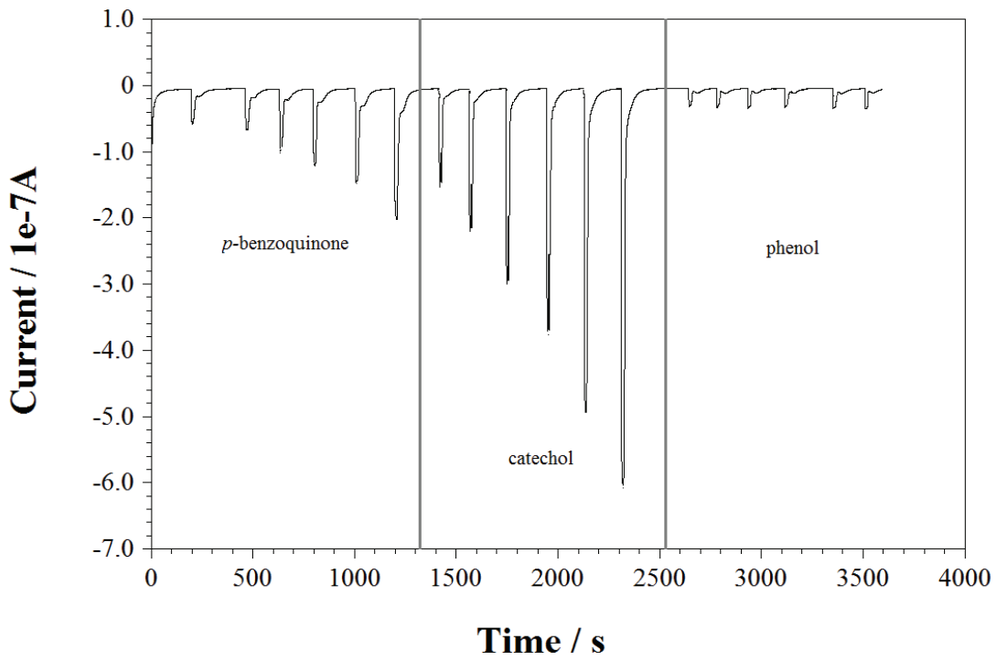

Fig. 5. Poly(GA-*co*-Py)/HRP composite film electrode response to 75-125-200-300-500-750 µM *p*-benzoquinone, catechol and phenol injections. Applied potential was -50 mV (vs. Ag/AgCl, 3 M NaCl).

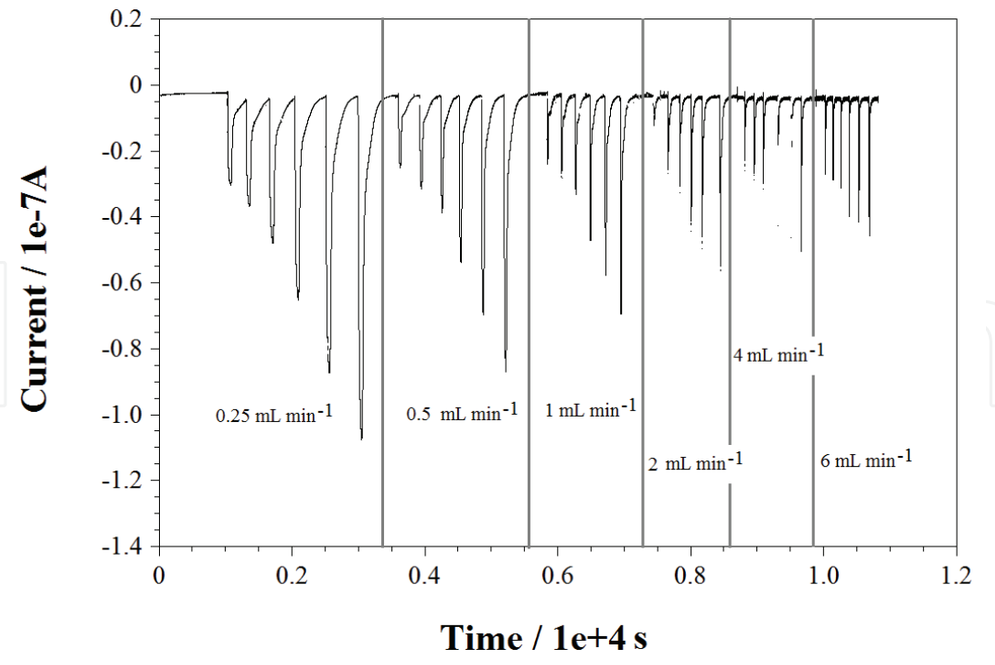

Fig. 6. Effect of 0.25-0.5-1-2-4-6 mL min^{-1} flow rate on Poly(GA-*co*-Py)/HRP composite film electrode response to 75-125-200-300-500-750 µM *p*-benzoquinone injections. Applied potential was -50 mV (vs. Ag/AgCl, 3 M NaCl).

Stability of Poly(glutaraldehyde-*co*-pyrrole)/HRP composite film electrode was evaluated by the 20 repetitive analyses of *p*-benzoquinone at a concentration of 2.5 μM recorded at 1 min intervals over a prolonged period. Well-defined reduction responses were obtained with a standard deviation of ±0.23 nA. Poly(GA-*co*-Py)/HRP composite film electrode could be used for one month without loosing its initial response. The high operational and storage stability of the electrode can be a result of removing of the enzymatic phenolic products by continuous flow. Owing to the copolymerization of pyrrole with glutaraldehyde, a sufficient electron transfer was provided between enzyme and the electrode since PGA contains conjugated electroactive aldehyde groups. These active aldehyde groups incorporated to the conductive polymeric backbone by the copolymerization with pyrrole. Therefore, strong chemical bonds were formed between HRP and the copolymeric film via the aldehyde groups of the copolymer. There have been a few reports of FIA phenol biosensor in literature. Poly(GA-*co*-Py)/HRP composite film electrode showed lower detection limit and wider linear range in comparison to those reports (Table 1). This can be attributed to the electrocopolymerization of glutaraldehyde with pyrrole monomer since the aldehyde groups of PGA both electroactive and capable to bind the enzyme chemically.

Analyte	**Biosensor**	**Detection limit (μM)**	**Linear range (μM)**	**Reference**
p-Cresol	Laccase/Graphite	39	10-1000	(Wilkolaza et al., 2005)
4-Chlorophenol	Laccase/Graphite	346	1000-10000	(Wilkolaza et al., 2005)
Hydroquinone	Laccase/Graphite	0.58	1-10	(Wilkolaza et al., 2005)
Hydroquinone	Laccase/ECH Sepharose	-	0-500	(Vianello et al., 2006)
4-Aminophenol	Laccase/Graphite	0.61	1-10	(Wilkolazka et al., 2005)
4-Methoxyphenol	Laccase/Graphite	7.9	1-100	(Wilkolazka et al., 2005)
p-Benzoquinone (*at a flow rate of 1mL min*$^{-1}$)	Poly(GA-*co*-Py)/HRP	2	2.5-750	This study

Table 1. Analytical parameters of some FIA biosensors for phenolic compounds.

4.2 Amperometric detection of phenolic compounds in batch operation

4.2.1 CNT/PPy/HRP nanocomposite film electrode

Electropolymerization CVs of CNT/PPy/HRP nanocomposite film and PPy/HRP (without CNT) electrode were shown in Fig. 7. Typical polypyrrole voltammograms were obtained for both electrodes. Oxidation current of pyrrole was much higher for CNT/PPy/HRP nanocomposite film electrode than the other electrode fabricated without CNT. This can be attributed to the enhanced pyrrole oxidation process since the electron transfer mechanism was facilitated by the incorporation of CNT into the PPy film structure.

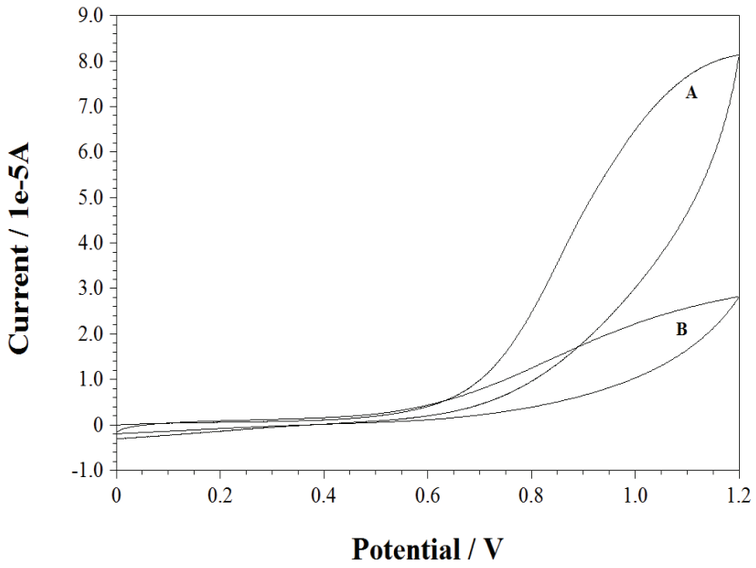

Fig. 7. Cyclic voltammogram of CNT/PPy/HRP (A), and PPy/HRP (B) electrode at a scan rate of 100 mV s^{-1}.

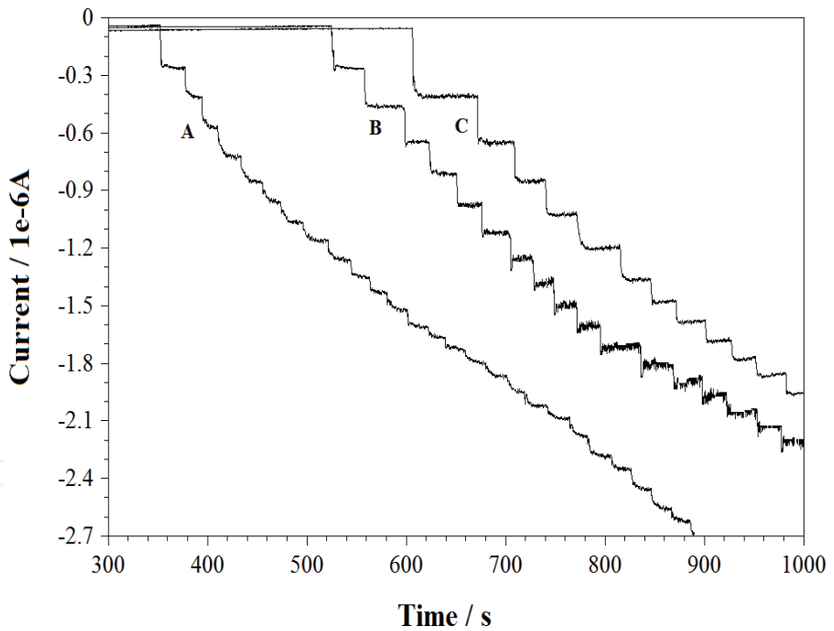

Fig. 8. Amperometric response of CNT/PPy/HRP electrode to 4-methoxyphenol (A), hydroquinone (B) and 2-aminophenol (C) additions.

Eighteen phenolics were tested for CNT/PPy/HRP nanocomposite film electrode with an applied potential of -50 mV. Fig. 8 illustrates typical amperometric responses of the electrode after the addition of successive aliquots of some of the phenolic compounds under a constant stirring at batch operation. Table 2 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 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 phenolics. It was previously reported that the phenolic compounds with electron-donor substituents in an *ortho*-position gave no response (Kane & Iwuoha, 1998). CNT/PPy/HRP nanobiocomposite film electrode did not give any response to *o*-cresol. The highest sensitivity was obtained for 4-methoxyphenol since 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 1-50 nA μM^{-1} for the phenolics tested.

Analyte	r	Sensitivity (nA μM^{-1})	Linear range (μM)	LOD (μM)	%RSD
Phenol	0.99	1	16-144	3.52	2.89
p-Benzoquinone	0.99	3	0.02-0.16	0.027	4.43
Hydroquinone	0.99	8	16-240	6.42	6.5
2,6-Dimethoxyphenol	0.99	7	1.6-19.2	0.29	1.8
2-Chlorophenol	0.99	8	1.6-8	0.26	1.7
3-Chlorophenol	0.99	6	1.6-12.8	0.2	1.1
4-Chlorophenol	0.99	8	1.6-14.4	0.3	1.87
2-Aminophenol	0.99	40	8-60.8	1.53	5.4
4-Methoxyphenol	0.99	50	1.6-81.6	1.06	2.8
Pyrocatechol	0.99	8	1.6-446.4	6.27	6.7
Guaiacol	0.98	9	1.6-9.6	0.3	1.92
m-Cresol	0.99	9	8-20.8	1.5	2.84
o-Cresol			no response		
p-Cresol	0.98	5	128-832	24	2.5
Catechol	0.98	2	1.6-8	0.93	3.8
4-Acetamidophenol	0.99	3	1.6-16	1.11	2.57
Pyrogallol	0.98	1	1.6-22.4	1.24	1.2
2,4-Dimethylphenol	0.98	1	64-240	27.9	2.2

Table 2. Analytical characteristics of CNT/PPy/HRP nanobiocomposite film electrode for various phenolic compounds. Applied potential; -50 mV, 0.1 M phosphate buffer (pH 7) containing 16 μM hydrogen peroxide.

Fig. 9 illustrates the amperometric responses of CNT/PPy/HRP and PPy/HRP working electrodes to increasing concentrations of hydroquinone additions into the 0.1 M, pH 7 phosphate buffer at an applied potential of -50 mV (vs. Ag/AgCl). No reproducible signals were observed for PPy/HRP electrodes. 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 (Gorton et al., 1992). However, in this study, the available responses could only be obtained by CNTs-based electrode due to its ability to promote electron transfer reaction

with HRP. 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 previously reported procedure (Vojinovic et al., 2004). 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. HRP was mainly entrapped into the polymeric film structure during the pyrrole electropolymerization process, and chemically linked via the carboxylated groups of CNTs.

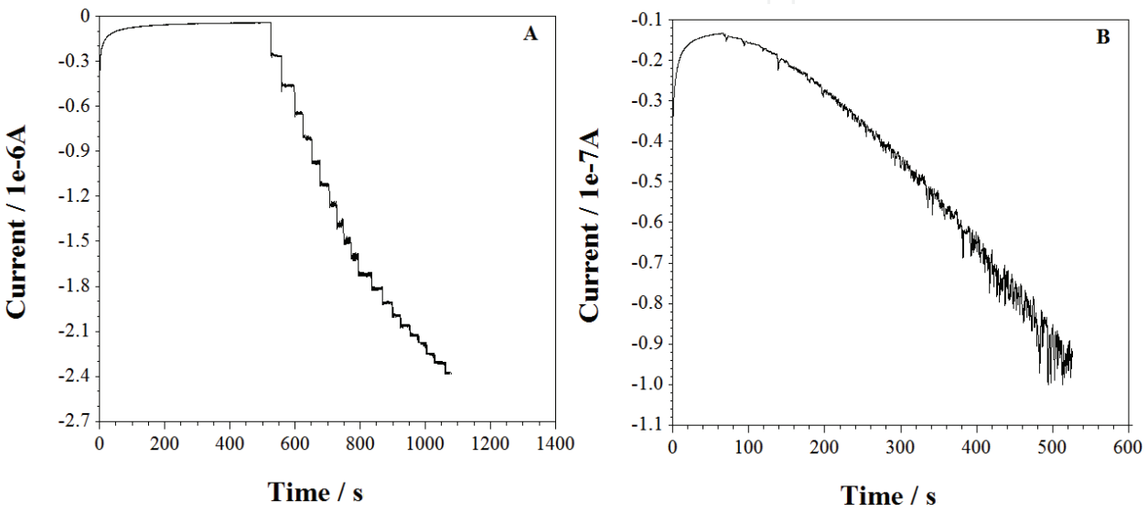

Fig. 9. Amperometric responses of CNT/PPy/HRP (A) and PPy/HRP working electrode (B) to the successive additions of hydroquinone.

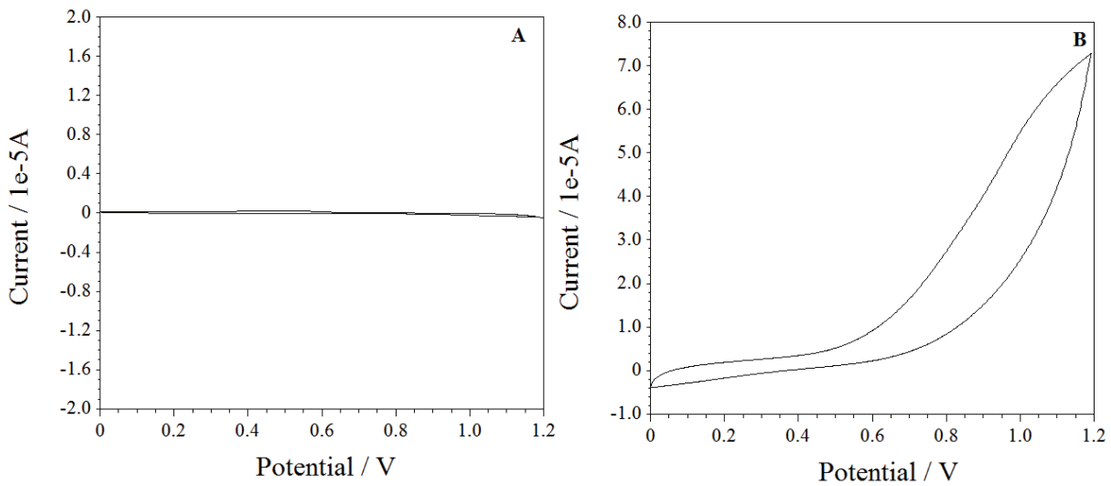

Fig. 10. Cyclic voltammogram of Poly(GMA-*co*-MTM) film electrode (A) and Poly(GMA-*co*-MTM)/PPy/CNT/HRP composite film electrode (B) in 0.1 M, pH 7 phosphate buffer at a scan rate of 100 mVs^{-1}.

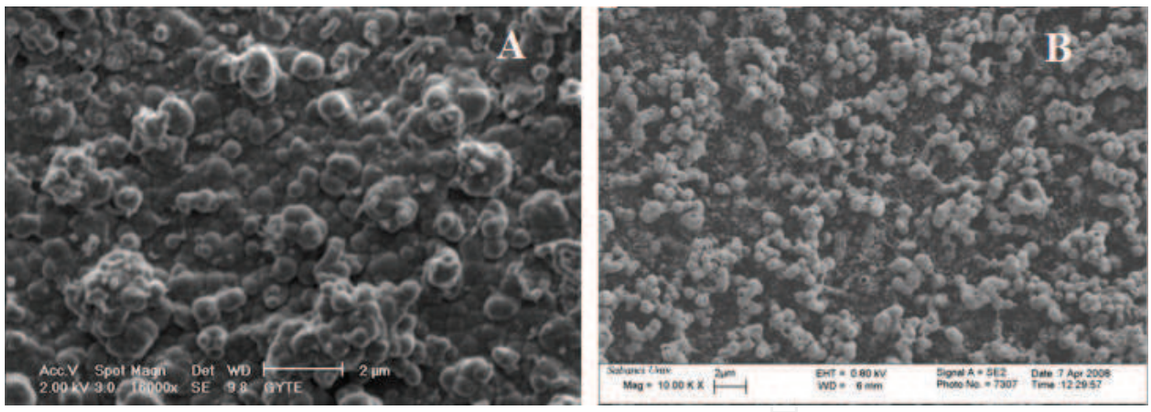

Fig. 11. SEM images of Poly(GMA-*co*-MTM) film (A) and Poly(GMA-*co*-MTM)/PPy/CNT composite film (B).

4.2.2 Poly(GMA-*co*-MTM)/PPy/CNT/HRP composite film electrode

The electrochemical properties of the composite electrodes of Poly(GMA-*co*-MTM) (Fig. 10A) and Poly(GMA-*co*-MTM)/PPy/CNT (Fig. 10B) were evaluated through cyclic voltammetry in 10 mL of 0.1 M phosphate buffer solution (pH 7) contained 0.7 mg mL^{-1} of lithium chloride. CV obtained with Poly(GMA-*co*-MTM) film electrode revealed, in both scan directions, that no voltametric peak in the scanning potential range (0 to 1.2 V vs. Ag/AgCl) is obtained. It means that the thiophene groups on the copolymer did not show any electroactivity. In the case of pyrrole present in the system, the usual pyrrole polymerization peaks were drastically shifted (Fig. 10B). It is an indication for the electropolymerization reaction between pyrrole and the thiophene moiety of the copolymer.

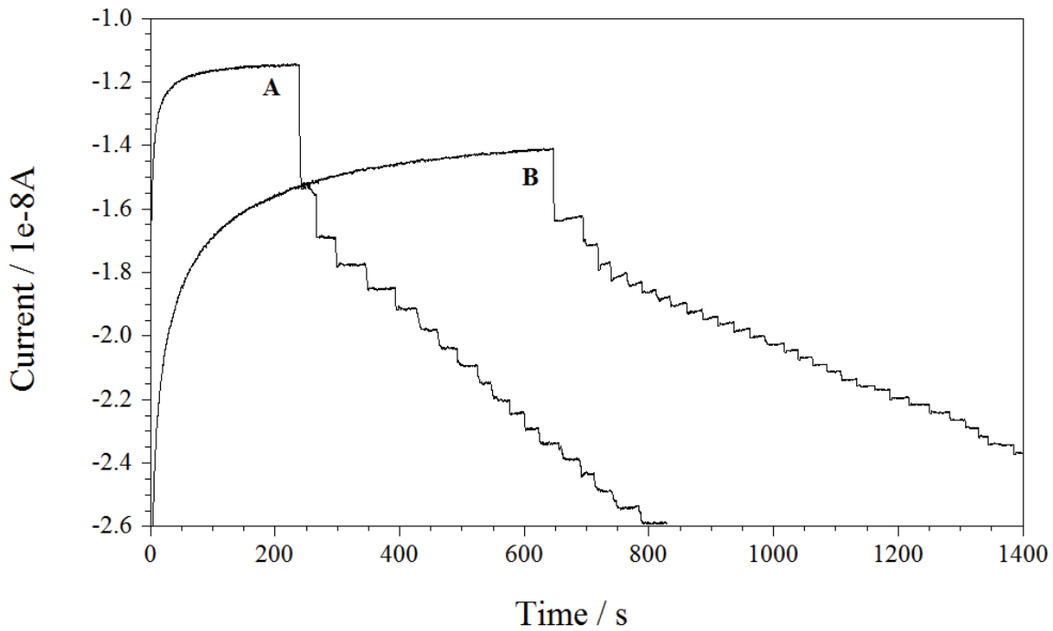

Fig. 12. Amperometric response of Poly(GMA-*co*-MTM)/PPy/CNT/HRP composite film electrode to the successive additions of guaiacol (A) and pyrogallol (B).

Fig. 12 shows amperometric response of Poly(GMA-*co*-MTM)/PPy/CNT composite film electrode to some of the phenolics. The biosensor responded rapidly to the concentration increments of the various phenolics. Rapid response indicates a fast electron exchange between HRP and its substrates, indicating that the catalytic properties of the enzyme were not hindered by Poly(GMA-*co*-MTM)/PPy/CNT/HRP composite film. Analytical parameters were presented in Table 3. Wide linear range was observed for 2-chlorophenol (1.6-68.8 μM), 3-chlorophenol (1.6-81.6 μM) and 4-chlorophenol (1.6-86.4 μM) with the correlation coefficient of 0.999. The biosensor was also tested by the phenolics recovery experiments, which showed satisfactory results, with recoveries from 95% to 107% for the all tested phenolics. The available responses could only be obtained by the copolymeric film of Poly(GMA-*co*-MTM)/PPy. No reproducible response was observed by the electrode only coated with Poly(GMA-*co*-MTM) since it was not an electroactive polymer.

Analyte	**r**	**Sensitivity (nA μM^{-1})**	**Linear range (μM)**	**LOD (μM)**	**%RSD**
Phenol	0.99	0.7	1.6-72	0.732	7.5
p-Benzoquinone	0.99	5	1.6-25.6	0.409	13
Hydroquinone	0.99	9	1.6-25.6	0.336	8.8
2,6-Dimethoxyphenol	0.99	0.8	1.6-36.8	0.382	8.38
2-Chlorophenol	0.99	1	1.6-68.8	0.249	4.7
3-Chlorophenol	0.99	1	1.6-81.6	0.441	9.9
4-Chlorophenol	0.99	1	1.6-86.4	0.336	6.9
2-Aminophenol	0.99	2	1.6-44.8	0.247	6.6
4-Methoxyphenol	0.99	2	1.6-35.2	0.312	6.35
Pyrocatechol	0.99	1	1.6-49.6	0.516	11
Guaiacol	0.99	0.3	3.2-52.8	0.490	10
m-Cresol			no response		
o-Cresol			no response		
p-Cresol			no response		
Catechol	0.99	2	1.6-44.8	0.304	7.5
4-Acetamidophenol	0.99	3	1.6-22.4	0.624	12
Pyrogallol	0.99	0.1	4.8-48	0.660	11
2,4-Dimethylphenol	0.99	0.4	1.6-40	0.382	7.8

Table 3. Analytical characteristics of Poly(GMA-*co*-MTM)/PPy/CNT/HRP composite film electrode for various phenolic compounds. Applied potential; -50 mV, 0.1 M phosphate buffer (pH 7) containing 16 μM hydrogen peroxide.

The most possible linkages between HRP and the functional groups of the composite film were C-N bonds. The enzyme HRP was chemically immobilized via the epoxy groups of the Poly(GMA-*co*-MTM) and the carboxyl groups of the CNTs. The bonding mechanisms are illustrated in Fig. 13. Theoretically, it is possible for an enzyme molecule to bind to the

composite film through the two different mechanisms simultaneously. Such multiple linkages might be resulted an increased steric hindrance on the enzyme molecule (Korkut Ozoner et al., 2010; Bayramoğlu & Yakup Arıca, 2008). Moreover, Kobayashi et al. 2005 reported that their results suggested the magnitude of the effect of steric hindrance depended on the disubstitution of phenol derivatives (Kobayashi et al., 2005). It is the fact that the enzyme molecules directly bond onto the CNTs, acting as an electron transferring bridge or a wire, may stabilize the microenvironmental conditions for the desired electrochemical reaction. Hence, the enzyme was also immobilized chemically to the composite film of Poly(GMA-*co*-MTM)/PPy/CNT supported by a conductive copolymer.

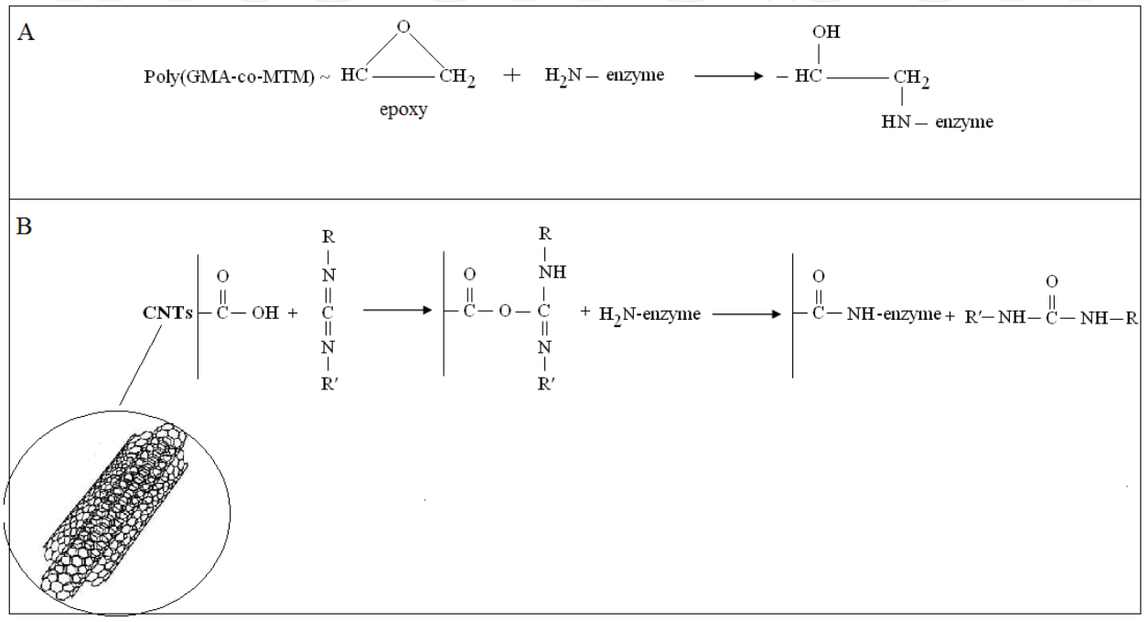

Fig. 13. The proposed immobilization mechanism of HRP on Poly(GMA-*co*-MTM)/PPy/CNT composite film via the epoxy groups of Poly(GMA-*co*-MTM) (A) and via the carboxyl groups of CNTs (B).

4.2.3 Poly(GMA-*co*-MTM)/PPy/HRP composite film electrode

The typical amperometric responses and the calibration curves of the electrode F are illustrated in Fig. 14 and Fig. 15, respectively after the addition of successive aliquots of phenolic compounds at an applied potential of -50 mV under continuous stirring at 600 rpm. Poly(GMA-*co*-MTM)/PPy/HRP composite film electrode reached to the steady-state current of 95% in less than 3 s.

Table 4 summarizes the characteristics of the calibration plots obtained from the current-time recordings of phenol derivatives. The detection limit ranged between 0.13 and 1.87 µM for the tested phenol derivatives. The different sensitivities varied between 3-200 nA μM^{-1} for the tested phenolics can be related to the formation of *o*-quinones during the enzymatic reaction. The maximum sensitivity was found to be 200 nA μM^{-1} for hydroquinone. In addition to this, 4-methoxyphenol and 4-acetamidophenol showed higher sensitivity than the other phenolics. This can be dialed with the presence of $-OCH_3$ group of 4-

methoxyphenol which enhances oxidation of the phenolic by HRP. Due to the strong ability of electron-donor conjugation of hydroquinone and 4-acetamidophenol, the corresponding conjugation structure could be easily formed. The higher sensitivity can be attributed to the favorable microenvironment of the immobilization matrix and enzyme immobilization procedure, which was performed by both chemical bonding via the epoxy groups and entrapment during the electropolymerization step. However, the type of the electrode material played an important role on the value of the sensitivity. Glassy carbon electrodes (GCEs) have been widely used compared with metal electrodes due to its biocompatibility with tissue, having low residual current over a wide potential range and minimal propensity to show a deteriorated response as a result of electrode fouling (Jin et al., 2008). Recently reported papers have stated that HRP is more compatible with carbon electrode materials (Santos et al., 2007; Carvalho et al., 2007; Huang et al., 2008). Rabinovich and Lev have claimed that the response of a phenol biosensor is usually limited by the electrochemical back reduction of the quinone leading to the diphenolic compound. Carbon electrode material affects significantly the sensitivity of the biosensor, because the limiting electrochemical back reduction of the enzymatic products takes place on the grain of the carbon materials (Rabinovich & Lev, 2001).

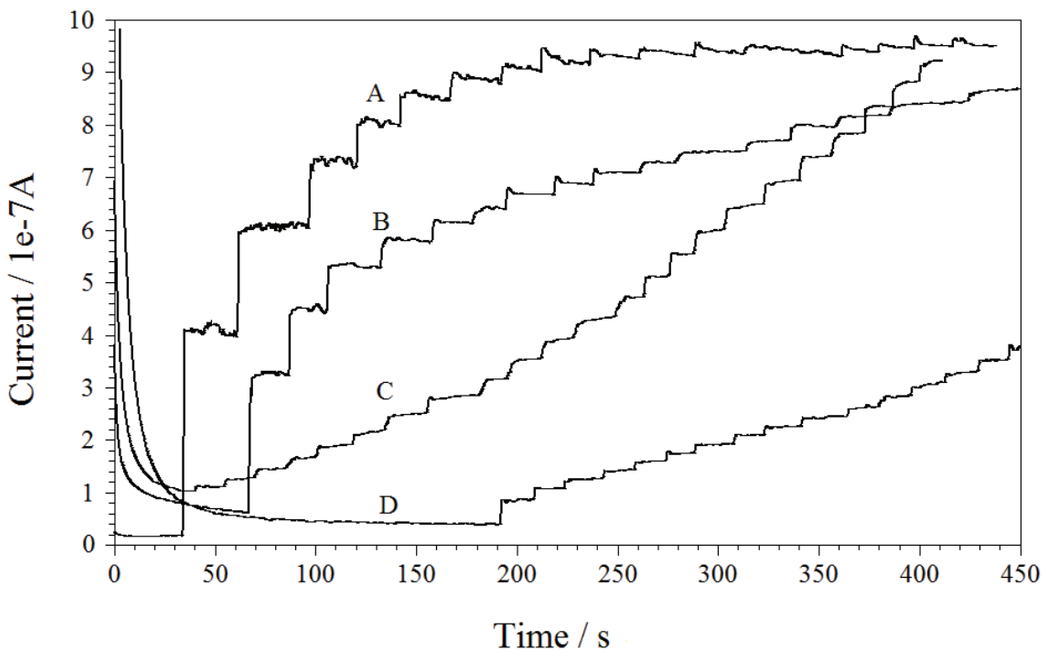

Fig. 14. Amperometric response of Poly(GMA-*co*-MTM)/PPy/HRP composite film electrode to the successive additions of catechol (A), *p*-benzoquinone (B), *p*-cresol (C) and *m*-cresol (D).

No response was obtained for 2,4-dimethylphenol, as expected, for the one having the *ortho*-position occupied by a methyl group. Not only o-cresol and 2,4-dimethylphenol but also 2-aminophenol, pyrogallol and 2,6-dimethoxyphenol gave no response. The operational stability of the electrode was monitored for a series of 20 succesive additions of 2 μM phenolic compounds. High operational stability was observed with relative standard deviations (RSD) ranging between 2% and 5.1% as seen in Table 4.

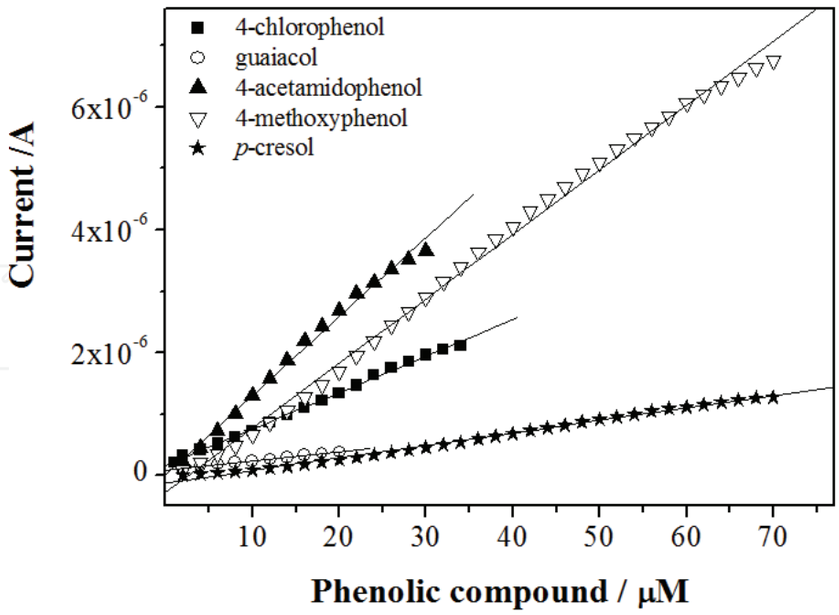

Fig. 15. Calibration curves of Poly(GMA-*co*-MTM)/PPy/HRP composite film electrode to increasing phenolic concentrations (initial phenolic concentration is 2 µM).

Analyte	r	Sensitivity (nA μM^{-1})	Linear range (µM)	LOD (µM)	%RSD
Hydroquinone	0.99	200	2-34	0.13	2.3
Catechol	0.92	30	2-12	0.87	4.5
p-Benzoquinone	0.92	30	2-10	0.85	5
2-Chlorophenol	0.97	10	4-10	1.62	4.1
3-Chlorophenol	0.98	20	2-12	1.31	5
4-Chlorophenol	0.99	60	1-34	0.55	2
2-Aminophenol			no response		
Phenol	0.98	90	2-12	0.3	2.1
Guaiacol	0.99	10	2-20	1.2	3.8
2,6-Dimethoxyphenol			no response		
4-Acetamidophenol	0.99	100	2-30	0.21	2.3
4-Methoxyphenol	0.99	100	2-70	0.25	3.2
2,4-Dimethylphenol			no response		
Pyrogallol			no response		
Pyrocatechol	0.98	3	2-22	1.87	2.8
m-Cresol	0.99	10	2-88	1.43	3.8
o-Cresol			no response		
p-Cresol	0.99	20	2-70	1.28	5.1

Table 4. Analytical characteristics of Poly(GMA-*co*-MTM)/PPy/HRP composite film electrode for various phenolic compounds. Applied potential; -50 mV, 0.1 M phosphate buffer (pH 7) containing 20 µM hydrogen peroxide.

5. Conclusion

In this study a series of working electrode was fabricated for the amperometric detection of different phenolic compounds. For the fabrication of the working electrodes Poly(GMA-*co*-MTM) was synthesized as target spesific regarding to its chemically enzyme immobilization capacity and electropolymerizable thiophene groups with a conductive polymer such as polypyrrole. Different electrode designs conducted by using the same polymers of Poly(GMA-*co*-MTM) and polypyrrole showed different measurement results for the tested phenolics due to the differences of enzyme immobilization techniques, film electroactivity and variety of composite/copolymeric film structures of the fabricated electrodes. Electron transfer promoting effect of CNTs was distinctly observed for some of the fabricated electrodes.

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Environmental Biosensors

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This book is a collection of contributions from leading specialists on the topic of biosensors for health, environment and biosecurity. It is divided into three sections with headings of current trends and developments; materials design and developments; and detection and monitoring. In the section on current trends and developments, topics such as biosensor applications for environmental and water monitoring, agro-industry applications, and trends in the detection of nerve agents and pesticides are discussed. The section on materials design and developments deals with topics on new materials for biosensor construction, polymer-based microsystems, silicon and silicon-related surfaces for biosensor applications, including hybrid film biosensor systems. Finally, in the detection and monitoring section, the specific topics covered deal with enzyme-based biosensors for phenol detection, ultra-sensitive fluorescence sensors, the determination of biochemical oxygen demand, and sensors for pharmaceutical and environmental analysis.

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