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# Amperometric Phenol Biosensor Based on Horseradish Peroxidase Entrapped PVF and PPy Composite Film Coated GC Electrode

**Meral Topcu Sulak · Elif Erhan · Bülent Keskinler**

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**Abstract** Polyvinylferrocene (PVF) was used as a mediator for the fabrication of a horseradish peroxidase (HRP)-modified electrode to detect phenol derivatives via a composite polymeric matrix of conducting polypyrrole (PPy). Through an electropolymerization process, enzyme HRP was entrapped with PPy in a three-electrode system onto a glassy carbon electrode previously covered with PVF, resulting in a composite polymeric matrix. Steady-state amperometric measurements were performed at −200 mV vs. Ag/AgCl in aqueous phosphate buffer containing NaCl 0.1 M (pH 6.8) in the presence of hydrogen peroxide. The response of the HRP-modified PVF electrode was investigated for various phenol derivatives, which were 4-chlorophenol, phenol, catechol, hydroquinone, 2-aminophenol, pyrogallol, *m*-cresol, and 4-methoxyphenol. Analytical parameters for the fabricated PVF electrode were obtained from the calibration curves. The highest sensitivity was obtained from the calibration of 4-chlorophenol as 29.91 nA/μM. The lowest detection limit was found to be 0.22 μM (S/N=3) for catechol, and the highest detection limit was found to be 0.79 μM (S/N=3) for 4-methoxyphenol among the tested derivatives. The biosensor can reach 95% of steady-state current in about 5 min. The electrode is stable for 2 months at 4 °C.

**Keywords** Phenol biosensor · Conducting polymer · HRP · PVF · Polypyrrole

## Introduction

The importance of using biosensors for environmental surveillance becomes more prevalent in literature with the emphasis to phenol determination and control [1]. The accurate determination of phenols is of immense importance in environmental analysis, and the detrimental effect of phenols on human health requires a strict mandate for the

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quantification of such compounds. The concentration of these compounds in natural water or soil may vary to some degree, but on the whole, they are present at the parts per billion level. A number of phenol compounds are listed in the European Community (EC) Directive 76/464/EEC concerning dangerous substances discharged into the aquatic environment [2] and in the US-EPA list of priority pollutants due to their toxicity and persistence in the environment [3, 4]. Their toxicity affects directly a great variety of organs and tissues, primarily lungs, liver, kidneys, and genitourinary system [5].

Amperometric biosensors for the detection of phenolic compounds have been introduced based on a number of approaches. Most of them for the detection of phenolic compounds have been studied as a mono-enzyme system using either tyrosinase or horseradish peroxidase (HRP) [6, 7]. Many methods and techniques are available for the determination of phenolic compounds, including gas chromatography, spectrophotometric analyses [8, 9], and sensors/biosensors [10, 11] direct electrochemical detection [12]. However, some of these methods suffer from demanding sample pretreatment, which makes them unsuitable for on-line monitoring and do not having the potential for detoxification. To solve this problem, the enzyme electrode has been developed because of its simplicity and high selectivity [13].

In biosensors studies, composite working electrodes have been investigated to enhance especially the stability of the enzyme immobilized within a film basing on a conductive polymer. Polypyrole-modified phenol biosensors can be focused for the composite electrode preparation due to the wide application of polypyrole, including the advantages of relatively high conductivity and easy electropolymerization in aqueous solution etc. [14, 15]. However, improvements are expected from composite materials where the conducting polymer exhibits the desired conductivity, while the composite matrix provides the possibility for the enzyme immobilization, the needed mechanical properties, and easy application. There have been various types of PPy composites existing, which were investigated for better structural and electrical properties [16–18]. In addition to this, multi-walled carbon nanotubes (MWCNTs) were introduced into polyvinylferrocene (PVF) film [19]. This method was successfully transferred to prepare a PVF-MWCNT paste electrode, which was applied to glucose detection in diluted laked horse blood. A homopolymer of polyvinylferrocene was introduced into the PPy to develop enhanced electrical and thermal stability of PPy film as a first [20]. PPy homopolymers and PVF/PPy composites indicated different properties as depending on synthesis media. The authors showed that PVF/PPy composites had been improved for their stability and conductivity values. The chemical oxidative modification of PPy by PVF was also reported that the resulted composites with conductivity in the range of $1.94–4.5\times10^{-1}$ S $cm^{-1}$ were found to be higher than that of their homopolymer counterparts. Previously, PVF, as a homopolymer, has been investigated due to its organometallic properties for various enzyme electrodes for the amperometric determination of glucose, sucrose, urea, cholesterol, and hydrogen peroxide [21–23]. Most of those electrodes utilized immobilized enzymes in a redox polymer polyvinylferroceniumperchlorate ($PVFClO_4^-$) by means of ion exchange interactions. The enzyme molecule existed in the form of an anion ($E^-$) since the pH was above the isoelectric point of the enzyme, facilitating its ion exchange interaction with the oxidized polymer ($PVF^+$). The interaction between the negatively charged enzyme species and PVF matrix was shown as follows [24, 25].

$$PVF + ClO_4^- + E^- \longrightarrow PVF + E^- + ClO_4^-$$

In addition to this, it was also reported that $PVF^+$ centers acted as catalytic sites for $H_2O_2$ oxidation according to the following reactions, resulting in higher sensitivity values for the electrode [26]:

$$2PVF^+ + H_2O_2 \longrightarrow O_2 + 2PVF + 2H^+$$

$$PVF \longrightarrow PVF^+ + e^-$$

Mobile species transfer accompanying the redox switching of PVF in aqueous perchlorate of four cations (hydronium, sodium, rubidium, or tetraethylammonium) was investigated using the electrochemical quartz crystal microbalance [27]. The mass transients could not be explained solely in terms of anions and solvent transport processes. The role of $OH^-$ ion in the redox processes of PVF in $NaClO_4$ solutions was investigated, and it was demonstrated that the $OH^-$ ions share an important portion in the counterion doping/undoping processes in neutral $NaClO_4$ [28]. Schlindwein et al. showed that the PVF film had a "solvent sensitivity" effect, and the charge density changed according to the solvent used. A reversible charge sensitivity effect when the films were cycled between propylene carbonate- and tetramethylene sulfone-based electrolytes was reported [29]. Although PVF was a well-defined homopolymer and a promising material with its capacity for enzyme electrodes in the literature [30], utilizing its composites, which can be derived with intrinsically conducting polymers such as PPy, is still widely lacking.

A new PVF and PPy composite film electrode in which enzyme was entrapped by means of electropolymerization in a one-step process was fabricated in this study. PVF, dissolved in methylenechloride, was dropped onto the glassy carbon (GC) electrode and left for evaporation. PVF precoated electrode was introduced into the aqueous solution (citrate buffer) of enzyme (HRP) and PPy through an electropolymerization process. The new HRP-PPy/PVF composite GC electrode was investigated for detecting various phehols. This PVF-modified electrode was expected to offer a stronger biocatalytic film adhesion and minimize enzyme denaturation by contact with the GC electrode as well as utilizing its mediating capacity in the electrochemical reaction.

## Experimental

### Reagents

HRP (E.C.1.14.18.1 from mushroom, 4,200 units $mg^{-1}$) and sodium dodecyl sulfate (SDS) were obtained from Sigma. $Na_2HPO_4$, $NaH_2PO_4$, $H_2O_2$ solution (% 35), and citric acid were obtained from Merck. Dichloromethane was obtained from Riedel-de Haen. Phenol was purchased from Fluka. All other chemicals were of analytical grade. PVF was prepared by the chemical polymerization of its monomer, vinylferrocene (Aldrich) at 70 °C for 24 h using 2,2-azo-bisizobutironitrile as an initiator [31].

### Preparation of the Working Electrode (GC/PVF/PPy-HRP)

Glassy carbon 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 biosensor was constructed with the following procedure: (1) 10.0 μL of PVF

methylene chloride solution (3 mg/mL) was pipetted onto the surface of the electrode and dried in atmosphere (PVF/GC). (PPy-HRP) film was coated onto the surface of the (PVF/GC) working electrode by electrochemical polymerization in a three-electrode cell. The polymerization medium contained 5 mL of 100 mM pH 4.5 citrate buffer, including 0.01 M pyrrole, 3.5 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 electrolytes for electropolymerization of pyrrole in aqueous medium [15]. The composite film consisting polypyrrole and HRP enzyme was electrochemically coated onto a glassy carbon electrode at a voltage of 0–1.0 V and at a scan rate of 100 mV/s vs. Ag/AgCI. The coated electrodes were washed with buffer solution and dried and finally stored at 4 °C.

### Electrochemical Measurements

Electrochemical measurements were performed with a three-electrode system. The GC/PVF/PPy-HRP electrode, an Ag/AgCl (saturated KCl) electrode, and a platinum wire were used as the working, the reference, and the auxiliary electrode, respectively. A CHI 842 potentiostat/galvanostat was used for the experiments in a stirred system by applying a potential of −200 mV to the working electrode. In this low potential, the oxidation of some interference present in the real samples can be minimized. In more negative potential values, the enzyme molecules could be inactivated by the formation of HRP, decreasing the activity, because the operational stability of HRP biosensor is greatly affected by the polarization potential.

Aliquots of a constant amount of $H_2O_2$ at the ratio of two times for the used phenol concentration and phenol were added to the cell containing buffer solution. Amperometric measurements were carried out in the experimental batch setup. Current–time data were started to record after a steady-state current had been carried out.

## Results and Discussion

### GC/PVF/PPy-HRP Enzyme Electrode Characterization

The morphologies of PVF/GC, PVF–PPy/GC, and HRP-PPy/PVF/GC were characterized by scanning electron microscopy (SEM, Philips XL30 SFEG). A typical SEM picture of PVF film displays a microporous structure (Fig. 1a). This microporous structure provides a template for the polymerization of polypyrrole (Fig. 1b). After PPy and HRP coated onto PVF matrix by in situ electropolymerization method, the micropores of PVF film disappeared, and the enzyme was observed at the vicinity of the surface of the polymeric composite film (Fig. 1c). It can be seen that the three forms have distinctly different morphologies. This porous structure of polypyrrole may have a significant role toward the high enzyme stability within the polymer matrix and good reproducibility of the enzyme electrode. In addition to this, PVF can act as a mediator by facilitating the electron transfer between electrode and phenols. The CVs of PVF, PPy-HRP, and HRP-PPy/PVF on glassy carbon electrodes are given in Figs. 2, 3, and 4. When the CV of HRP-PPy/PVF GC electrode was compared with the CV of PVF-coated GC electrode, they were both seen completely different from each other, which was an evidence of formation of the HRP-PPy/PVF composite film on GC electrode. The CV of HRP-PPy/PVF/GC in Fig. 4 reflects basely the profile of PPy-HRP system since the electroconductivity is supplied mainly by the PPy coverage but PVF might be in the role of a mediator through the supposed reaction.

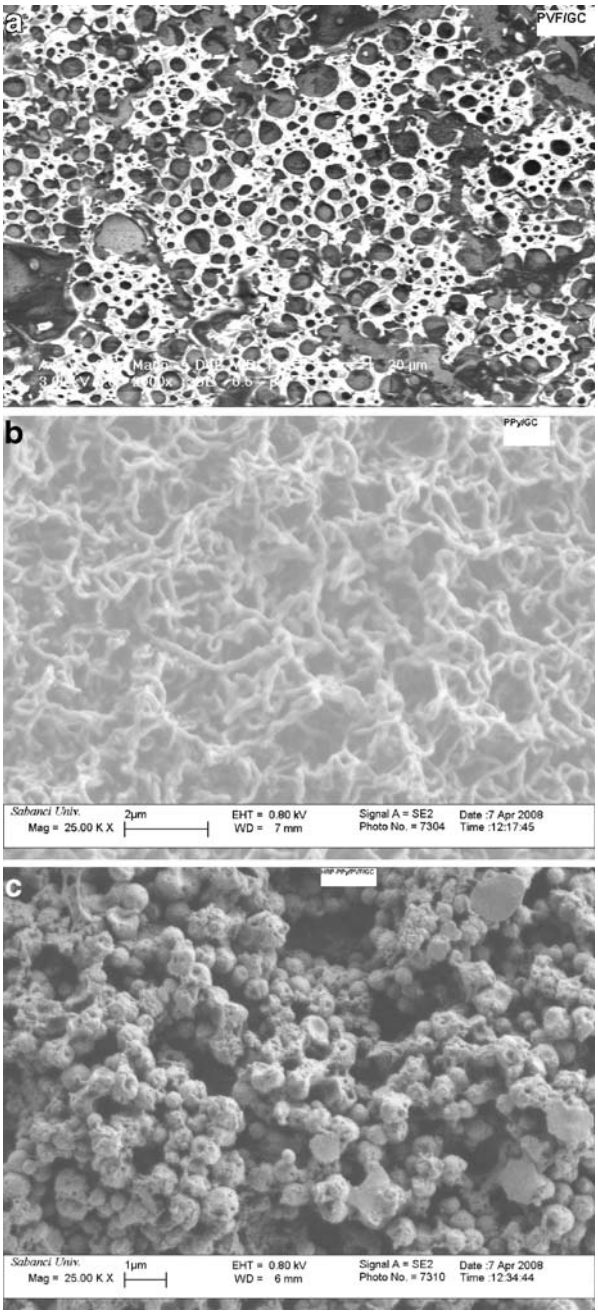


**Fig. 1 a–c** The SEMs of PVF/GC, PVF–PPy/GC, and HRP-PPy/PVF/GC film electrodes, respectively

## Effect of the Applied Potential

The applied potential has an important influence over the biosensor response because the sensitivity and selectivity of the system partially depends on the applied potential. The effect of potential on biosensor response at steady state is shown in Fig. 5. Sensitivity

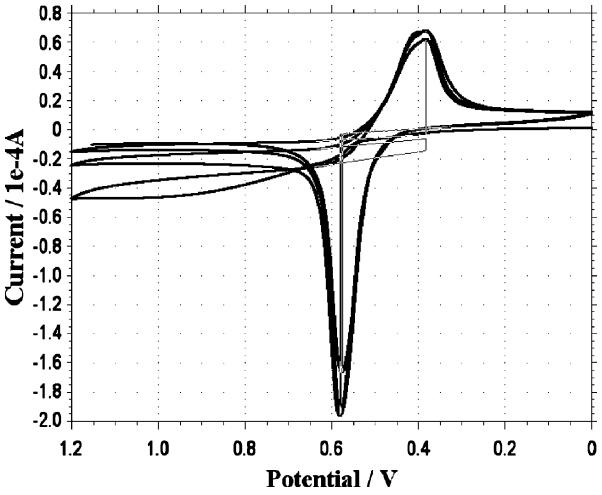


**Fig. 2** The CV of PVF/GC electrode

response decreases with the increased potential, which is in agreement with those given in the literature for phenol biosensing. The potential was fixed at −0.2 V for the rest experiments in the study since the current reached its highest value at that potential. The oxidation of some interference present in the real samples can be minimized due to the low potential. At higher values of negative potential, the enzyme molecules could be inactivated by the formation of HRP, decreasing the activity because the operational stability of HRP biosensor is greatly affected by the polarization potential.

## The Effect of pH

It is known that pH is one of the critical parameter of the enzymatic activity and the stability in aqueous media. The effect of pH was determined for the GC/PVF/PPy-HRP in 0.1 M phosphate buffer by adjusting the range between 5.0 and 8.0 for the phenol concentrations of $5 \times 10^{-6}$ M. The optimum current was obtained at pH 6.8 as seen in Fig. 6.

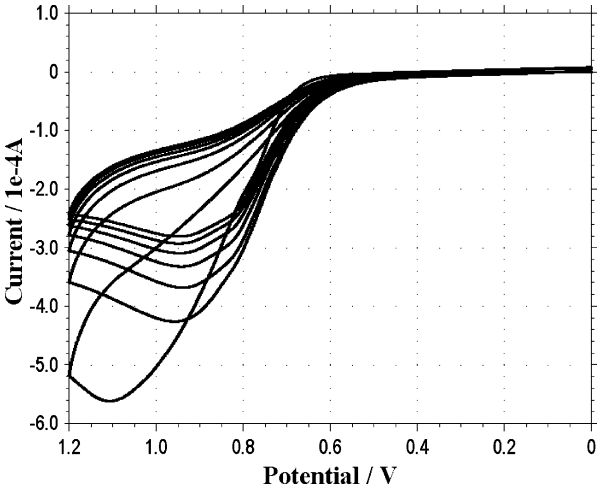


**Fig. 3** The CV of PPy-HRP/GC electrode

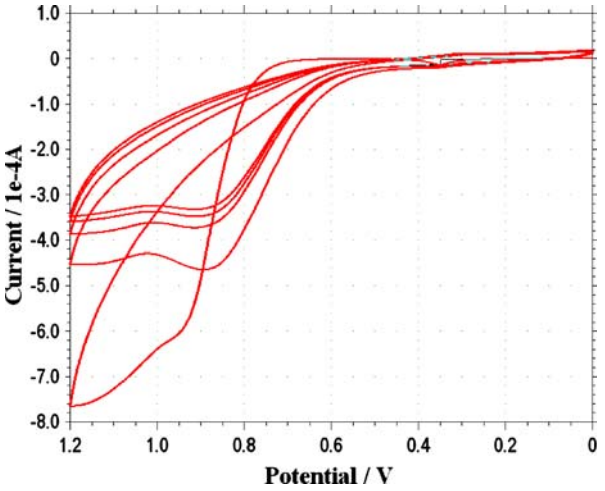


**Fig. 4** The CV of the finalized HRP-PPy/PVF/GC electrode

Further experiments were conducted in phosphate buffer at pH 6.8 cited previously as an optimum value for the catalytic activity of horseradish peroxidase in the decomposition reaction of phenol [32, 33].

## The Effect of Enzyme Amount

Various amount of HRP, 0, 5–4 mg/mL, were dissolved in the electrochemical cell in the presence of constant pyrrole concentration of 0.01 M at −200 mV. The immobilization mechanism of HRP in this study is entrapment of the enzyme into PVF–PPy composite matrix through the electropolymerization process. The obtained currents for the entrapped HRP at four various concentrations of the modified working electrode are shown in Fig. 7. The optimized enzyme concentration for immobilization was found to be 2 mg/mL in electrochemical cell. It can be seen from the figure that a plateau existed after the concentration of 2 mg/mL. Therefore, excess amount of enzyme could not be entrapped due

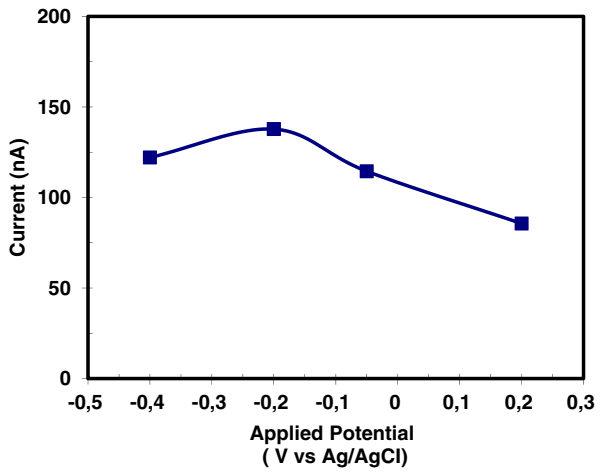


**Fig. 5** Effect of potential (vs. Ag/AgCl, 3 M KCl) on the biosensor response to the amount of $5\times10^{-6}$ M phenol in 0.1 M phosphate buffer, 30 μg PVF, 5 s electropolymerization time, 1 mg HRP, 25 °C

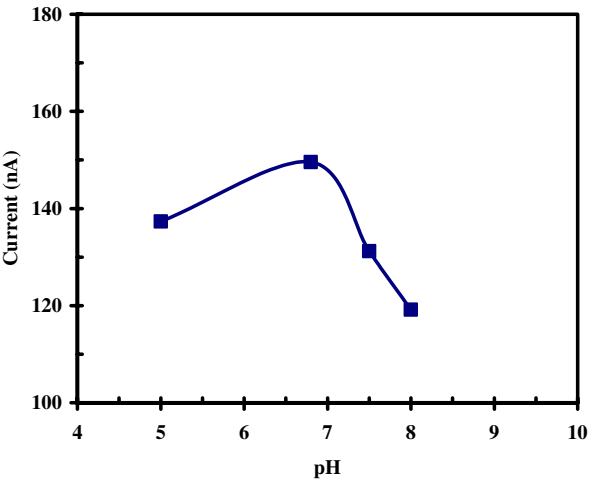


**Fig. 6** Effect of pH (vs. Ag/AgCl, 3 M KCl) on the biosensor response to the amount of $5\times10^{-6}$ M phenol in 0.1 M phosphate buffer at −200 mV, 30 μg PVF, 5 s electropolymerization time, 1 mg HRP, 25 °C

to the supporting capacity of the formed polymeric matrix of the fabricated GC/PVF/PPy working electrode under electropolymerization conditions.

## Amperometric Response of the Biosensor

The electrochemical reaction between HRP and the electroactive groups of PVF in the form of ferrocene is given in Fig. 8. Because of its electron-transferring abilities, ferrocene has found application as "redox mediator" in amperometric biosensors, especially in those based on conducting polymer matrixes [34, 35]. It is very well known that HRP reaction with phenols is hydrogen peroxide dependent. $H_2O_2$ is used to produce the oxidized form of HRP and turns into $H_2O$ at the end of this reaction. Since $H_2O_2$ presence in the medium decreases due to the reaction between HRP and phenols, sufficient amount of it is always added at the beginning of the reaction to the reaction medium.

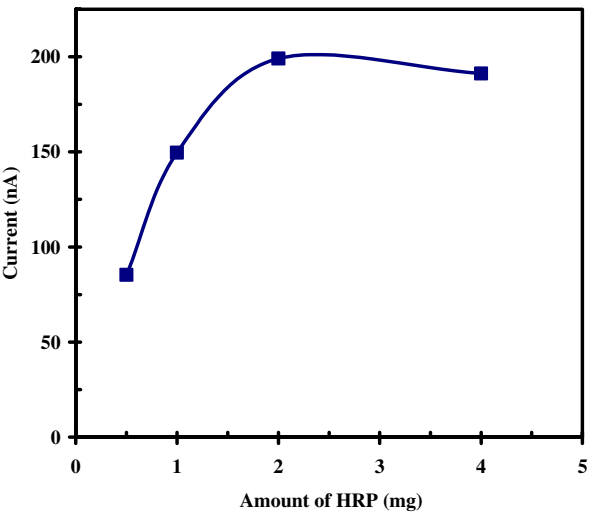


**Fig. 7** Effect of enzyme amount (vs. Ag/AgCl, 3 M KCl) on the biosensor response to the amount of $5\times10^{-6}$ M phenol in 0.1 M phosphate buffer at pH 6.8 at −200 mV, 30 μg PVF, 5 s electropolymerization time, 25 °C

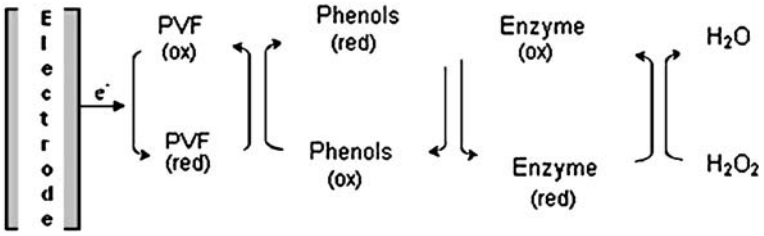


**Fig. 8** The proposed reaction between HRP and ferrocene through the electrochemical reaction

The active mediating role of PVF in the reaction is clearly seen. According to this reaction, various phenolics representing different substitutions, which were phenol, catechol, hydroquinone, 4-chlorophenol, pyrogallol, *m*-cresol, and 4-methoxyphenol, were detected by the newly constructed PVF/PPy-HRP-modified GC electrode in 0.1 M phosphate buffer solution (pH 6.8) at a working potential of −200 mV (vs. Ag/AgCl). Figures 9 and 10 show the amperometric response of the electrode upon successive additions of 4-methoxyphenol as a model application and the calibration plot obtained from that curve.

The sensitivity in the linear range increases at the following order: 4-chlorophenol, phenol, catechol, hydroquinone, 2-aminophenol, pyrogallol, *m*-cresol, and 4-methoxyphenol (Table 1). 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 3 $s_b/m$ criteria in Ref. [36], 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. 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 compound [37]. The highest sensitivity was obtained from the calibration of 4-chlorophenol. The lowest detection limit was found to be 0.22 μM (S/N=3) for catechol, and the highest detection limit was found to be 0.79 μM (S/N=3) for 4-methoxyphenol among the tested derivatives. Detection limit ranges between 0.00003 and 346 μM (S/N=3) for various phenol derivatives with different phenol oxidases in recently reported biosensors

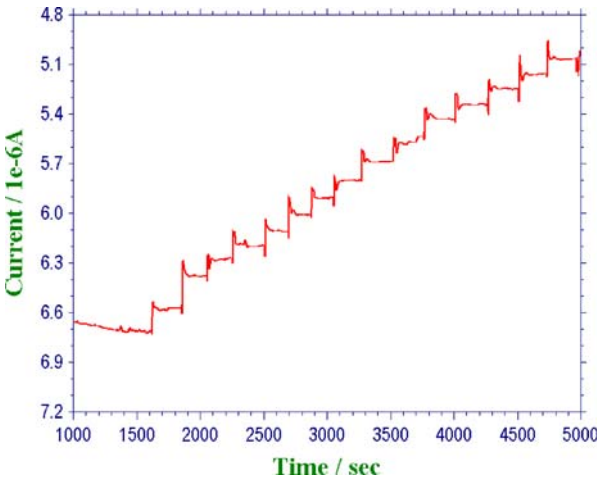


**Fig. 9** Typical steady-state current/time response curve of GC/PVF/PPy-HRP electrode for the increasing amount of 4-methoxyphenol ($1.78\times10^{-6}$ M 4-methoxyphenol in 0.1 M phosphate buffer at pH 6.8 at −200 mV, 30 μg PVF, 5 s electropolymerization time, 25 °C)

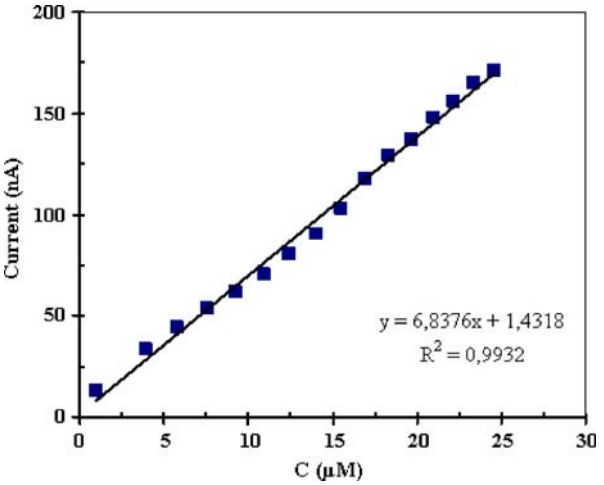


**Fig. 10** The obtained calibration curve of GC/PVF/PPy-HRP electrode for the increasing amount of 4-methoxyphenol ($1.78\times10^{-6}$ M 4-methoxyphenol in 0.1 M phosphate buffer at pH 6.8 at −200 mV, 30 µg PVF, 5 s electropolymerization time, 25 °C)

[38–50]. The trend of the sensitivity for phenol derivatives was consistent with the ability of their substituents for forming electrondonor conjugation. Usually, stronger ability of electrondonor conjugation resulted in higher sensitivity for the detection [51]. Wilkolazka et al. [38] reported that there was an obvious difference among the phenol derivatives with regard to the parameter of sensitivity. This biosensor showed a linear response up to 0.5–$10.10^{-6}$ M concentration of phenol with a correlation coefficient of 0.99. Koile and Johnson reported that a loss in linearity at higher concentration of phenolic compounds could be attributed to slow surface fouling by the reaction products since HRP reaction produced phenoxy radicals, which could possibly polymerize through the enzymatic reaction [52].

One of the other objectives of simultaneous co-immobilization of enzyme and PPy on PVF matrix is to achieve a high sensitivity response, which could be used repeatedly over a long period of time as well as maintaining the mediator capacity of ferrocene for phenol detection. It was also observed that the GC/PVF/PPy-HRP electrode retained 60% of its initial enzyme activity for 2 months when stored at 4 °C in a refrigerator.

## Conclusions

This study has demonstrated the feasibility of developing a conducting polyvinylferrocene and polypyrrole-based biosensor for monitoring phenol in aqueous medium. A new

**Table 1** Analytical characteristics of GC/PVF/PPy-HRP electrode for various phenolic compounds.

| | Linear range (µM) | *S* (nA/µM) | *R* | LOD (µM) | % RSD |
|---|---|---|---|---|---|
| 4-Chlorophenol | 0.3–5.0 | 29.91 | 0.998 | 0.17 | 3.21 |
| Phenol | 0.5–10 | 25.93 | 0.997 | 0.23 | 2.15 |
| Catechol | 0.5–8 | 18.0 | 0.995 | 0.22 | 2.84 |
| Hydroquinone | 1.6–15 | 15.32 | 0.998 | 0.6 | 5.60 |
| 2-Aminophenol | 1–20 | 15.25 | 0.997 | 0.74 | 4.45 |
| Pyrogallol | 0.5–30 | 15.2 | 0.987 | 0.25 | 2.56 |
| *m*-Cresol | 1.2–12 | 12.35 | 0.988 | 0.75 | 1.2 |
| 4-Methoxyphenol | 1–24.6 | 6.83 | 0.993 | 0.79 | 5.43 |

*S* sensitivity, *R* regression coefficient, *LOD* limit of detection, *% RSD* relative standard deviation

biosensor has been fabricated by simultaneous co-immobilization of enzyme, HRP in a thin conducting polypyrrole film by an electrochemical method on GC/PVF electrode. Precovering of PVF on GC surfaces may limit the polymerization of pyrrole monomer as polypyrrole due to the formed PVF–PPy composite polymer. This can allow the electron transfer much easer through the relatively thin and porous composite polymer. The use of PVF in the composite film contributed to the obtaining of relatively higher current values since ferrocene in the structure of PVF acted successfully as a mediator of the proposed reaction. This co-immobilization method provides an efficient entrapment of enzyme within the polymer film and reflects a long-term life stability of the biosensor for 2 months at 4 °C. The low cost and simple method of fabrication of biosensor is an additional advantage with respect to conventional electrode.

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