Amperometric tyrosinase based biosensor using an electrogenerated polythiophene film as an entrapment support

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Amperometric tyrosinase based biosensor using an electrogenerated polythiophene film as an entrapment support

CHRISTOPHE VÉDRINE[(1)], SILVIA FABIANO[(1)], CANH TRAN-MINH[(1)]*

[(1)] *Ecole Nationale Supérieure des Mines de Saint Etienne, Centre SPIN ; Département PMMC ; LPMG, UMR CNRS 5148, 158 Cours Fauriel ; 42023 Saint-Étienne Cedex 2, France*

Abstract

An amperometric enzyme sensor using tyrosinase, also called polyphenol oxidase (PPO), was constructed for determination of phenolic compounds and herbicides. The enzyme was entrapped in a conducting polymer, poly 3,4-ethylenedioxythiophene (PEDT), electrochemically generated on a glassy carbon electrode. Several experimental parameters in the electropolymerisation process and working conditions were determined to optimise biosensor performances. Mono-phenol and di-phenol were tested in oxygenated solutions, by amperometric measurements at −200 mV (vs. SCE) in a batch system. The limit of detection of these molecules ranges from 5 to 500 nM. Detection of herbicides was obtained from the inhibition of tyrosinase electrode responses. The limit of detection for atrazine and diuron was 1 and 0.5 mg l^{-1} respectively. These data suggest that PEDT film is a promising PPO immobilisation method.

Keywords:

Conducting polymer; Tyrosinase; Phenolic compounds; Herbicides

I. Introduction

Biosensors are devices capable of recovering analytical information by utilising biological component as part of the sensor [1]. The use of an amperometric enzyme electrode provides a promising way of development of a wide range of biosensors. This approach combines specificity of the biocomponent (selective molecular recognition) with advantages of electrochemical detection [2].

The essential task in construction of an amperometric biosensor is to efficiently and effectively immobilise the bioactive moiety onto the electrode surface. Electrochemical polymerisation of conducting polymer is a simple one-step process to construct bioelectrodes. Moreover, this immobilisation method allows to easily control the thickness of enzyme film [1 and 3].

The ability to synthesise conductive electroactive polymers under mild conditions enables to perform the immobilisation of a range of biological moieties (enzymes, antibodies, even whole living cells…). Many conducting polymers can be electrochemically generated [4]. In most polymers such as polypyrrole [5, 6, 7 and 8], polyaniline [9, 10 and 11] and polythiophene [12 and 13], redox charges are delocalised over some polymer groups that could facilitate the oxidation–reduction processes.

Polypyrroles can be formed under mildly oxidative conditions from aqueous media. Polyanilines are formed from similar conditions, although formation of the most highly conducting form requires the presence of acid. The monomers of simple polythiophenes are

* Corresponding author: tran@emse.fr

not water-soluble. Among the promising family of polythiophenes, poly 3,4-ethylenedioxythiophene (PEDT) shows remarkable stability, provides homogeneous films [14] and can be synthesised electrochemically even in aqueous medium [15]. Unlike pyrrole, 3,4-ethylenedioxythiophene monomer (EDT) does not require distillation before use. Previous works [16 and 17] showed the interest of using PEDT as a support for glucose oxidase (GOD) immobilisation.

Tyrosinase is a binuclear copper containing metalloprotein and catalyses, in the presence of molecular oxygen, the hydroxylation and oxidation of mono-phenols to *o*-quinones (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [18]. This enzyme, produced from mushrooms, has an isoelectric point of 4.5 [19] and carries a negative charge for pH >4.5 in aqueous solution. Attractive electrostatic interactions can occur with PEDT that is positively charged [15 and 20].

The PEDT/PPO biosensor is based on the detection of the electrochemical reduction at −0.2 V of *o*-quinone enzymatically produced. Thus *o*-diphenol electrochemically generated can undergo another enzymatic oxidation. It leads to a local increase of the concentration of *o*-diphenol [21]. This amplification mechanism explains high sensitivities of mono-phenol or o-diphenol detection with tyrosinase based bioelectrode. It was also reported that triazine and phenyl-urea herbicides could be determined from inhibition of tyrosinase response to a substrate [22, 23 and 24].

The present work describes a tyrosinase based biosensor. It attempts to demonstrate the potentialities of the PEDT immobilisation technique for analytical applications using tyrosinase (which has a more complex action mechanism than GOD). Some molecules of medical interest and pollutants potentially present in the environment were tested using this biosensor.

II. Experimental

II.1. Reagents

EDT was kindly provided by Bayer AG (France). Polyethylene glycol (PEG) of 15 000 MW was purchased from Aldrich. Tyrosinase (polyphenol oxidase, PPO) (EC 1.14.18.1, 6680 U mg^{-1} lot 109H7037) from mushroom was procured from Sigma (France).

Catechol, dopamine, epinephrine, L-dopa, *p*-cresol, 3-chlorophenol, 4-chlorophenol were purchased from Sigma. Phenol was obtained from Carlo Erba.

The following herbicides (Pestanal®): atrazine [2-ethylamino-4-chloro-6-isopropyl amino-1,3,5 triazine], diuron [3(3,4-dichlorophenyl)1,1 dimethylurea], were purchased from Riedel-de-Haen (France). They were prepared in anhydrous ethanol (HPLC grade, Carlo Erba) to get 10 g l^{-1} stock solutions which were stored in darkness at 4 °C for 1 month.

All other chemicals were of analytical grade. Phosphate buffer was prepared using di-sodium hydrogen phosphate and sodium di-hydrogen phosphate (Prolabo, France). All solutions were prepared using milliQ® pure water (Millipore, France).

II.2. Apparatus

Voltammetric and amperometric measurements were performed using a Tacussel PGP 201 potentiostat. The potentiostat output was recorded and processed by means of a VOLTA-MASTER 1 software. The electrochemical cell was a three-electrode cell where the PEDT/PPO modified glassy carbon disk electrode acted as the working electrode and a platinum wire as the counter electrode. All potentials were measured vs. SCE. The working electrodes were constructed using a BAS® glassy carbon electrode with a disk-shaped active surface of 3 mm diameter. Diamond fluid kit was provided by BAS® (England) to polish the electrode surface.

II.3. Immobilisation procedure

Bare electrodes used for preparing the biosensors consisted of glassy carbon disks. The working electrode surface was polished before use with diamond solution of 15 μm, then 3 μm and finally 1 μm particle size. At last, it was carefully rinsed with distilled water.

Two immobilisation procedures were used (Figure 1).

II.3.1. Method A, for direct determination of substrates

The electropolymerisation solution was prepared from 1 mg of tyrosinase dissolved in 200 µl of a 10^{-2} M EDT aqueous solution. Five microlitres of this solution were deposited on a glassy carbon disk and dried at room temperature.
The dry electrode was dipped in a PEG 10^{-3} M phosphate buffer solution (0.02 M, pH 6.2) and the EDT electropolymerisation was carried out using a +1200 mV constant potential for 2 min.

II.3.2. Method B, for determination of inhibitors

The electrode was dipped in a 0.02 M pH 6.2 phosphate buffer solution containing 10^{-2} M of EDT, 10^{-3} M of PEG and 5 mg ml^{-1} of tyrosinase. Electrochemical growth of PEDT was carried out using a +1200 mV constant potential for 2 s.

II.4. Measurements

Amperometric measurements of substrates and inhibitors, with the PEDT/PPO biosensor were carried out in a 20 ml beaker containing 10 ml of phosphate buffer solution (0.1 M, pH 6.5). The electrochemical cell was completed with a SCE reference electrode and a platinum wire counter electrode. The solution was continuously stirred in the open air with a magnetic stirrer to maintain a constant oxygen concentration in the solution for the enzymatic reaction to operate properly. The temperature was controlled using a thermostat (Lauda-Thermostat, Germany).
For substrates determination a potential of −200 mV was applied to the working electrode to detect *o*-quinone formation. When a baseline was stable, substrate was spiked. The current generated on the biosensor was measured when steady state was reached.
For determination of inhibitors, dopamine 25 µM was used as a substrate, when the current was stable the inhibitor was spiked and the current decrease was measured.
Since tyrosinase catalyses the oxidation of several mono-phenols and *o*-diphenols to *o*-quinones in the presence of oxygen, its concentration should be kept constant because it is a co-substrate of the enzymatic reaction.
Unless otherwise stated all experiments were carried out at 25 °C with an applied potential of −200 mV vs. SCE, in a phosphate buffer (0.1 M, pH 6.5) containing 0.1 M KCl.

III. Results and discussion

Several immobilisation methods have been used to construct PPO based biosensors (Table 1). Entrapment of PPO in a PEDT film is an easy and fast immobilisation method. There is no need to synthesise the monomer since it is commercially available. This polymer is directly electrogenerated on a glassy carbon electrode. Contrary to cryo-hydrogel or silica-gel immobilisation methods, it does not require any incubation step in a specific solution or in defined temperature conditions.
PEDT/PPO electrodes were constructed according to two methods. Electrodes prepared with the method A were used to detect substrates. Biosensors constructed with method B were used for inhibitor determination. The main difference between these two methods lies in the film thickness achieved.

III.1. Substrate determination

III.1.1. Optimisation of the enzyme immobilisation

Method A (Figure 1) was used to achieve entrapment of tyrosinase for phenolic compound determination.
The influence of enzyme loading in the polymer on the sensor response to 5 µM catechol was examined (Figure 2, solid line). Response increases when the amount of enzyme deposited on the electrode increases from 5 to 25 µg. At higher amount of deposited enzyme, response decreases. *O*-quinone production takes place mostly at the region closer to the film/solution interface. Therefore, most of *o*-quinone molecules produced in this way are diluted in the bulk solution hence their transfer to the electrode surface is decreased. Similar behaviour was reported for polypyrrole–GOD electrode [25].

The optimal amount of enzyme (25 μg) was used to assess the effect of electropolymerisation time. While 5 s of electropolymerisation time were found sufficient to ensure maximal response (Figure 3, solid line). It was observed that in this case the film was fragile, friable and could easily be detached from the electrode during handling. With 2 min electropolymerisation corresponding to complete polymerisation of the polymer, a more adherent film is obtained. Biosensor response decreases slightly. It is probably caused by the diffusional limitation of a polymeric layer.

III.1.2. Effects of pH and temperature on the biosensor response to catechol

Appropriate pH ensures efficient entrapment of the enzyme while preventing loss of its activity under electropolymerisation conditions. An optimal pH of 6.2 was used: the same pH had previously been used for PEDT electropolymerisation [17].

Working pH, corresponding to the amperometric response of the PEDT/PPO electrode to catechol 5 μM was also investigated for pHs ranging from 5 to 8 in 0.1 M phosphate buffer solution (Figure 4). Maximal response is obtained for pH 6.5. This agrees with data reported by Cosnier and Innocent [26] for a bioelectrode using a polypyrrole tyrosinase film.

Effects of temperature on the enzyme electrode were studied with 5 μM catechol at temperature ranging from 15 to 40 °C (Figure 4). The biosensor response increases with temperature from 15 to 25 °C then a decrease is observed. The optimal temperature 25 °C also corresponds to the biosensor maximal enzyme activity.

III.1.3. Long-term stability

The main objective of enzyme immobilisation on a transducer, for analytical purposes, is to stabilise the enzyme for the biosensor to be used repeatedly over a long period of time. The long-term stability of the biosensor was monitored by measuring its response to 5 μM catechol solution everyday. The PEDT/PPO electrode was washed and stored, between measurements, at 4 °C in phosphate buffer solution. The enzyme electrode retained about 30 % of its initial activity after 12 days (Figure 5).

III.1.4. Response of the biosensor to different substrates

The two catalytic activities of tyrosinase (monophenolase and diphenolase) were tested using mono-phenolic and *o*-diphenolic compounds [27].

III.1.4.1. Detection of o-diphenols

Responses of the tyrosinase sensor to *o*-diphenols was first investigated. After each addition of aliquots of the catechol stock solution into air-saturated phosphate buffer solution the signal reaches a stable plateau, so that a calibration curve can be plotted (Figure 6). Dose–response curves for dopamine, epinephrine, -dopa are also shown in this figure. Sensitivity corresponding to the linear range for catechol, dopamine, -dopa, epinephrine are about 1999, 133, 104, 56 mA M^{-1} cm^{-2} respectively. This sensitivity gradually decreases at higher substrate concentrations. The same sensitivity order was reported by Cosnier and Innocent [26], and Besombes *et al.* [28]. The PEDT/PPO electrode sensitivity to dopamine is 2.2 times higher than that of polypyrrole/PPO electrode [29].

Tyrosinase exhibits a particular behaviour for L-dopa as a substrate. Sensitivity to L-dopa detection is higher than to epinephrine for low concentrations. At higher concentrations, sensitivity to epinephrine is better compared to L-dopa. Duckworth and Coleman [30] and Pomerantz and Warner [31] reported that two apparent values of Km for L-dopa have been observed (two order of magnitude of difference). These data could explain the particular behaviour of the PEDT/PPO electrode when L -dopa is used.

III.1.4.2. Detection of mono-phenols

Calibration curves obtained for mono-phenolic compounds vary in sensitivities depending on the analyte involved (Figure 7). Sensitivities (calculated in the linear range) decrease in this order: *p*-cresol>4-chlorophenol>phenol>3-chlorophenol. The main characteristics of the biosensor including sensitivity, linear range and detection limit are listed in Table 2.

The overall high sensitivity of the PEDT/PPO electrode compared to PPO biosensors using other matrices can be attributed to the biocompatible microenvironment for the enzyme. The difference in sensitivity between each mono-phenolic compound might depend on the hydrophobic characteristics of the immobilisation matrix [32] and molecular steric hindrance. In fact, 3-chlorophenol showed only a weak response. Transition state of this compound is difficult to form because of large steric restriction [33]. Polypyrrole/PPO bioelectrode showed similar detection profile towards chlorinated compounds. Moreover, sensitivities of PEDT/PPO electrodes towards phenol, 4-chlorophenol, 3-chlorophenol are 2.5, 3.8, 0.7 times of the polypyrrole/PPO electrode detection sensitivities respectively [34]. Compared to a sol–gel/PPO electrode described by Wang *et al.* [35], our electrode is 5.6 and 3.3 times more sensitive towards *p*-cresol and phenol respectively.

No significant difference of response time has been observed for *o*-diphenolic and mono-phenolic compounds. While a time-lag is necessary for the sample solution to be homogeneous after substrate addition, a 20–40 s response of the PEDT/PPO biosensor is observed for any substrate. This response time is consistent with results reported by Wang *et al.* [35].

III.2. Determination of inhibitors

Detection of atrazine by inhibition of a tyrosinase bioelectrode has already been reported [22]. Atrazine acts as a competitive inhibitor on the enzyme active site [28]. Similar inhibition with phenyl-urea herbicides was observed by McArdle and Persaud [22]. Since diuron is a phenyl-urea herbicide, both inhibitory effects of atrazine and diuron were determined with the PEDT/PPO electrode.

Bioelectrodes prepared using the electropolymerisation method A did not show any inhibition when they were incubated with atrazine or diuron. Numbers of parameters act on the PEDT/PPO electrode behaviour such as inhibitor, substrate, and product diffusion, kinetic of immobilised tyrosinase, polymer film permeability [36]. For instance, Wang *et al.* [37] showed that the thicker the polymer film the lower the permeability.

A new electropolymerisation method B was adopted to reduce the polymer film thickness while maintaining enough enzyme for a significant response (Figure 1). Since tyrosinase to catechol affinity is higher than to dopamine [38 and 39], this latter substrate was used for inhibitor determination.

The influence of enzyme loading in the polymer on the sensor response to 25 μM dopamine was examined (Figure 2, dashed line). Similar results were observed with method A. Optimal response is achieved when the enzyme solution concentration is 5 μg μl^{-1}. Two seconds of electropolymerisation were found optimal to ensure maximal response (Figure 3, dashed line). It is the best compromise between thickness and amount of immobilised enzyme.

The enzyme chemical environment is the same for both methods A and B. It was assumed pH and temperature optimisation performed on PEDT/PPO electrodes (method A) was still valid for bioelectrodes prepared according the method B. Inhibition tests were carried out using the following conditions: 25 μM dopamine, 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 °C, −200 mV vs. SCE.

Calibration curves for atrazine and diuron are reported in Figure 8. These results agree with those given by other authors [22, 28 and 40]. Diuron has a greater inhibitory action than atrazine on tyrosinase electrode, which is consistent with detection limits 0.5 and 1 mg l^{-1} respectively.

IV. Conclusion

In conclusion, tyrosinase can effectively be immobilised in PEDT electrogenerated film to produce sensitive biosensors. Optimisation of temperature and pH enables the biosensor to detect mono-phenolic and *o*-diphenolic compounds as substrates with high sensitivity and fast response. Compared to osmium hydrogel film, poly-pyrrole amphiphilic, cryo-hydrogel, silica sol–gel based biosensor, sensitivity of phenol detection of the PEDT/PPO electrode is higher than other unmediated immobilisation methods (Table 1).

Detection of herbicides (atrazine, diuron) by enzyme inhibition has also been achieved at μM concentration levels. These results are in agreement with those reported by Besombes *et al.* [28] and Wang *et al.* [40].
Further investigations are required to achieve detection limits of 0.1 nM for catecholamine [41] and 0.1 μg l−1 for a single herbicide in drinking water, 0.5 μg l−1 for the phenol index in water required for medical and environmental applications respectively. The use of mediators would lower detection limits and reduce interferences [27]. Long-term stability could also be improved under dry storage conditions. Biosensors miniaturisation and incorporation in a flow-injection-analysis system are currently underway.

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Figures

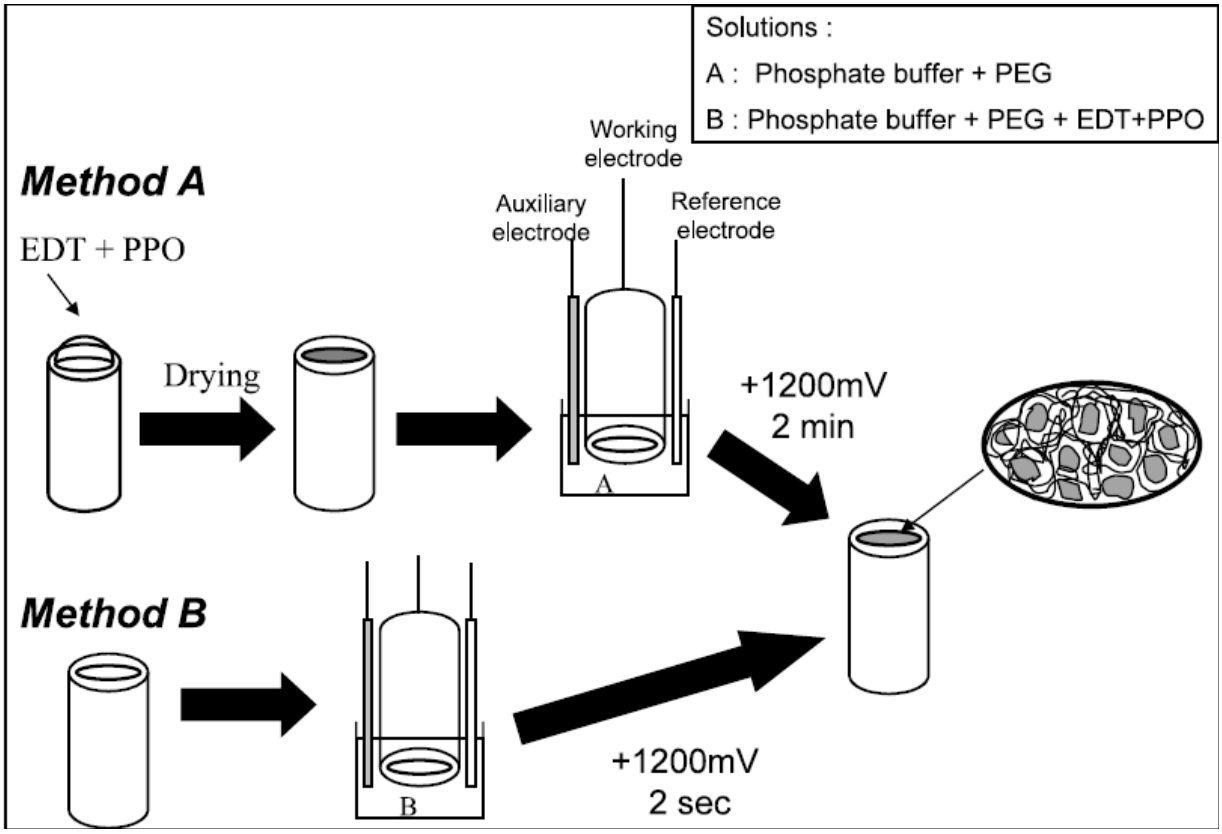

Figure 1: Immobilisation methods for preparation of PEDT/PPO electrode. Method (A) for direct determination of mono-phenolic and o-diphenolic compound and method (B) for inhibitor determination.

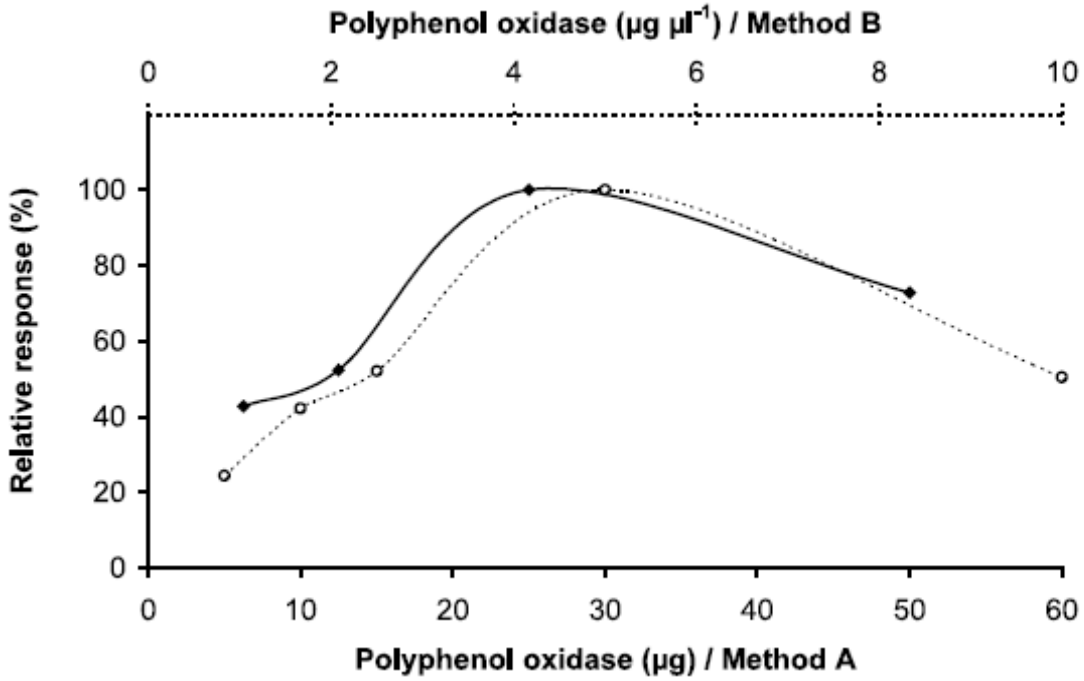

Figure 2: Effect of enzyme loading on PEDT/PPO electrode response to 5 µM catechol (——) using preparation method A (2 min of electropolymerization) and to 25 mM dopamine (-----) using preparation method B (2 s of electropolymerization). Conditions: 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

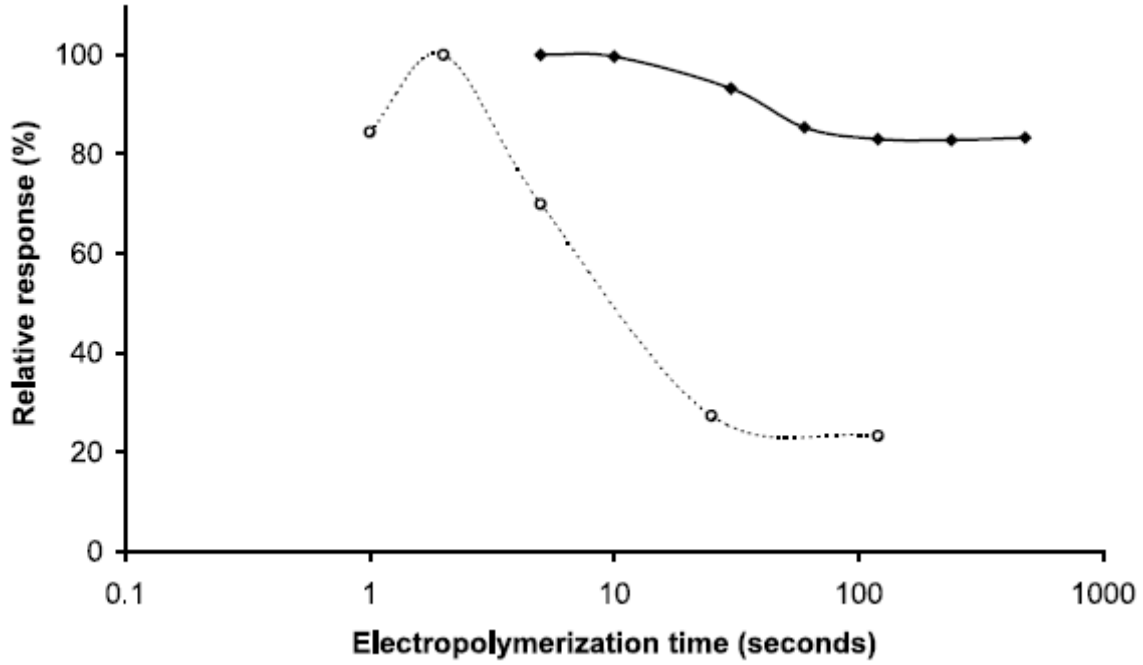

Figure 3: Effect of electropolymerization time on PEDT/PPO electrode response to 5 µM catechol (——/) using preparation method A and to 25 mM dopamine (-----) using preparation method B. Conditions: 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

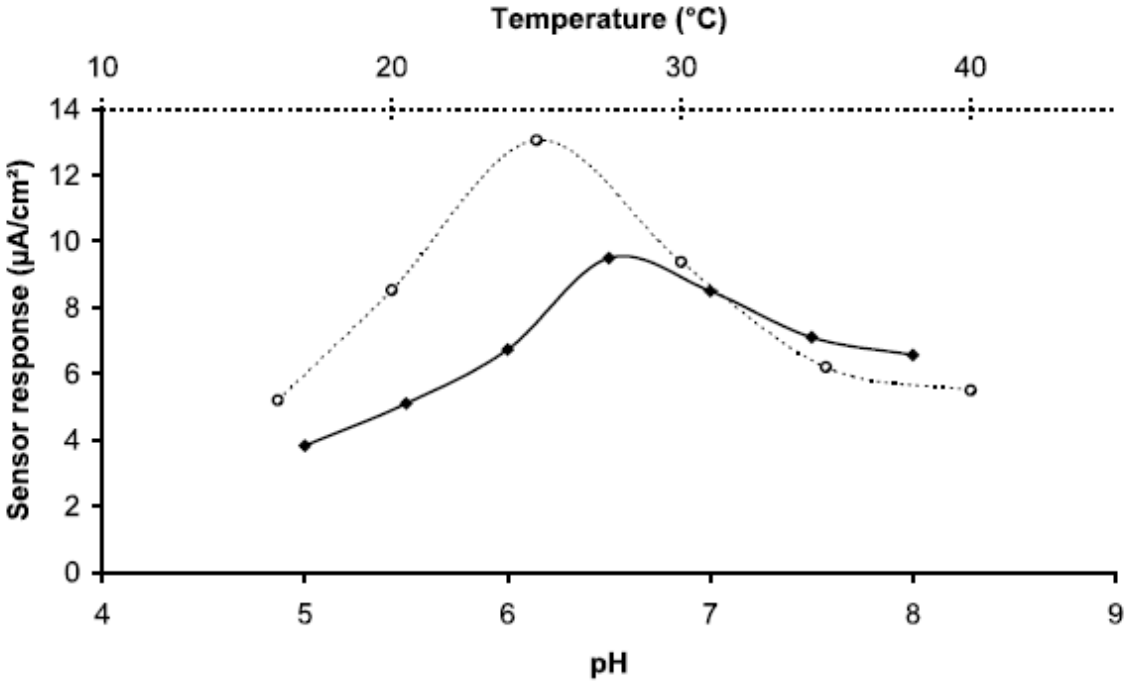

Figure 4: Effect of temperature (-----) and working pH (——) on PEDT/PPO electrode response to 5 µM catechol using preparation method A. Conditions: 0.1 M sodium phosphate buffer and 0.1 M KCl, -/200 mV vs. SCE.

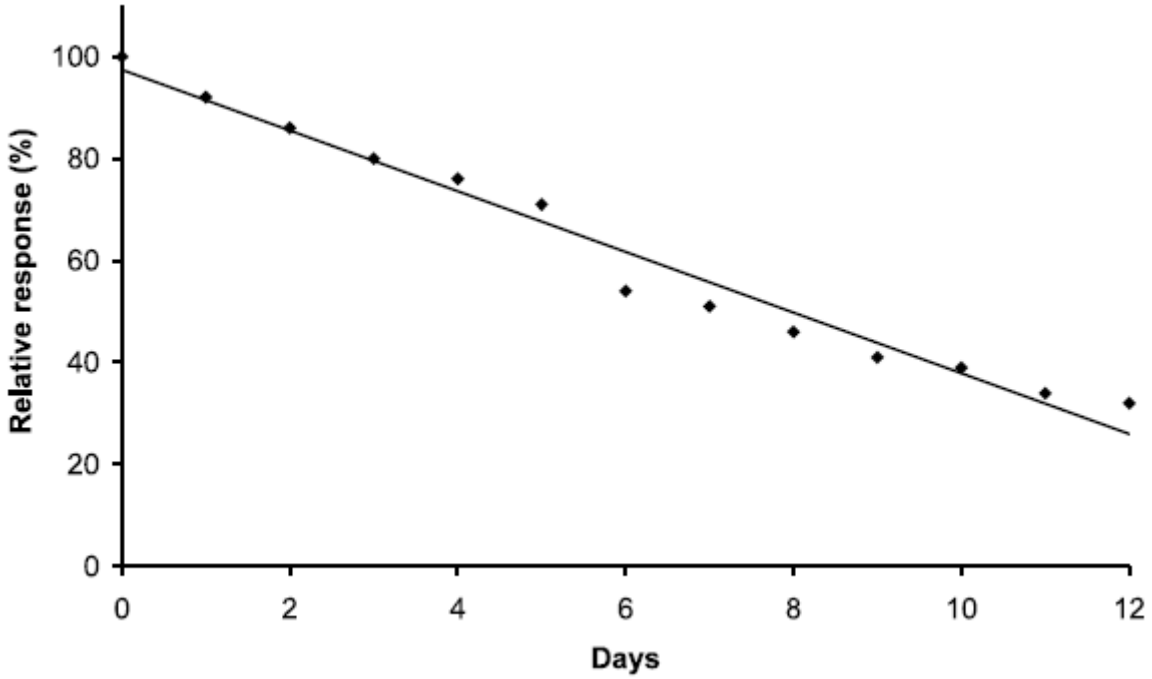

Figure 5: Long-term stability of a PEDT/PPO electrode using preparation method A. Conditions: 5 µM catechol, 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

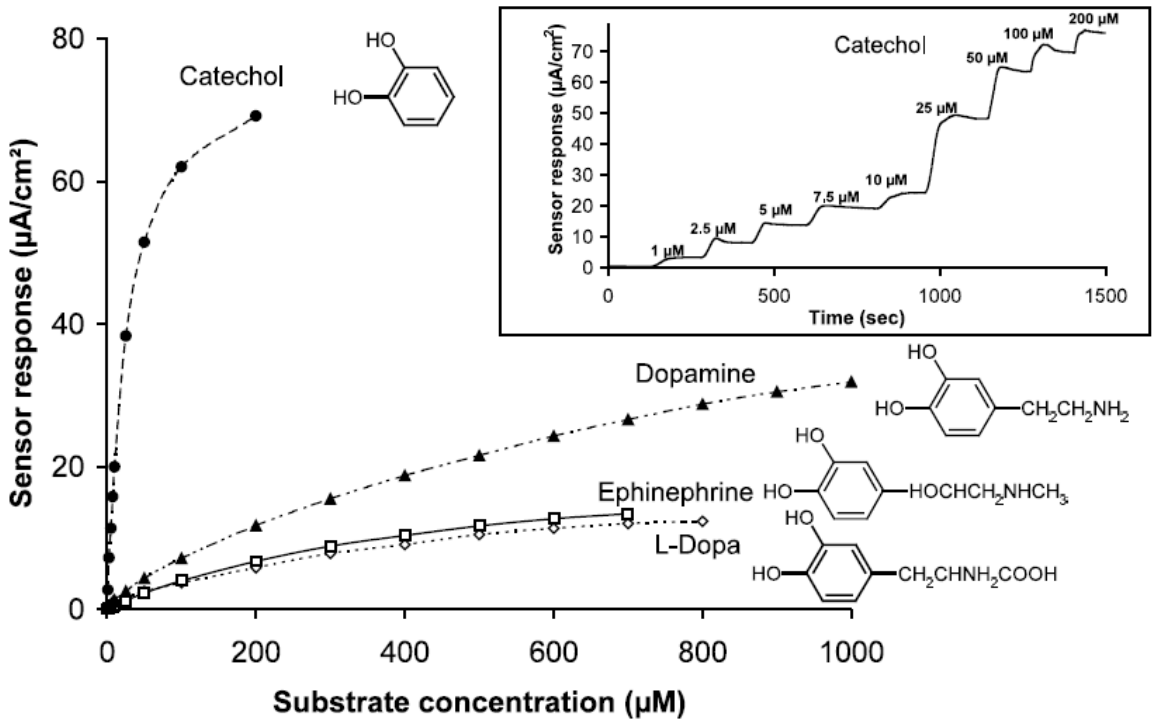

Figure 6: Dose-/response curves of a PEDT/PPO electrode using preparation method A for o-diphenol derivatives. Inset: Typical response of a PEDT/PPO electrode to catechol in an air-saturated buffer. Conditions: 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

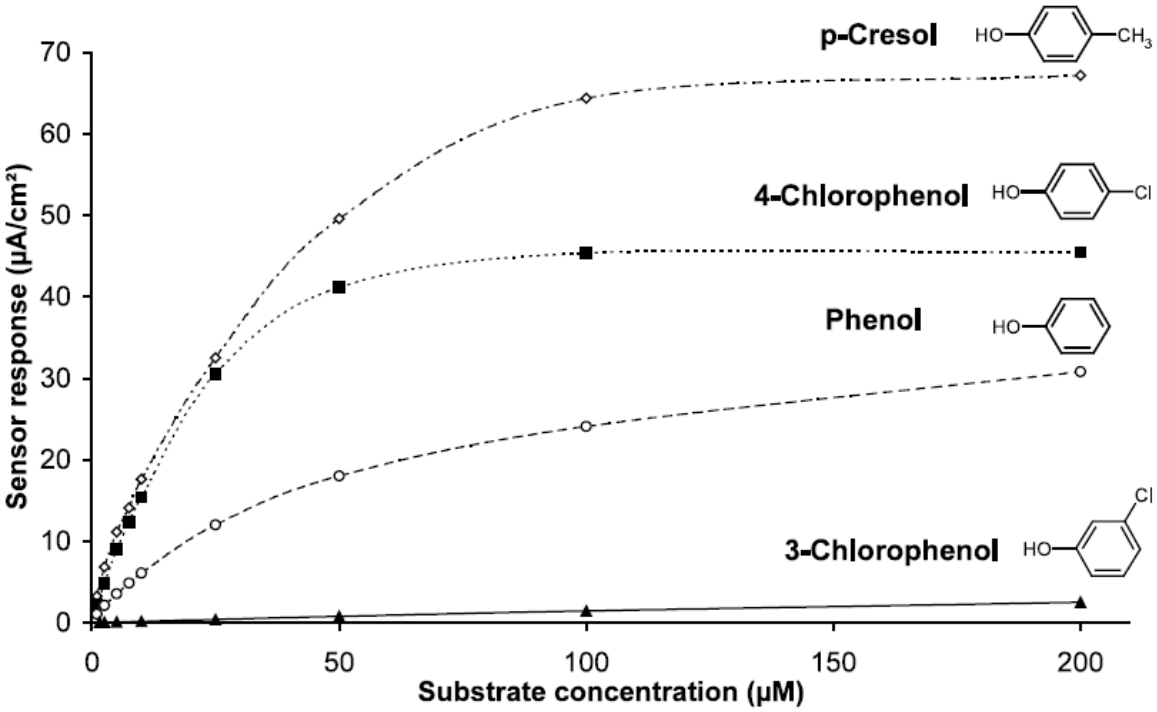

Figure 7: Dose-response curves of a PEDT/PPO electrode using preparation method A for mono-phenol derivatives. Conditions: 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

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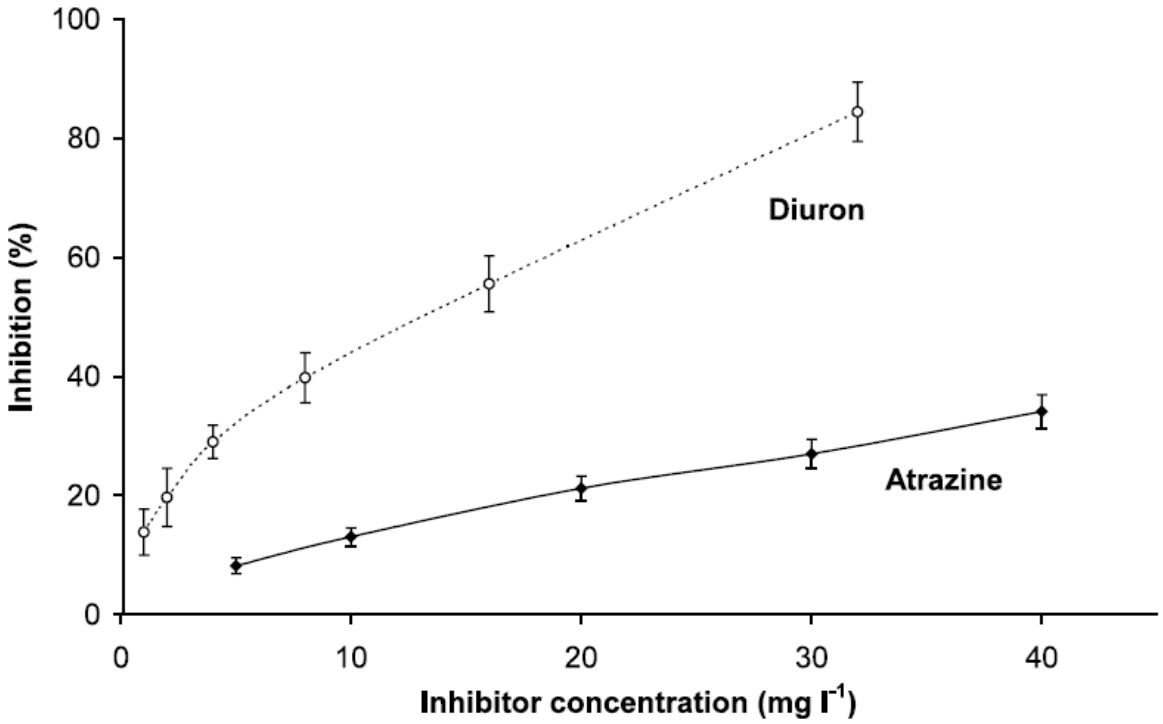

Figure 8: Inhibition effect of atrazine and diuron on the PEDT/PPO electrode response to 25 µM dopamine. Conditions: 0.1 M, pH 6.5 sodium phosphate buffer and 0.1 M KCl, 25 8C, -/200 mV vs. SCE.

Tables

Table 1: Sensitivity of phenol detection of different unmediated biosensors

Immobilisation methods	Sensitivity (mA M-1 cm-2)	Time required for immobilisation procedure (min)	Reference
Osmium hydrogel film	9.6	Several minutes	[27]
Polypyrrole amphiphilic	356	20 min of electropolymerisation	[21, 26]
Cryo-hydrogel	180	39 h of freezing-/thawing cycles	[42]
Silica sol-/gel	184	24 h at 4 8C=/drying of the electrode	[35]
PEDT film	608	2 min	—

Table 2: Response characteristics of the tyrosinase biosensor to several phenolic compounds

	Analyte	Sensitivity (mA M-1 cm-2)	Linear up to (µM)	Limit of detection (nM)
Ortho-diphenol	Catechol	1999	25	—
	Dopamine	133	200	100
	Epinephrine	56	200	500
	L-Dopa	104	10	500
Mono-phenol	p-Cresol	1759	10	5
	4-Chlorophenol	1538	10	5
	Phenol	608	25	50
	3-Chlorophenol	13	100	500