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A mediated polyphenol oxidase biosensor immobilized by electropolymerization of 1,2-diamino benzene

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ABSTRACT

A biosensor based on a partially purified polyphenol oxidase (PPO) enzyme was developed by using electropolymerization of [(2,2′-bipyridine)(chloro)(p-cymene)rutenium(II)]chloride] mediator complex and 1,2-diamino benzene (DAB) on a screen printing Pt electrode (1 mm diameter). The electropolymerization was carried out at +0.7 V for 45 min in phosphate buffer (50 mM, pH 7.0) which contained 14.0 U/10 mL polyphenole oxidase, 200 mM DAB and 2.5 mM Ru-mediator complex solutions. Measurement is based on the detection of the oxidation current of the Ru-mediator complex that related to the enzymatic reaction catalyzed by PPO at +0.65 V. The phosphate buffer (50 mM, pH 7.0 containing 0.1 M KCl) and 30 °C were established as being the optimum working conditions. Under the optimum experimental conditions a linear calibration curve was obtained between 5 and 100 μM catechol concentration. The detection limit of the biosensor is 2.385 μM. In the characterization studies of the biosensor some parameters such as effect of Ru-mediator types on the biosensor response, substrate specificity, reproducibility and storage stability were studied. From the experiments, the average value (x), standard deviation (SD) and coefficient of variation (CV%) were found to be 48.75 μM, ± 1.56 μM, and 3.2% respectively for 50 μM catechol standard.

1. Introduction

Tyrosinases (monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1) are copper containing metalloproteins and widely found in some microbes, plants and mammals. These enzymes are known as type 3 copper proteins having a diamagnetic spin-coupled copper pair in the active centre [1,2]. Tyrosinases catalyze the o-hydroxylation of monophenols and subsequent oxidation of o-diphenols to quinones [2]. Molecular oxygen is used as an electron acceptor in the catalysis, with subsequent reduction of oxygen to water. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved [3]. The binuclear active site of tyrosinases is known to exist in three states: oxy-tyrosinase, met-tyrosinase and deoxy-tyrosinase. Both met- and oxy-states of tyrosinases can catalyze the diphenolase reaction, whereas the hydroxylation reaction that is involved in the monophenolase catalytic cycle requires the oxy-form of the active site, in which dioxygen is bound as a peroxide. Deoxy-tyrosinase is a reduced and instable form and binds oxygen to give the oxy-form [4,5]. In plants, polyphenol oxidase (PPO) is predominantly located in the chloroplast thylakoid membranes, and the enzyme catalyzes rapid oxidation of the phenols [6].

Pyrocatechol, resorcinol, hydroquinone, pyrogallol, and gallic acid exhibited the characteristics of reducing agents but their reduction potentials and their ability to remove copper oxide from copper metal are not known. There are many biosensoric methods for the detection of catechol based on electrosynthesized poly(aniline-co-p-aminophenol) [7] , polyacrylamide microgels [8], hydrophilic films of electropolymerized polypyrrole [9], self-assembly of polyphenol oxidase and chitosan [10], tyrosinase-modified screen-printed arrays [11], Fe_3O_4 nanoparticles-coated carbon nanotubes nanocomposite [12], and poly(dicarbazole) electrodes [13].

Different redox mediators have been described and combined with a variety of different redox enzymes in biosensors. Redox mediators have a fast electron-transfer rate between the active site of the enzyme and the electrode surface. Interaction between an enzyme and a redox mediator is investigated by means of voltammetric methods in the presence of the mediator and also the substrate of the enzyme [14].

Osmium and ruthenium complexes have been evaluated with respect to their ability to act as redox mediators in the biosensor studies that are based on different oxidoreductases. Especially Ru-complexes such as $[Ru(XX)_2Y_2]$, where XX can be 1,2-bypiridine or 1,10-phenantroline and Y is an acido ligand, were found to undergo rapid interaction with some oxidoreductases, such as glucose oxidase and horseradish peroxidase [15,16]. These mediators could be bound to the redox proteins by means of a ligand exchange reaction with the histidine residues [17]. Mediator modified enzymes showed improved electron-transfer rates compared with the native enzyme.

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In biosensor studies non-conducting polymers have been considered as a useful support matrix for the immobilization of biomolecules because of some advantages such as good permselectivity and high reproducibility [18,19]. They can be used to eliminate electrochemical interference result from ascorbate, urea, acetaminophen and other oxidizable species. Some phenol derivatives such as phenol, 3-aminophenol, 3-methyphenol, 3-nitrophenol and 1,3-dihydroxybenzene, 1,2-dihydroxybenzene, 1,4-dihydroxybenzene, 4-aminophenol, 1,3,5-trihydroxybenzene and 1,2,3-trihydroxybenzene, 1,2-diaminobenzene, 1,3-diaminobenzene etc. have been used as immobilization matrices for enzyme biosensors [20–24]. A fast response time and high selectivity could also be expected for non-conducting polymer based enzyme biosensors.

This study presents a new biosensor which has been developed by using electropolymerization of partially purified polyphenol oxidase (PPO) enzyme, [(2,2′-bipyridine)(chloro)(p-cymene) rutenium(II)] chloride] mediator complex, and 1,2-diamino benzene (DAB) on a screen printing Pt electrode.

2. Experimentals

2.1. Chemicals and apparatus

Polyphenol oxidase was isolated and partially purified from potato *(Solanum tuberosum)* [25], mononuclear p-cymene ruthenium complex was obtained in the usual manner from the reactions of dimeric chloro bridged complexe [{(p-cymene)RuCl(μ-Cl)}$_2$] with N^N in methanol and was isolated in pretty good yield following an earlier method [26] (Scheme 1).

Catechol, 1,2-diamino benzene (DAB), tyrosine, phenol, glucose, L-ascorbic acid, KH_2PO_4, K_2HPO_4 ,KCl, and all other chemicals were purchased from Sigma Chemical Co. (USA).

All the electrochemical experiments were carried out on a PalmSense Instruments potentiostat (Holland). A corundum ceramic based screen-printed platinum electrode (1.0 mm) from BVT Technologies (CZ) was used as working electrode that is combined with the reference Ag/AgCl and the auxiliary Pt electrode. In the experiments, Gibson P100 and P1000 automatic pipets (France), a Yellow line model magnetic stirrer (Germany) and a Nuve model thermostat (TR) were used. Data from the measurements have been obtained with a computer system connected to the potentiostat. All the buffer solutions used in the experiments were prepared by using ultra pure water obtained from Mili-Q and Milipore RIOS-DI 3 UV System (USA).

2.2. Preparation of the biosensor

Immobilization procedure of the enzyme and the mediator is given below:

First of all 14.0 U polyphenol oxidase was dissolved in 10 mL phosphate buffer (50 mM, pH 7.0). 2.5 mL of 200 mM DAB (prepared in 50 mM, pH 7.0 phosphate buffer), 2.5 mL of 2.5 mM Ru-mediator complex (prepared in 50 mM, pH 7.0 phosphate buffer) and also 5 mL of 14.0 U/10 mL polyphenol oxidase solution were added into the reaction medium. Immobilization of the enzyme was formed via the electropolymerization method, which was performed at +0.7 V for 45 min through using the chronoampeorometric method [27]. After the electropolymerization the electrode was washed and rinsed in ultra pure water to remove unfixed enzyme molecules and dried at room temperature.

2.3. Measurements

The principle of the measurements by the biosensor is based on the detection of electrocatalytic oxidation current of Ru-mediator complex at +0.65 V potential related to the enzymatic reaction of polyphenol oxidase in the presence of catechol. In the enzymatic reaction, PPO converts catechol to 1,2-benzoquinone and it was converted to catechol again via the reduced form of the ruthenium mediator complex at +0.65 V potential. At the end of the procedure the oxidized form of the mediator was reduced on the screen-printed Pt electrode surface (Scheme 2).

3. Results and discussion

3.1. Electrochemical characteristics of the biosensor

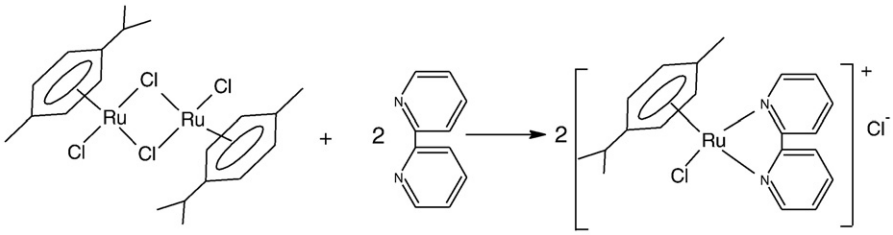
Cyclic voltammetry was used for the investigation of the electrochemical characteristics of the biosensor. Fig. 1 shows the cyclic voltammograms of the biosensor at different stages in the potential range of (−0.5)–(+1.0) V in phosphate buffer solution containing 0.1 M KCl at a scan rate of 0.1 Vs^{-1}. According to the figure no redox peak appeared in the cyclic voltammogram of the bare screen-printed Pt electrode. PPO–DAB–Ru-med modified electrode showed a very small redox peak pair at about +0.75 and −0.35 V vs Ag/AgCl in the absence of catechol. If the catechol was injected into the reaction medium the modified electrode showed a very clear redox peak pair at about +0.65 and −0.35 V vs Ag/AgCl.

Cyclic voltammograms obtained for different catechol standards are given in Fig. 2. From the figure it can be said that an increase in catechol concentration resulted in clear increases in the biosensor responses. A cathodic peak was observed at a potential of nearly −0.35 V potential and this can be accepted as a proof of the reduction of the Ru-med complex on the electrode surface.

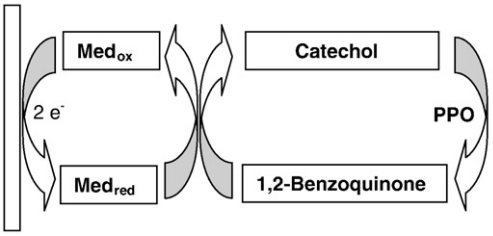
Fig. 3 displays a typical amperometric response of the PPO–DAB–Ru-med modified biosensor to successive standard additions of 50 μM of catechol at +0.65 V applied potential. The sensor responds rapidly to the change in the substrate concentration, producing a steady-state current within 30 s. Such a fast response is attributed to the fast diffusion of the substrate molecule in the thin and suitable bioactive surface of the biosensor. After the 4th addition, because of the susbstrate saturation of the enzyme there was a deterioration in the amperometric signal.

3.2. Optimization of the bioactive layer constituents of the biosensor

For the optimization of the bioactive layer constituents of the biosensor the effects of enzyme amount, concentration of DAB and Ru-mediator complex, and the electropolymerization time on the biosensor response were investigated.



Scheme 1. The synthesis of [(2,2′-bipyridine)(chloro)(p-cymene)rutenium(II)]chloride].



Scheme 2. Principle of the measurement by the modified PPO biosensor.

3.2.1. Enzyme activity of the biosensor

The enzyme amount used in the biosensor preparation, is the key factor for the biosensor sensitivity because the responses depend upon the enzyme activity. For the determination of the effect of enzyme activity on the biosensor response 7.0, 14.0, and 21.0 U/10 mL solutions of the enzyme were used in the biosensor preparation. According to the results, when the bioactive layer of the biosensor contained 14.0 U/10 mL PPO, the most useful calibration curve was obtained. In addition, cathecol was detected with a linear range between 5.0 and 100 μM concentrations by this biosensor. An increase from 14.0 to 21.0 U/10 mL resulted in higher biosensor responses and this was an expected result but in this case the standard curve obtained was nearly the same as with the 14.0 U/10 mL. When the bioactive layer of the biosensor contained 14.0 U/10 mL of polyphenole oxidase we could observe a wide current range and also linearity. In this case, if we consider the results obtained from the experiments it can be said that the most suitable biosensor responses were obtained by using 14.0 U/10 mL solution of PPO.

3.2.2. DAB concentration

For the determination of the effect of DAB concentration on the biosensor responses 100, 200, and 400 mM DAB concentrations were used for the electropolymerization procedure. From the results it was obvious that the most suitable biosensor responses were obtained when 200 mM DAB concentration was used in the electropolymerization. Current response of the biosensor is also affected by the DAB monomer concentration in the electropolymerization medium. The best responses and also linear graph were obtained by using 200 mM DAB. The response current increased when the DAB monomer concentration is increased from 100 to 200 mM, but it decreased gradually when the DAB monomer concentration was higher than 200 mM. 400 mM DAB formed a strict polymeric membrane on the electrode surface, and this membrane has been proven to act as a serious diffusion barrier for the substrate molecules. This illustrates

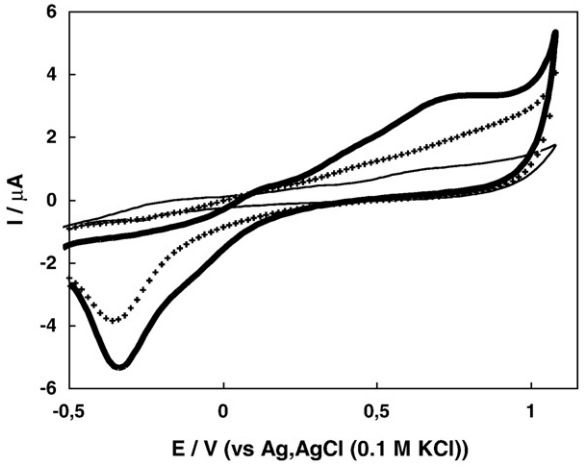


Fig. 1. Cyclic voltammograms obtained from the experiments (phosphate buffer, 50 mM, pH 7.0, *T:* 30 °C), (—) : bare electrode, (+) : PPO-DAB-Ru-med without catechol, (—) : PPO–DAB–Ru-med with 50 μM catechol. Scan rate 0.1 Vs^{-1} vs Ag/AgCl.

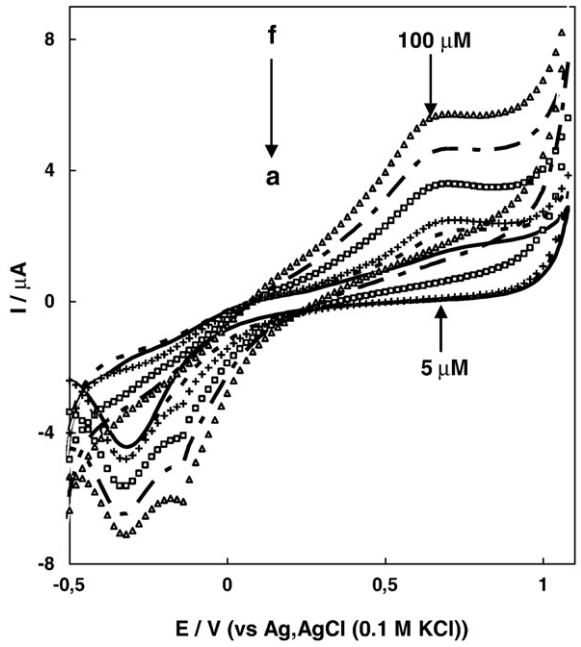


Fig. 2. Cyclic voltammograms obtained for different catechol standards (phosphate buffer, 50 mM, pH 7.0, *T:* 30 °C), inside to outside respectively a) 5, b)10, c) 25, d) 50, e) 75, and f) 100 μM catechol. Scan rate 0.1 Vs^{-1} vs Ag/AgCl.

that too high DAB monomer concentration may result in thick and compact films, which do not facilitate the determination of catechol [28]. In fact, a useful standard curve was obtained using 100 mM DAB but its slope and current responses are not better than 200 mM DAB and also 100 mM DAB did not allow sufficient polymer formation for the immoblization. From the experiments it is obvious that 200 mM DAB concentration is a rather critical concentration for obtaining the most useful and linear responses. Thus, 200 mM DAB was chosen as the best monomer concentration in the electropolymerization process.

3.2.3. Mediator concentration

For the investigation of the effect of the mediator concentration on the biosensor response 1.0, 2.5, and 5.0 mM concentrations of Ru-mediator complex were used in the preparation of the biosensor. According to the results obtained from the experiments, 2.5 mM of Ru-med complex gave the most useful and effective results for the

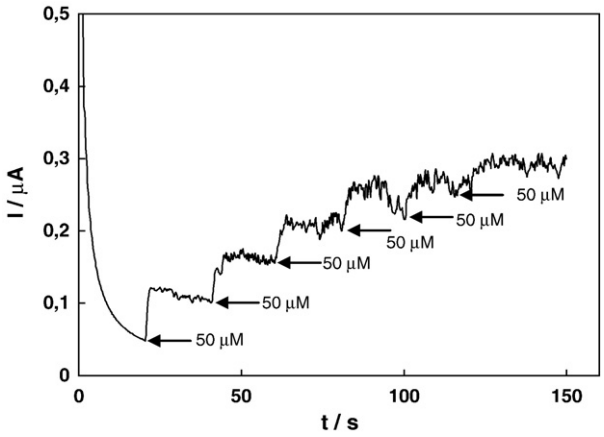


Fig. 3. Typical amperometric reponses recorded at screen-printed Pt electrode coated with PPO–DAB–Ru-med complex to the successive additions of 50 μM catechol. Applied potential is +0.65 V, (phosphate buffer, 50 mM, pH 7.0, *T:* 30 °C).

biosensor. Above and below this concentration we didn't obtain a suitable biosensor response for catechol.

3.2.4. Electropolymerization time

The amount of DAB on the electrode surface can be controlled by adjusting the polymerization time because it has a direct effect on the resulting current values. For the investigation of the effect of the electropolymerization time on the biosensor responses the electropolymerization procedure was carried out for 30, 45, 60, and 90 min at +0.7 V potential. According to the results obtained from the experiments it can be said that the optimum electropolymerization time was 45 min. The best current and also biosensor responses were obtained by this electropolymerization time. However, below and above this time decreases in the biosensor responses were observed and these decreases affected stability and sensitivity of the biosensor. This result could be due to insufficent or excessive film structure formed on the electrode surface related to the electropolymerization time.

3.3. Optimization of working conditions of the biosensor

3.3.1. Effect of pH on the biosensor response

Biosensors based on an enzyme need a suitable buffer system and pH medium for obtaining the best responses. To detect the effect of the pH value on the biosensor response different buffer systems were investigated. For this aim 50 mM concentration of acetate (pH 5.0–5.5), phosphate (pH 6.0–6.5–7.0–7.5) and Tris-HCl (8.0–8.5) buffers were used in the experiments. The optimum pH value was 7.0. Below and above this pH value decreases in the biosensor responses were observed.

3.3.2. Effect of scan rate on the biosensor response

For the determination of the effect of scan rate on the biosensor responses measurements were carried out at 0.025, 0.05, 0.1, 0.2, and 0.4 V s^{-1}. Cyclic voltammograms obtained from the experiments showed that 0.1 V s^{-1} was the optimum scan rate for the detection of catechol considering the fastest response and maximum reduction current.

3.3.3. Effects of different Ru-mediator complexes on the biosensor responses

In this part of the study to investigate the effects of Ru-mediator types that were synthesized in our laboratory, a few biosensors were prepared by using PPO–DAB and different Ru-mediator complexes. The redox properties of the mediators were determined by means of cyclic voltammetry in phosphate buffer (50 mM, pH 7.0) as electrolyte. Close to reversible redox behavior was observed for all the different redox species,but as could be expected from the ligand properties the redox potential of (2,2′-bipyridine)(chloro)(p-cymene) was higher than the others. (2,2′-bipyridine)(chloro)(p-cymene) type Ru-mediator complex was a more efficient redox mediator for the reduction of o-benzoquinone. Cyclic voltammograms in the presence of 50 μM catechol obtained by the biosensors prepared with different Ru-mediator complexes are given in Fig. 4.

The other Ru-complexes except 2,2′-bipyridine type ligands, could also mediate the reduction of o-benzoquinone but the current responses were lower for the same concentration of catechol.

3.4. Effect of storage stability on the biosensor response

One of the most important performance parameters of a biosensor is its long-term operation and storage stability. The use of an enzyme based biosensor is usually limited by the life time of the immobilized enzyme.

The long-term stability of the biosensor was monitored by measuring its response to 50 μM catechol solution. The biosensor was washed and stored at 4 °C in a phosphate buffer solution after each measurement. From the experiments, the biosensor retained about 86% of its initial activity after 21 days. This result indicates that the immobilization procedure of the biosensor provides a positive effect in the long-term stability of the biosensor.

The electrodes were stored in a volumetric flask that contains very little phosphate buffer which was treated with nitrogen gas to prevent the drying of its bioactive layer and also the negative effect of oxygen.

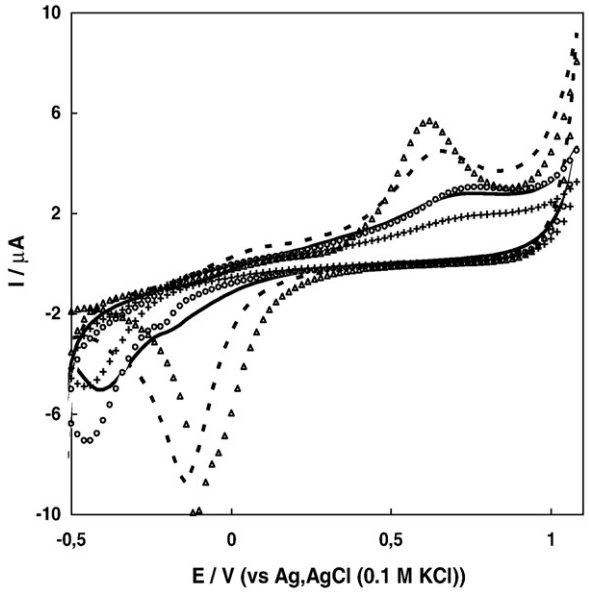


Fig. 4. Cyclic voltammograms obtained by the biosensors prepared with different Ru-mediator complexes in the presence of 50 μM catechol (phosphate buffer, 50 mM, pH 7.0, *T:* 30 °C). Scan rate 0.1 Vs^{-1} vs Ag/AgCl. (△△): [(2,2′-bipyridine)(chloro)(p-cymene)utenium(II)]chloride, (••): [(2-aminomethylaniline)(chloro)(p-cymene) rutenium(II)]chloride, (○○): [(4,4′-dimethyl-2,2′-bipyridine)(chloro)(p-cymene) rutenium(II)]chloride, (—): [(1,10-phenanthrolin)(chloro)(p-cymene)rutenium(II)] chloride (+ +): [(8-aminochinolin)(chloro)(p-cymene)rutenium(II)]chloride.

3.5. Analytical characteristics of the biosensor

3.5.1. Linear range of the biosensor

Under the optimum experimental conditions a linear calibration curve was obtained at concentrations between 5.0 and 100 μM catechol. The responses obtained for 125 and 150 μM concentrations were nearly the same with the response of 100 μM as a result of the substrate saturation of the enzyme. The limits of quantification and

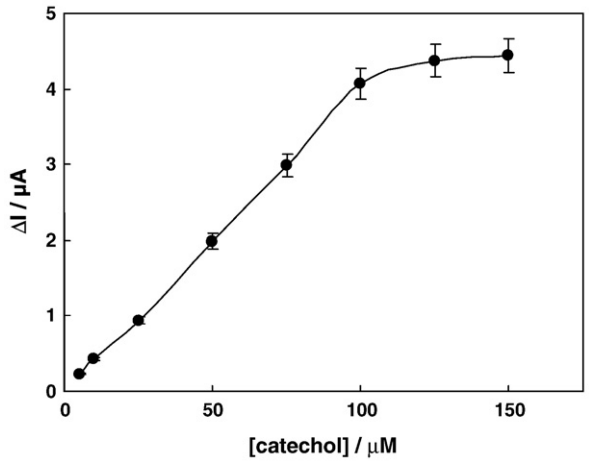


Fig. 5. Calibration curve for catechol obtained by screen-printed Pt electrode coated with PPO–DAB–Ru-med complex (phosphate buffer, 50 mM, pH 7.0, *T:* 30 °C).

Table 1
Substrate specificity of the biosensor.

Substrate	[a]Biosensor Response (%)
Catechol	100
Tyrosine	27
Phenol	19
Glucose	17
Ascorbic acid	50

[a] Average of five measurements.

detection were calculated according to the 10 × standard deviation) and $3S_b/m$ criteria, where m is the slope of the calibration curve and S_b is the standard deviation ($n = 10$) of the signal from 5.0 μM catechol. From the results of the calculation the detection limit and quantification limit of the biosensor were determined to be 2.385 μM and 0.318 μM, respectively. Fig. 5 shows the linear curve of the biosensor for catechol.

3.5.2. Reproducibility

Determination of the reproducibilty of the biosensor experiments were also studied for 50 μM catechol concentration ($n = 5$). From the experiments the average value (X), standard deviation (SD) and coefficient of variation (CV%) were found to be 48.75 μM, + 1.56 μM, and 3.2% respectively. From those results it can be said that the reproducibility of the biosensor response is good.

3.5.3. Substrate specificity

In the substrate specificity experiments 25 μM of catechol, tyrosine, phenol, glucose and ascorbic acid was used. The biosensor response obtained for catechol was accepted to be 100% and compared with the responses of the other subtrates. All the measurements were carried out at least in triplicate. Table 1 shows the results obtained from the experiments. In the experiments the best and highest biosensor responses were obtained for catechol. The biosensor allows negligible responses for tyrosine and phenol and this shows that partially purified PPO has a diphenolase activity.

Table 2 shows the performance of various sensors based on tyrosinase for the determination of catechol [29–36]. All of them determined catechol sensitively. However some of them need very expensive materials and equipments. By considering the advantages of our biosensor such as the simplicity of preparation and use, relative low cost and high selectivity and stability it can be a reason for the use of this type of biosensor.

4. Conclusions

In this study PPO and Ru-med complex were immobilized by the electropolymerization of DAB to construct a catechol biosensor. The cyclic voltammograms obtained from the experiments showed that ruthenium mediator complex positively affected the biosensor responses for catechol determination. The responses of the biosensor are therefore proportional to the oxidation peaks of the Ru-med complex at + 0.65 V potential related to the enzymatic reaction which takes place at the biosensor's interface. This work demonstrates that the biosensor which is based on a mediator system of enzymatic analysis has good analytical properties such as fast and clear response, storage stability and a good detection range and it is suitable for the routine analysis of catechol.

Table 2
Performance of various sensors based on tyrosinase.

Type	Substrate	Linear range	Detection limit	Ref.
Conducting polymer film	Catechol	1.3–110.1 μM	0.7 μM	[29]
Fe_3O_4 nanoparticles–chitosan nanocomposite	Catechol	0.08–70.0 μM	0.025 μM	[30]
Graphite–teflon–Au nanoparticles	Catechol	0.01–8.0 μM	3.0 nM	[31]
$Si0_2$ sol gel	Catechol	0.1–100.0 μM	0.04 μM	[32]
Au nanoparticles	Catechol	0.5–50.0 μM	0.15 μM	[33]
FIA based on tyrosinase/DNA	Catechol	1.0–50.0 μM	1.0 μM	[34]
MWCN–Nafion	Catechol	1.0–23.0 μM	0.22 μM	[35]
Agarose–Guar gum	Catechol	60.0–800.0 μM	6.0 μM	[36]
Present work	Catechol	5.0–100.0 μM	2.385 μM	

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