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Development of a biosensor for endocrine disrupting compounds based on tyrosinase entrapped within a poly(thionine) film

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Abstract

Preparation of semiconducting films by electropolymerisation of a monomer which is itself a redox mediator is an attractive and simple method for biosensor fabrication. A polymeric film of the redox dye thionine (phenothiazine) enables the stable immobilisation of polyphenol oxidase (tyrosinase) while acting as mediator for the enzymatic process. The immobilisation method is based on an inner crosslinked tyrosinase layer which contains thionine with an electropolymerised film of poly(thionine) on top. This method gave the most stable redox couple for poly(thionine) and exhibited the greatest response stability. The sensor was tested using a range of synthetic oestrogens and phenolic compounds, which are suspected endocrine disruptors/oestrogen mimics. The device responded well to all compounds tested with limits of detection ranging from 1 to 23 μM (based on three times S/N ratio). The tyrosinase/poly(thionine) electrode response to phenol was 3 orders of magnitude greater than the unmediated response in the absence of poly(thionine).

Keywords: Thionine; Mediator; Electrocatalysis; Endocrine disruptors

1. Introduction

A group of chemicals known collectively as endocrine disrupting compounds (EDCs) are suspected of interfering with the normal function of the endocrine system causing effects adverse effects in humans and wildlife. These include a range of synthetic oestrogens, pesticides, plasticisers and phenolics (Corborn et al., 1996). Concerns have been raised in particular about the contribution of alkylphenols (breakdown products of detergents alkylphenol polyethoxylates) which have been identified in drinking water supplies and waste streams (Blair et al., 2000). They possess phenolic rings (a common structural feature also found in natural oestrogens) and show both in vitro and in vivo estrogenic potential, as demonstrated by Matthews et al. (2000).

Gas chromatography–mass spectrometry (GC–MS) (Markham et al., 1998; Gonzalez-Casado et al., 1998) and capillary electrophoresis (CE) (Regan et al., 2002, 2003; Fogarty et al., 2000) have been the main analytical tools employed for the separation and measurement of these compounds. However tyrosinase (polyphenol oxidase) based electrochemical sensors have the potential to provide a faster, selective alternative to chromatographic methods with potential in water/effluent screening applications.

Tyrosinase is a binuclear copper containing metalloprotein which catalyses the hydroxylation and oxidation of mono and diphenols to *o*-quinones. It can exist as mono and diphenolase (Hedenmo et al., 1997). The mono form can be inactive (met) or active (oxy) and formation of a significant amount of the active form depends on the presence of catechol. When phenol is used as substrate only a small part of the enzyme is active due to the absence of the diphenol 'activator'.

$$\text{Phenol} + \text{met-tyrosinase} \rightarrow \text{catechol} + \text{deoxytyrosinase} \quad (1)$$

$$O_2 + \text{deoxytyrosinase} \rightarrow \text{oxytyrosinase} \quad (2)$$

$$\text{Catechol} + \text{oxytyrosinase} \rightarrow 1,2\text{-benzoquinone} + \text{met-tyrosinase} \quad (3)$$

$$1,2\text{-Benzoquinone} + 2e^- + 2H^+ \rightarrow \text{catechol} \quad (E_{app} = -0.2\,\text{V}) \quad (4)$$

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Since amperometric detection generates catechol (a substrate for the diphenolase activity) this can then undergo successive cycles of enzymatic oxidation followed by electrochemical reduction which induces an amplification effect and a low limit of detection (Cosnier et al., 1999). Drawbacks inherent to phenol biosensors include electrode fouling due to polymerisation of radicals, and enzyme inactivation by the generated *o*-quinone. Redox mediators can achieve a $2e^-$ transfer scheme of *o*-quinone avoiding intermediate radicals and helping to prevent deactivation of the sensor. There have been numerous reports on the analysis of phenolics based on direct measurement of the *o*-quinone product (Cosnier and Innocent, 1993; Serra et al., 2002; Cosnier et al., 1999) or indirectly via mediators (Hedenmo et al., 1997; Cosnier et al., 2001).

An essential task in the fabrication of amperometric sensors is the efficient and effective immobilisation of the biolayer. Electrochemical polymerisation of a film which also mediates the catalytic response has advantages over the use of mediators in solution. Azines (phenothiazines, phenoxines and phenazines) undergo reversible two electron reduction and have received attention for their good electron mediating and electrocatalytic properties. Surface confined and polymeric azines have been widely used as redox indicators and mediators in the fields of biomedical, environmental and biotechnological analysis (Komura et al., 2003). The cationic dyes consist of π conjugated azine rings which absorb visible rays strongly and fluoresce in high quantum yields. Phenazinium dyes have been shown previously to catalyse the reduction of *o*-quinones, however if these redox catalysts are physically adsorbed they suffer from mediator leakage.

Thionine (phenothiazine) is a redox dye which contains two amino groups in the α positions, and has been employed for the electrocatalytic oxidation of NADH (Ohtani et al., 1997; Hajizadeh et al., 1991) by crosslinking onto a graphite electrode. A hydrogen peroxide biosensor was developed by Chen et al. (2001), based on a thionine doped AQ_{55D} polymer. Cosnier et al. (2001) have reported the mediated electrochemical detection of catechols using thionine covalently bound to a poly(dicarbazole) backbone.

A tyrosinase based phenol sensor based on a functionalised polypyrrole layer with redox dye (thionine) covalently bound was developed by Kranz et al. (1998) in order to prevent electrode fouling caused by polymerised quinoid species. This method facilitated electron transfer between the enzyme and the electrode surface and allowed electrocatalytic conversion of compounds generated in the enzyme catalysed reaction, with decreased influence of interfering compounds. Diffusion zones within the layer enabled amplification by means of substrate recycling.

Studies into the electropolymerisation of azines have been reported by Karyakin et al. (1999) and the mechanism of electropolymerisation involves the formation of a cation–radical species after release of one proton from the monomer at high positive potentials. It was proposed by Schlereth and Karyakin (1995) that the monomer units in poly(thionine) could bind to the next monomer unit in the α/β aromatic position (*ortho* with respect to NH_2). Tyrosinase has an isoelectric point of 4.7 (Vedrine et al., 2003) and is therefore negatively charged at pH 7. This allows electrostatic attraction with the positively charged thionine along with interaction through hydrophobic regions.

To our knowledge the first study of the electrochemical behaviour and reaction mechanisms of environmental hormones was carried out by Ngundi et al. (2003) using a number of electrochemical techniques. Recently Notsu et al. (2002) employed a boron doped diamond electrode modified with tyrosinase to detect phenolic derivatives including bisphenol A and 17β-oestradiol (Notsu et al., 2002) but results demonstrated very small reduction currents for the oestrogenic substrates.

Our objectives are to examine the electropolymerisation of thionine incorporated on top of an inner layer of enzyme, in order to achieve a sensitive device for EDC analysis. Our work demonstrates the potential of a mediated reagentless biosensor in detecting a range of these compounds and to the best of our knowledge is the first such report of a mediated biosensor for EDCs.

2. Experimental

2.1. Reagents

Tyrosinase (polyphenol oxidase, PPO) (EC 1.14.18.1; 2590 units mg^{-1} from mushroom), potassium chloride, phenol, bisphenol A, nonylphenol, β-oestradiol, and 17α-ethinyloestradiol, sodium sulphate, acetic acid, glutaraldehyde dipotassium hydrogen phosphate and potassium dihydrogen phosphate were all purchased from Sigma–Aldrich (Dublin). Thionine acetate and bovine serum albumin (fraction V) was purchased form Fluka (Germany). Stock solutions were prepared in analytical grade solvents, acetonitrile for the phenolic compounds and methanol for the synthetic oestrogens. Phosphate buffer (PBS) was prepared using dipotassium hydrogen phosphate and potassium dihydrogen phosphate (0.1 M, pH 6.5) in de-ionised water with 0.1 M KCl.

2.2. Apparatus

Cyclic voltammetric and amperometric measurements were performed using a CH Instruments 750 bi-potentiostat. The electrochemical cell was a three-electrode cell where the enzyme modified glassy carbon macro electrode acted as the working electrode and a platinum wire as the counter electrode. All measurements are versus Ag/AgCl aq. reference electrode.

2.3. Fabrication of the tyrosinase/poly(thionine) biosensor (T/pTH)

The glassy carbon working electrode surface was polished before use with gamma alumina powder, starting with 1.0, 0.3 μm and lastly 0.05 μm powder. The electrode was then washed and sonicated in de-ionised water. The deposition solution was prepared from 2 mg of tyrosinase (2590 units mg^{-1}) and 4 mg of bovine serum albinum (BSA) dissolved in 0.4 mM thionine in PBS. 5 μl of this solution was mixed with 1 μl of 2.5% glutaraldehyde. This mixture was then deposited upon the electrode surface and allowed to cross-link to dryness at room temperature. This resulted in an maximum enzyme activity of 130 units on the surface, assuming no leakage. The polymerisation method was based on that of Yang et al. (1999). The electrode was then immersed into a 0.4 mM thionine solution (in PBS) in the three electrode cell and the potential held at +1.2 V for 2 min while the charge was monitored. The poly(thionine) film was then grown in the same solution by potential cycling between −0.4 V and +0.1 V versus Ag/AgCl at 100 mV s^{-1} for 50 cycles. When not in use the biosensor was stored in phosphate buffer at 4 °C.

2.4. Experimental procedures

Preparation of the buffers employed for the pH study involved the use of Na_2SO_4 (0.1 M) for neutral pH, Na_2SO_4 (0.1 M) + Na_2HPO_4 (20 mM) adjusted with NaOH for basic pH range and Na_2SO_4 (0.1 M) + CH_3COOH (20 mM) adjusted with H_2SO_4 for acidic range. Cyclic voltammetric and amperometric measurements with the biosensor were carried out in a 10 ml electrochemical cell containing 5 ml of phosphate buffer (0.1 M, pH 6.5). Cyclic voltammetry was used to monitor the redox couple of the immobilised thionine mediator. The potential was held at the reduction potential for thionine (−0.2 V versus Ag/AgCl) for hydrodynamic amperometric experiments. When the charging current had decayed and the baseline was stable, substrate was added at regular time intervals and a steady-state current response measured. Unless otherwise stated all experiments were carried out at room temperature in phosphate buffer (0.1 M, pH 6.5) containing 0.1 M KCl.

3. Results and discussion

3.1. Electrochemistry of chosen EDCs at bare electrodes

Each of the endocrine disrupting compounds under study (see Fig. 1 for chemical structures) were examined electrochemically at both glassy carbon and platinum electrodes in different solvents/solvent mixtures. The electrolyte/solvent system which gave the best electrochemistry for both for the synthetic oestrogens (17β-oestradiol, 17α-ethinyloestradiol) and the phenolic compounds (phenol, octyl and nonyl phenol and bisphenol A) was found to be 0.1 M PBS (pH 6.5) with voltammetric response measured at glassy carbon electrodes. As reported by Ngundi et al. (2003) the oxidation peak for phenols is strongly dependent on the type, position and number of substituents. Chemical reactions occur among intermediates and the phenoxonium ion to give side products which form semi-conducting or insulating dimers and polymers which have been known to passivate the working

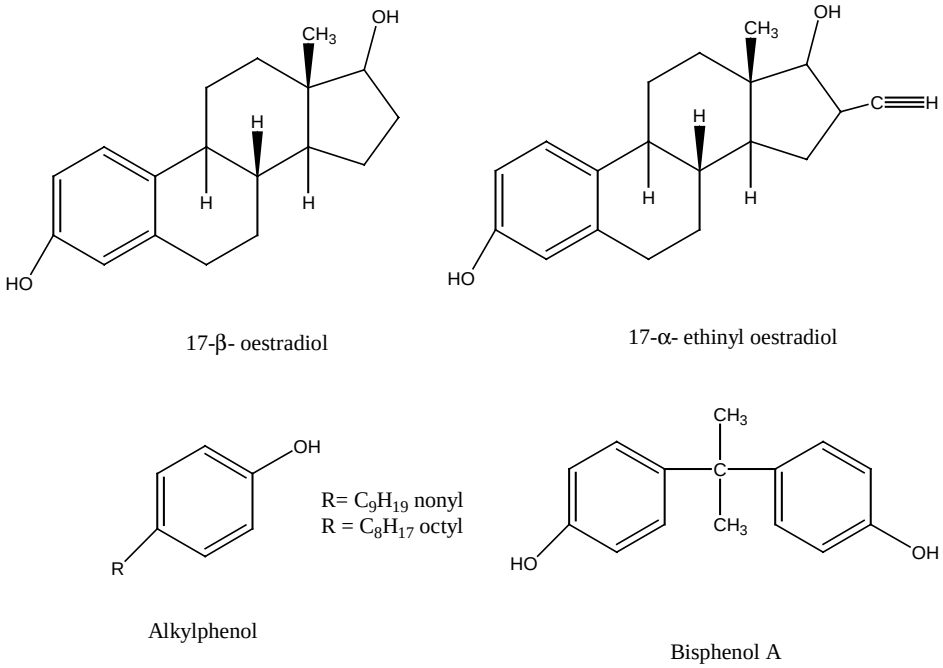


Fig. 1. Chemical structure of relevant suspected oestrogen mimics.

electrode. A cyclic voltammogram of 1 mM phenol was run in PBS at a glassy carbon electrode and showed an irreversible oxidation peak which decreased upon cycling as a result of passivation of electrode due to the growth of the insulating poly(phenol) layer.

Fig. 2 shows cyclic voltammograms for the synthetic oestrogens and nonylphenol over the electrochemically active range. The electrochemical oxidation of synthetic oestrogens was carried out in PBS and all those examined showed similar behaviour (Fig. 2a,c,d). The broad irreversible wave between 0.7 and 0.8 V versus Ag/AgCl decreased upon scanning in each case. This reduction in peaks currents can be attributed to the formation of a semiconductive quinone product which coats the electrode surface, as reported by Ngundi et al. (2003). In the case of the phenolics, oxidation of nonylphenol (Fig. 2b) exhibits the irreversible oxidation peak at 0.6 V versus Ag/AgCl due to anodic oxidation to a phenoxy radical, which can be either further oxidised or coupled with another radical/neutral nonylphenol molecule to form a dimer. This product appears to partially insulate the electrode surface as there is no significant decrease in current after the second scan. A similar CV was observed for octylphenol. The oxidation of bisphenol A also formed a non-conducting poly(bisphenol A) film on the electrode surface and failure to observe a peak during the second scan implied total insulation of the surface.

These results demonstrate the potential difficulties in direct electrochemical measurement of these compounds, as the rapid surface passivation inherent to their oxidation would prevent reproducible analysis. The common phenolic group should provide a site for the action of tyrosinase allowing a biosensor to be developed for EDCs with detection at lower potentials. The electrocatalytic action of the additional polymerised thionine layer will prevent formation of by-products by allowing rapid electron transfer between the enzymatically-generated product and the electrode surface.

3.2. Growth of poly(thionine) and its electrochemical characterisation

Fabrication of the T/pTH biosensor was carried out as described above with formation of a poly(thionine) film on top of the enzyme layer by potential cycling. The growth of poly(thionine) film is dependent on the preanodisation operation where a positive charge can be accumulated to create the thionine cation radical (Yang et al., 1999). These reactive cation radicals are linked through NH bridges to form polymerised thionine. Previous reports have shown that oxidation of thionine leads to a polymer that behaves as discrete units with redox properties similar to that of the monomer (Lee et al., 1990). Fig. 3a shows growth of the polymer at a tyrosinase modified glassy carbon electrode (50 cycles at 100 mV s^{-1}) with electroactivity being due to its heterocyclic nitrogen atoms, N bridges and free amino groups. During the electropolymerisation process the currents of the reversible redox peaks ($E_p(c) = -0.24$ V

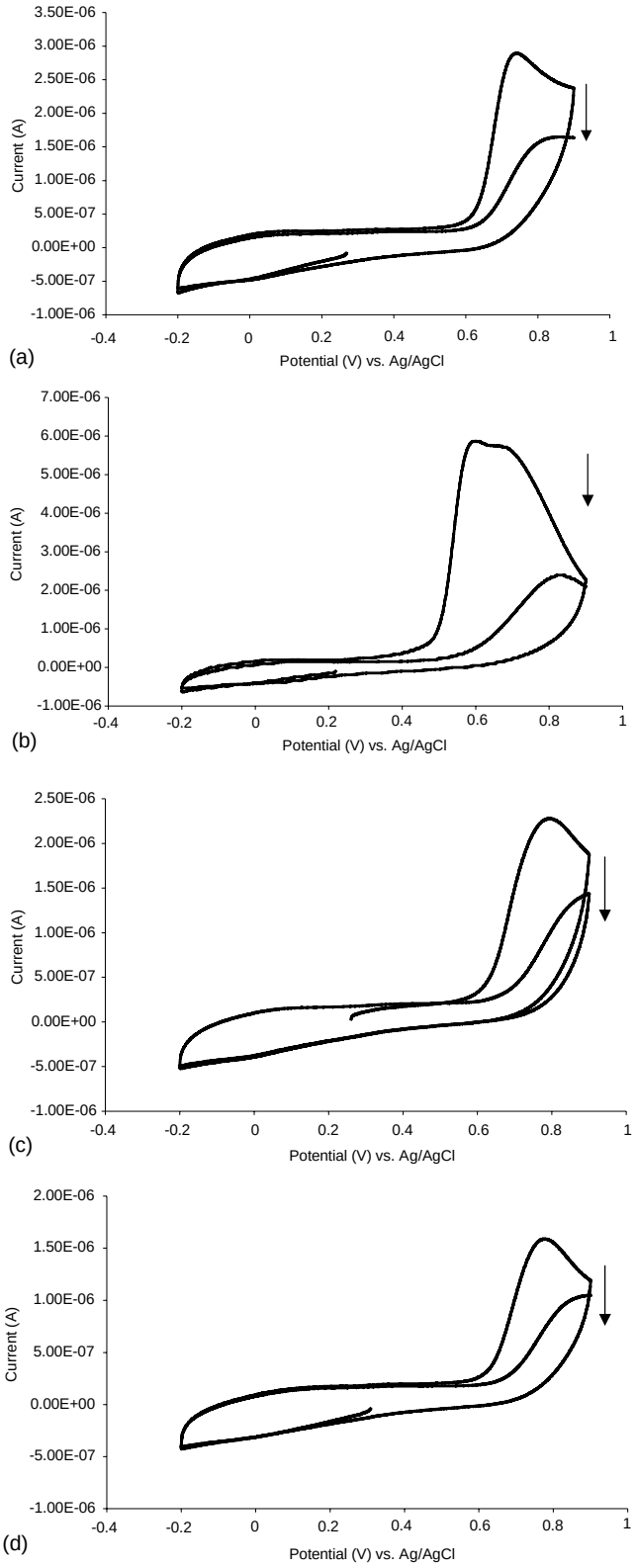
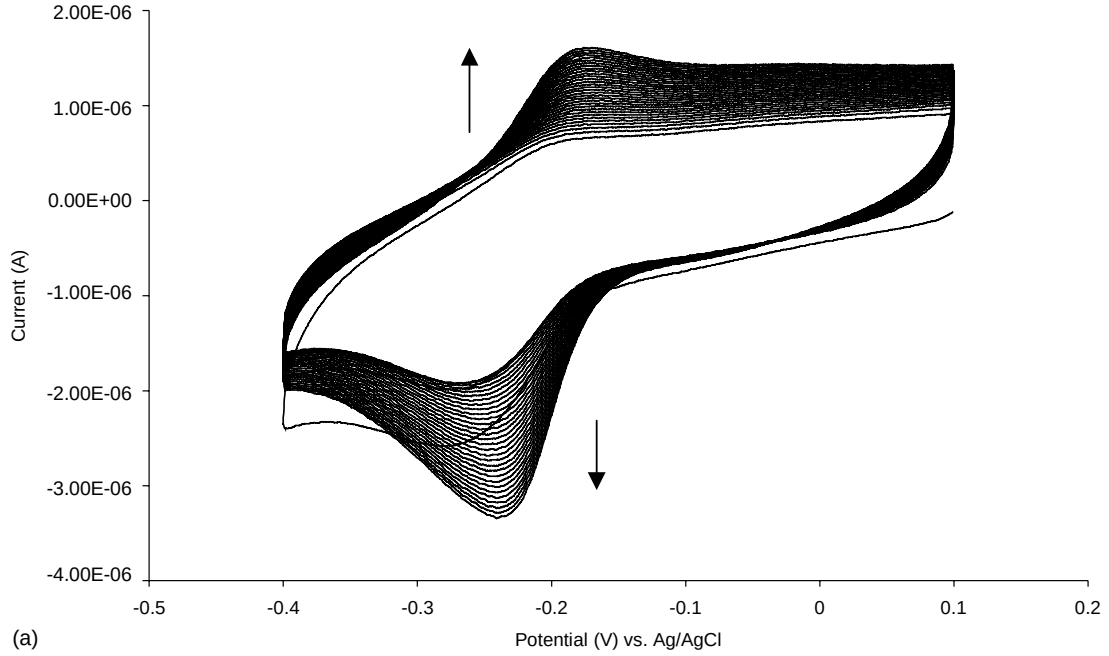


Fig. 2. Cyclic voltammogram of (a) 17β-oestradiol (b) nonylphenol (c) oestriol and (d) 17α-ethinyloestradiol at 1 mM in 0.1 M PBS at glassy carbon electrode; scan rate 100 mV s^{-1} showing first and second cycle.



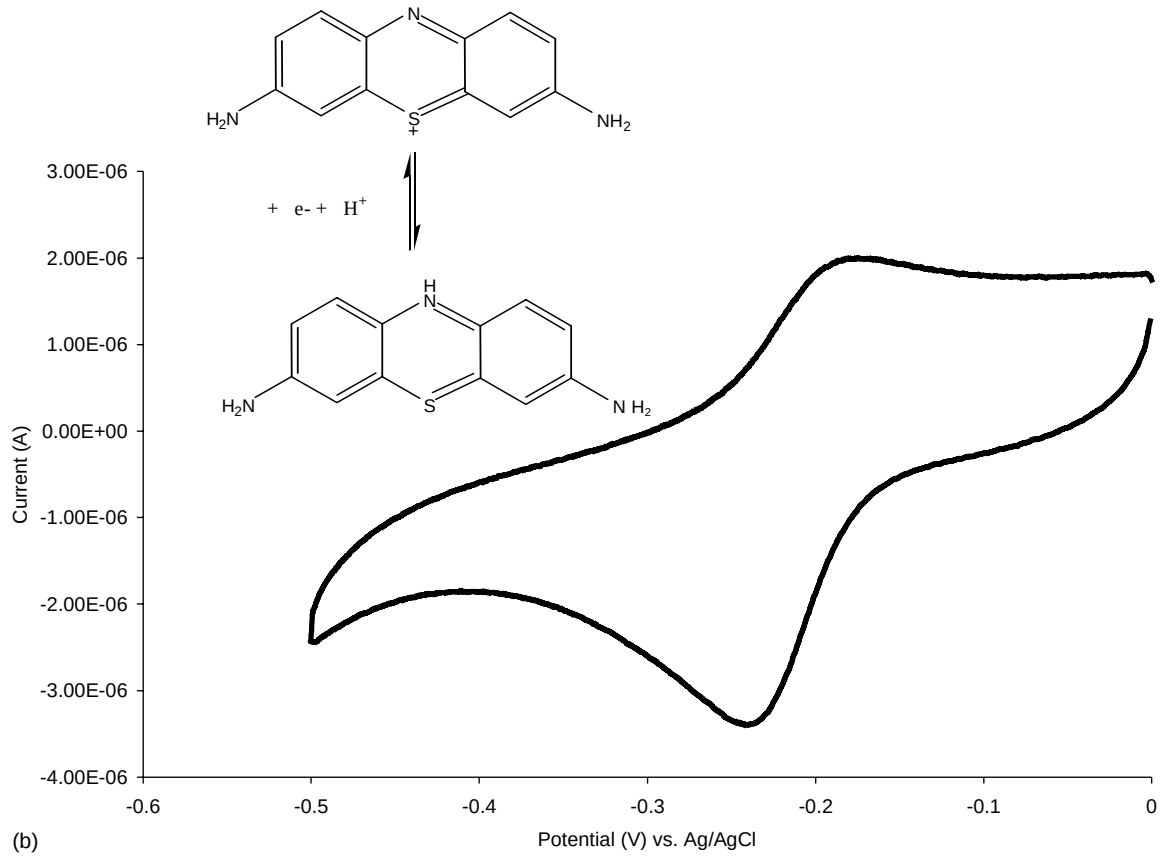


Fig. 3. (a) Cyclic voltammogram showing growth of poly(thionine) on glassy carbon electrode following charging at 1.2 V from solution of 0.4 mM thionine in 0.1 M PBS (pH 6.5) with 0.1 M KCl at 100 mV s^{-1}. (b) Cyclic voltammogram of poly(thionine) on glassy carbon surface after growth and stabilisation by cycling in 0.1 M PBS; scan rate: 100 mV s^{-1}.

and $E_p(a) = -0.18$ V versus Ag/AgCl) increased and then stabilised ($\Delta E_p = 0.057$ V). Following potential cycling, the electrode was rinsed, placed in buffer and cycled at 100 mV s^{-1} over the same potential range until the currents were stable (Fig. 3b). This confirmed the presence of surface attached electroactive material ($\Delta E_p = 0.055$ V versus Ag/AgCl at 5 mV s^{-1}) and the film was found to be stable after 100 cycles. The presence of thionine in the inner enzyme layer was found to improve the stability of the redox couple generated and hence the stability of the sensor.

In order to confirm the number of protons involved in the reduction of thionine a stable biosensor film was fabricated as described above and a plot of $E_{1/2}$ thionine versus pH over the range 3–7 generated. The more protons that are present in the solution the easier it is to reduce the poly(thionine) film, resulting in a negative shift in $E_{1/2}$ with increasing pH. A slope of 52 mV/pH unit was obtained which is close to the expected Nernstian value of 59 mV for a 1 electron 1 proton process. The optimum operating pH for tyrosinase has been found to be 6.5 by Vedrine et al. (2003), and therefore we used this as operating pH.

Surface coverage was calculated to be 6.04×10^{-11} mol cm^{-2} using the area under the cathodic wave at scan rate of 5 mV s^{-1}. A film of poly(thionine) alone without inner enzyme layer resulted in a surface coverage of 2.97×10^{-12} mol cm^{-2}. The difference may be due to the electrostatic attraction between the negatively charged tyrosinase layer and the positively charged thionine resulting in more of the redox polymer on the electrode which contains tyrosinase as the first layer.

A scan rate study was carried out on the T/pTH biosensor over the range 1–200 mV s^{-1}. The anodic and cathodic peak currents for thionine were found to be linear with respect to scan rate up to 30 and 50 mV s^{-1} ($r^2 = 0.9919$) respectively, indicating a surface confined species (see Fig. 4). Between 50 and 200 mV s^{-1} the cathodic current exhibited diffusion controlled behaviour and was linear with respect to square root scan rate ($r^2 = 0.9959$). Therefore it appears that slow charge transport through the polymer becomes the factor controlling the current at larger scan rates. This is in agreement with Chen et al. (2001) who found that Nafion and AQ_{55D} polymer films doped with thionine indicated diffusion controlled behaviour. The anodic peak current was found to be linear only between 9 and 40 mV s^{-1} ($r^2 = 0.9974$) after which the currents were independent of scan rate. As expected for a reversible couple the ΔE_p value did not change over the scan rate range investigated. The scan rate study was repeated for a poly(thionine) film in the absence of enzyme and the film was found to exhibit more typical thin film behaviour in terms of peak shape, smaller peak to peak separation and extended linearity of peak current versus scan rate plot. The relatively thicker biosensor film would be expected to exhibit more diffusion controlled behaviour as the counterions associated with the electron transfer need to move from the electrolyte through the film. In the case of poly(thionine) films alone the surface coverage is less and therefore diffusional resistances to counterion movement also will be less.

3.3. Testing of the T/pTH biosensor based on immobilised tyrosinase with poly(thionine) for determination of EDCs

In order to investigate the mediating ability of thionine towards the tyrosinase reaction, cyclic voltammograms

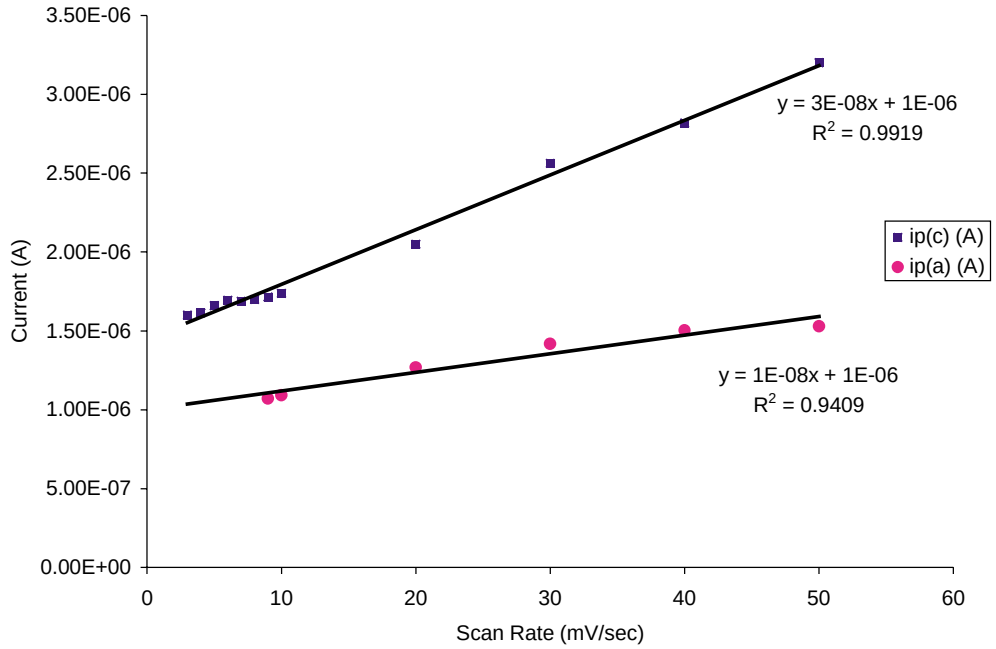


Fig. 4. Anodic and cathodic peak currents vs. scan rate plots for T/pTH electrode in 0.1 M PBS (pH 6.5) with 0.1 M KCl.

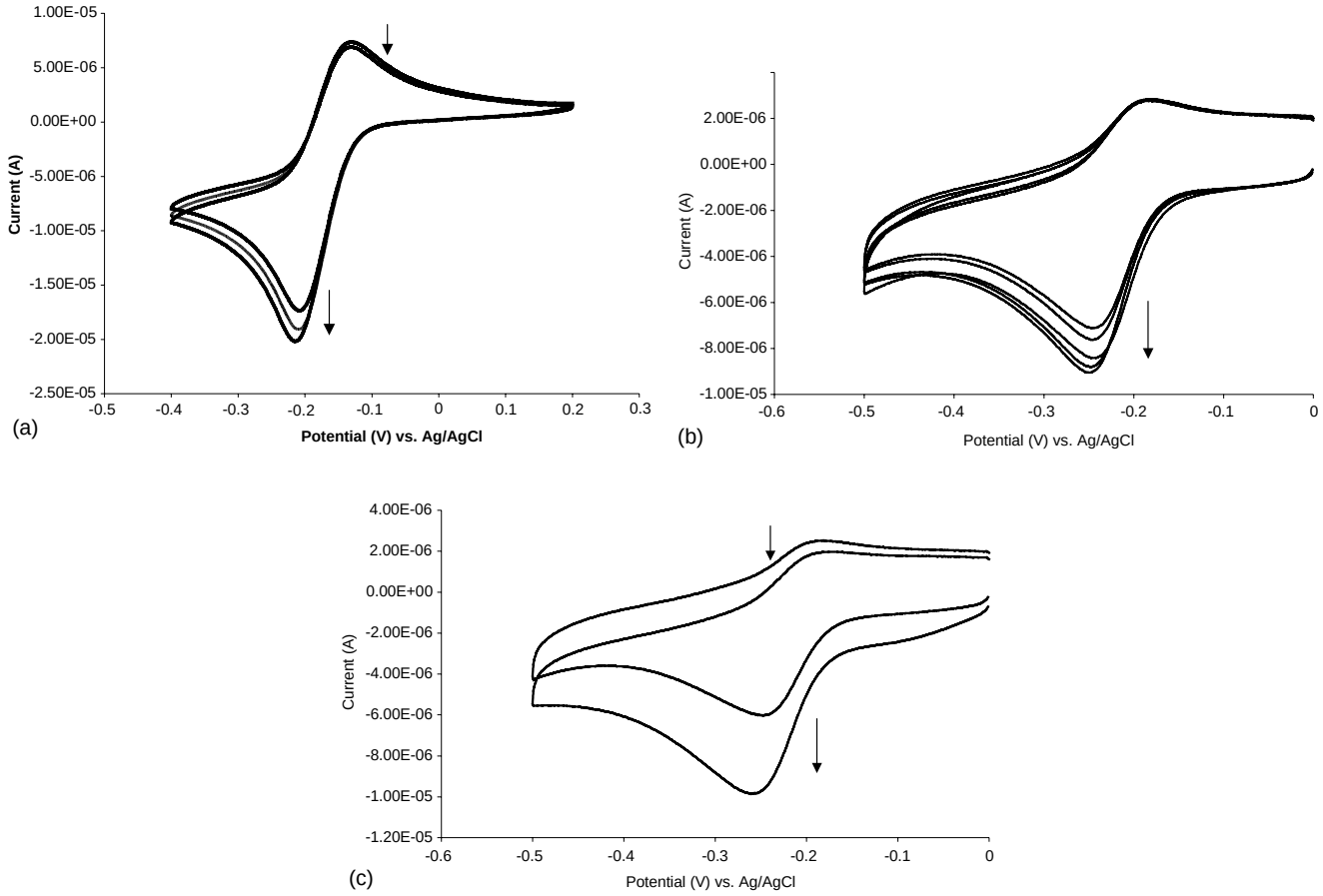


Fig. 5. (a) Electrocatalytic response to: (a) 1 and 2 mM additions of bisphenol A with 0.4 mM thionine in 0.1 M PBS solution at tyrosinase modified electrode; scan rate: 100 mV s^{-1}; (b) 0–4 mM nonylphenol at T/pTH electrode; and (c) 0 and 3 mM bisphenol A at T/pTH electrode.

were carried out using bisphenol A as substrate at a tyrosinase modified electrode with 0.4 mM thionine in solution (Fig. 5a). An increase in the reduction current upon addition of 1 and 2 mM bisphenol A was observed with a slight decrease in oxidation current. A stable T/pTH biosensor was then prepared and the immobilised electrocatalytic effect investigated using all substrates. Results for nonylphenol (0, 1, 2, 3, 4 mM additions) and bisphenol A (0 and 3 mM additions) are shown in Fig. 5b and c. In all cases an increase in reduction current for the thionine couple was observed upon addition of substrate with either no change or a slight decrease in oxidation current. CVs were carried out in the lower potential range well below the direct oxidation of the compounds to prevent electrochemical oxidation and subsequent fouling. In addition, a control electrode with poly(thionine) only was tested in the same manner with no observed change in current.

The postulated electrocatalytic mechanism is given below using phenol as an example with schematic in Fig. 6.

$$\text{Phenol} + \text{met-tyrosinase} \rightarrow \text{catechol} + \text{deoxytyrosinase} \quad (1)$$

$$O_2 + \text{deoxytyrosinase} \rightarrow \text{oxytyrosinase} \quad (2)$$

$$\text{Catechol} + \text{oxytyrosinase} \rightarrow \text{1, 2-benzoquinone} + \text{met-tyrosinase} \quad (3)$$

$$2\text{Thionine}_{(ox)} + 2e^- + 2H^+ \rightarrow 2\text{thionine}_{(red)} \quad (E_{app} = -0.2\,\text{V}) \quad (4)$$

$$\text{1, 2-Benzoquinone} + 2\text{thionine}_{(red)} \rightarrow \text{catechol} + 2\text{thionine}_{(ox)} \quad (5)$$

As the reduced form of thionine which is generated electrochemically (4), is used up in the reaction with the enzymatic product (5) and thionine$_{(ox)}$ is generated, this results in an enhanced cathodic current and a decreased anodic current for the thionine couple. This reflects the establishment of a fast catalytic two electron transfer process between the quinone and the electrode surface via the redox catalyst.

Hydrodynamic amperometry was carried out at $E_{app} = -0.3$ V versus Ag/AgCl for the synthetic oestrogens and at $E_{app} = -0.2$ V for the phenolic compounds (see examples of responses in Fig. 7a and b). The cathodic peak current for thionine reduction appeared to shift from −0.2 to −0.3 V

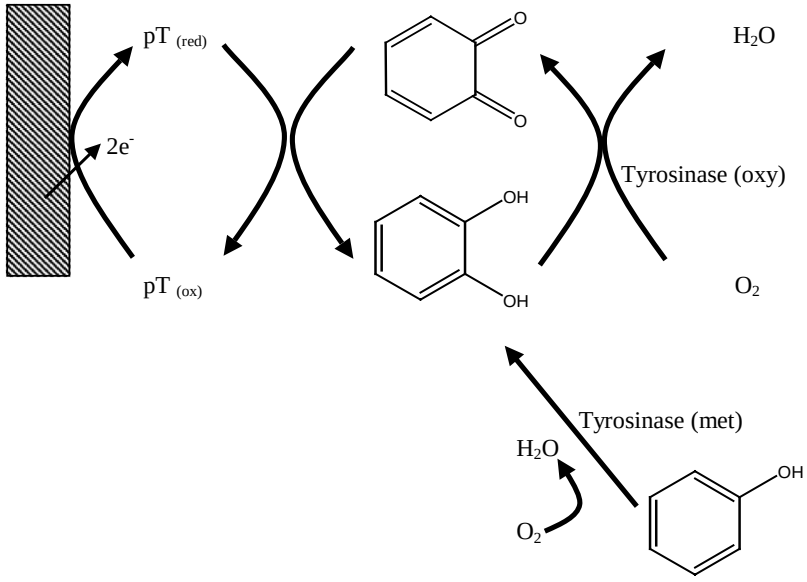


Fig. 6. Schematic for proposed surface processes at biosensor layer.

when the synthetic oestrogens were used as substrates, perhaps due to steric/size effects. This allows a certain degree of selectivity in the detection potential. As a control the response of a similarly prepared electrode without thionine was investigated in a similar manner. The steady state responses of the unmediated and mediated electrodes were compared using phenol as substrate over the range 1–5 mM. The sensitivity for the unmediated phenol sensor was 2.0×10^{-8} A mM^{-1} compared with 4.47×10^{-5} A mM^{-1} for the mediated sensor. This three order of magnitude increase in sensitivity reflects the electrocatalysis achieved by the presence of thionine, which allows a more efficient recycling of the *o*-quinone. Kranz et al. (1998) reported a tyrosinase based phenol sensor based on a polypyrrole layer with covalently attached thionine. The low limits of detection reached were explained by the establishment of a diffusion zone within the layer which hinders the escape of reaction products from the enzyme containing reaction layer to the electrolyte, and allows amplification of the signal as a result of catechol regeneration. In our case, the stable tyrosinase inner layer with poly(thionine) outer layer may also prevent catechol escape resulting in greater current response.

The steady-state currents were used to generate standard curves and Table 1 summarises the analytical data.

While a direct comparison of the analytical data generated using this method with previous reports using tyrosinase (mediated and unmediated) is not possible due to the novel analytes employed, Table 2 summarises some characteristics of recent phenol sensors compared to our sensor. Overall the device reported here compares favourably with other reported tyrosinase sensors in terms of the parameters outlined. The simple production and dual function of the poly(thionine) layer is advantageous in terms of enzyme immobilisation and enhanced electrocatalytic effect.

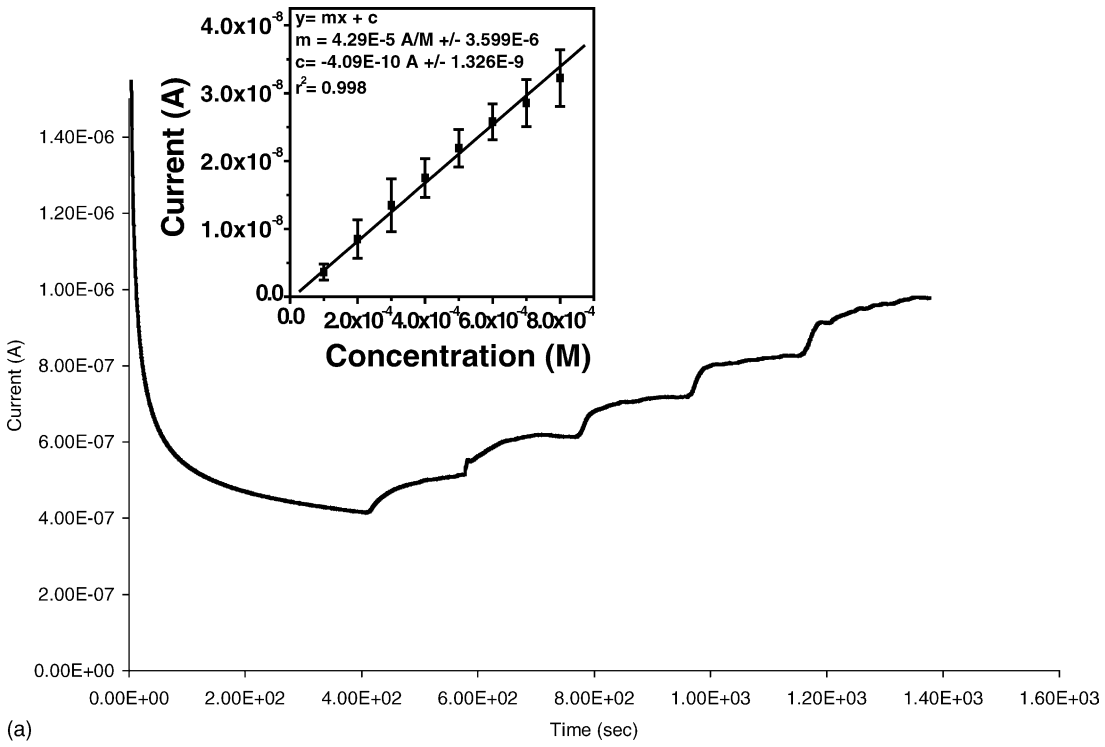
Previous reports of EDC analysis carried out in our laboratory by Fogarty et al. (2000) involved the use of capillary electrophoresis (CE) with UV detection for analysis of mixtures of analytes. This involved lengthy and complex buffer preparation, pre and post capillary rinses resulting in long total analysis times, relative to the method reported here which involves reproducible and convenient electrode preparation, with fast (<1 min) response times by amperometry.

The low linear range of these compounds may be due to the limited solubility of these compounds in PBS. However in terms of environmental analysis of these compounds the limits of detection in the μM range are of importance (LOD based on three times S/N ratio). Calibration plots were generated from amperometric experiments for $n = 5$ measurements over the range 2×10^{-4} to 8×10^{-4} M for β-oestradiol ($y = 4.29 \times 10^{-5}$ A M$^{-1} \pm 3.59 \times 10^{-6} - 4.1 \times 10^{-10}$ A) ($r^2 = 0.998$) (see Fig. 7a). Similar data was generated for phenol (Fig. 7b) over the range 2×10^{-5} to 8×10^{-5} M ($y = 0.0447$ A M$^{-1} \pm 2.3 \times 10^{-3} - 1.4 \times 10^{-7}$) ($r^2 = 0.996$).

Table 1
Response characteristics of tyrosinase biosensor to selected endocrine disruptor compounds

Analyte	Sensitivity[a] (A mM^{-1})	Linear range[a] (mM)	Limit of detection[b] (μM)
17α-Ethinyloestradiol	2×10^{-6}	0.4	15.1
17β-Oestradiol	4.3×10^{-8}	0.8	14.9
Phenol	4.47×10^{-5}	0.08	1.0
Nonylphenol	1×10^{-6}	0.5	10.0
Bisphenol A	4×10^{-7}	0.4	23.0

[a] Calculated from slopes of calibration curves generated from amperometric data.
[b] Based on three times S/N ratio.



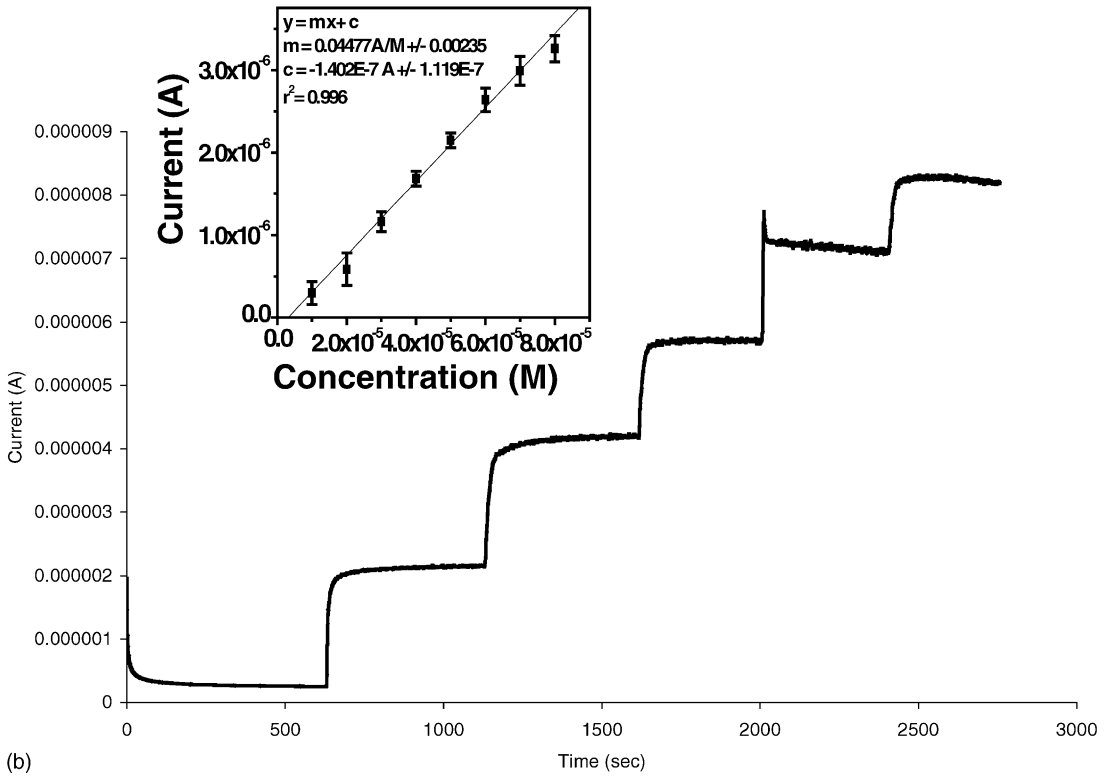


Fig. 7. Hydrodynamic amperometric data for (a) 17β-oestradiol ($E_{app} = -0.3$ V vs. Ag/AgCl) representing 0.1 mM additions and (b) phenol ($E_{app} = -0.2$ V vs. Ag/AgCl) 10 μM additions.

Table 2
Comparison of recent mediated/unmediated tyrosinase biosensors

Mediation/immobilisation method	Analyte	Sensitivity	Response time $t_{0.95}$ (s)	Stability	Limit of detection (M)	Reference
Unmediated/immobilisation in conducting polymer	Phenol	6.08×10^{-1} A M^{-1} cm^{-2}	20–40	−60% response after 12 days	5×10^{-8}	Vedrine et al. (2003)
Unmediated/boron doped diamond electrode	Phenol	–	–	12 h	1×10^{-7}	Notsu et al. (2002)
Unmediated/pyrrole monomer	Catechol	3×10^{-4} M^{-1}	3	–	2×10^{-9}	Cosnier and Innocent (1993)
Unmediated/RVC epoxy	Phenol	3.4×10^{-3} A M^{-1}	–	–	1.1×10^{-2}	Serra et al. (2002)
Osmium (4,4-dimethyl-2,2-bipy) (1,10-phenanthroline)	Phenol	3.9×10^{-3} A M^{-1} cm^{-2}	3–66	Unchanged after 1 week	–	Hedenmo et al. (1997)
Thionine grafted to poly(dicarbazole)	Catechol	1.46×10^{-1} A M^{-1} cm^{-2}	10	−55% after 5 days	–	Cosnier et al. (2001)
Poly(thionine)	Phenol	0.63 A M^{-1} cm^{-2}	33	−43% after 5 days	1×10^{-6}	–

Electrode stability was tested daily using amperometry over a 3-week period. This was achieved by injecting 50 μM phenol following application of −0.2 V versus Ag/AgCl to the electrode surface with subsequent current decay. In between measurements the electrode was stored at 4 °C in PBS. The percent decrease in current response was 43% after 5 days and 94% after 10 days testing demonstrating that leaching of enzyme from the film was taking place with time.

If we assume that the kinetic process limiting current is not substrate mass transport but the enzymatic reaction then apparent kinetic parameters may be determined from the electrochemical Eadie–Hofstee form of the Michaelis–Menton equation (Cosnier and Innocent, 1993):

$$J = J_{\max} - K_{\mathrm{m}}^{\mathrm{app}} \left(\frac{J}{C} \right)$$

where J is the steady state current density, $J_{\max}$ is the maximum current density under saturated substrate conditions, C is substrate concentration and $K_{\mathrm{m}}^{\mathrm{app}}$ is the apparent Michaelis–Menton constant which is characteristic of the enzyme electrode. The kinetic parameters and catalytic efficiency data ($J_{\max}/K_{\mathrm{m}}^{\mathrm{app}}$) are given in Table 3 with the lowest apparent Michaelis–Menton constant obtained for phenol, corresponding with the most sensitive response. This is not surprising as the other phenolic and oestrogenic compounds are less amenable substrates for the enzyme, due to size and steric effects.

Table 3
Kinetic parameters for phenolic endocrine disruptors based on data from Eadie–Hofstee plots

Analyte	$K_{\mathrm{m}}^{\mathrm{app}}$ (mM)	$J_{\max}$ (A m^{-2})	$J_{\max}/K_{\mathrm{m}}^{\mathrm{app}}$ (A m^{-2} mM^{-1})
Bisphenol A	0.222	5.40×10^{-3}	2.4×10^{-2}
Nonylphenol	0.2897	1.43×10^{-2}	5.1×10^{-2}
Phenol	0.111	3.60×10^{-1}	3.2

4. Conclusions

In conclusion, we have successfully demonstrated the ability of a tyrosinase biosensor to respond to a number of oestrogenic substrates. In order to prevent electrode fouling due to build up of quinoid enzymatic products a mediated approach using a polymerised layer of thionine on top of the cross-linked enzyme was used. This served both as electrocatalyst for quinone and also allowed for stable and simple immobilisation of the inner tyrosinase layer. The dual role of thionine, obviates any requirement for chemical attachment of mediator to conducting polymers with subsequent stability and loading issues. The simple two step procedure allows reproducible sensors to be fabricated and allows for signal enhancement over that of the unmediated approach. Amperometric data demonstrates good sensitivity, low LODs and fast response times compared with other tyrosinase based devices (using phenol for comparison) and a certain degree of selectivity in terms of choice of operating potential, while at this point not allowing for simultaneous multi-component analysis. Further work in our laboratory will explore the combination of this electrochemical detector with a microchip capillary electrophoresis separation method, allowing individual EDCs to be measured within environmental mixtures.

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