Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 68 (2006) 791–799
Talanta
www.elsevier.com/locate/talanta

# A comparative study of immobilization methods of a tyrosinase enzyme on electrodes and their application to the detection of dichlorvos organophosphorus insecticide

Juan C. Vidal *, Silvia Esteban, Javier Gil, Juan R. Castillo

*Department of Analytical Chemistry, Analytical Spectroscopy and Sensors Group (GEAS), Science Faculty, University of Zaragoza, Pza. San Francisco s/n, 50009 Zaragoza, Spain*

Received 29 December 2004; received in revised form 29 April 2005; accepted 8 June 2005
Available online 22 August 2005

**Abstract**

The use of several designs of amperometric enzymatic biosensors based on the immobilized tyrosinase enzyme (Tyr) for determining dichlorvos organophosphate pesticide are described. The biosensors are based on the reversible inhibition of the enzyme and the chronocoulometric measurement of the charge due to the charge-transfer mediator 1,2-naphthoquinone-4-sulfonate (NQS). Tyr becomes active when reducing the quinone form of the mediator molecule (NQS) to the reactive *o*-diol form substrate of Tyr ($H_2$NQS) at the working electrode, thus permitting modulation of the catalytic activity of the enzyme and measurement of the inhibition produced by the pesticide. The full activity of the enzyme reversibly recovers after removal of the pesticide and re-oxidation of $H_2$NQS.

Tyr was immobilized onto electrodes using different procedures: (i) entrapment within electropolymerized conducting and non-conducting polymers, (ii) covalent attachment to self-assembled monolayers (SAM), (iii) cross-linking with glutaraldehyde (and nafion covering) and (iv) dispersion within carbon-paste electrodes. The mediator was co-immobilized onto the working electrode next to the enzyme and reagentless biosensors were subsequently constructed. In the SAM design (ii) NQS was added to the solution. The analytical properties of the different biosensors based on the competitive inhibition produced by dichlorvos were then compared. A detection limit of about 0.06 μM was obtained for dichlorvos with entrapment of NQS and Tyr within electropolymerized poly(*o*-phenylenediamine) polymer (*o*PPD), which was the design that proved to have the best analytical performance.


*Keywords:* Amperometric biosensor; Organophosphorus pesticides; Dichlorvos; Tyrosinase enzyme inhibition

## 1. Introduction

Organophosphorus (OP) and carbamate (CAR) derivative pesticides are nowadays increasingly used as insecticides owing to their shorter persistence in the environment if compared with organochlorate derivatives, for example, which are more much important contaminating agents. The toxicity of OP and CAR pesticides is due to inhibition of the cholinesterase and the interference in neuronal pulse transmissions in living organisms [1,2]. Many OP derivatives used as pesticides also produce delayed neuropathy in humans several weeks after exposure caused by inhibition of the acetylcholine enzyme [2]. For this reason, significant progress has been made in recent years in the development of traditional analytical methods for the determination of small concentrations (ng $mL^{-1}$) of this kind of pesticide in environmental samples [3], food samples [4,5] and in monitoring biological samples [6].

Bioanalytical tools (enzyme activity/inhibition assays) and affinity-based assays using antibodies or receptors for the study of pesticides complement classical analytical methods of analysis (gas-chromatography (GC) and mass-spectrometry), and have certain advantages: simplicity, sensitivity, selectivity, portability, low cost, no sample

* Corresponding author. Fax: +34 976 761292.
*E-mail address:* jcvidal@unizar.es (J.C. Vidal).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.06.038

pre-treatment is needed and analysis times are short [7]. As a result, there has been a significant increase in the number of new biosensors in recent years [8]. Although amperometric transduction predominates, the use of quartz-crystal microbalance [9] and surface acoustic-wave impedance [10] has also increased considerably, and conductometric biosensors [11] are also used in pesticide determinations.

From the mid 1980s onwards, a significant number of amperometric enzymatic biosensors for determining pesticides have appeared, for the most part based on the inhibition produced in acetylcholine esterase (AChE) [12] and butyrylcholine esterase enzymes (BuChE) [13].

Unfortunately, a problem arises in practice as inhibition is produced by an irreversible complex of the enzyme (e.g. AChE) with the pesticide, and this irreversibility of the inhibition requires reactivation of the enzyme immobilized in the amperometric biosensors. This is usually carried out using nucleophilic reagents [8], although in some cases regeneration of the biosensor is impossible and in this case disposable biosensors are designed.

Although the use of biosensors based on the inhibition of immobilized cholinesterases predominates, new, mediated bioelectrocatalytic biosensors with less traditional enzymes for the detection of both carbamate and organophosphorus pesticides are rapidly emerging with enzymes like tyrosinase (Tyr), alkaline and acid phosphatase, glucose oxidase in the presence of acid phosphatase, ascorbic acid oxidase or organophosphorus hydrolase with favourable analytical properties [14,15]. In some cases, non-inhibition sensors have been described with some of these enzymes [14].

Tyr (monophenol monoxygenase) is a binuclear copper monooxygenase enzyme containing metalloprotein, it is widespread in nature throughout the phylogenetic scale from bacteria to mammals, and takes part in a large number of biological reactions. Tyr catalyzes the *o*-hydroxylation of monophenols to form *o*-diphenols (cresolate activity) and the oxidation of *o*-diphenols to *o*-quinones (cathecolase activity), or both, at the expense of oxygen as an electron-acceptor [15]. Tyr has a binding site with affinity for aromatic compounds (the substrate site) and another with affinity for a metal binding agent (oxygen site). Consequently, the activity of Tyr is affected by a large variety of inhibitors, some of them having copper-chelating properties. For example, Tyr is inhibited by carbamate, organophosphorus and dithiocarbamate pesticides, atrazines, thioureas, aromatic carboxylic acids, triazine and phenyl-urea herbicides, pyridinones, chlorophenols and copper chelating agents, but it is not inhibited by metals [8]. The use of Tyr for the study of the inhibitory effects of organic pollutants is recent with the first articles appearing in the middle of noventies, but is becoming more widespread.

*O*-Quinones are easily reduced to *o*-diphenols at low overpotential without needing an electrochemical catalyst and so Tyr has been used in most cases for determining phenolic compounds based on amperometric transduction with monitoring of the enzymatically formed quinonic compounds [16–18]. However, problems do arise at times as quinoid products inhibit Tyr due to the irreversible union produced and because the electrode surface is passivized by the formation of insulating polymer films [19].

Another way of applying the enzyme consists of monitoring the inhibition caused by numerous compounds on Tyr, measuring the inhibition produced by the analyte during the reaction of the enzyme in the presence of a substrate at a constant known concentration in the presence of molecular oxygen. This procedure has been the subject of more recent papers dealing with environmental contaminants. In the study of OP and CAR pesticides, for example, where the presence of the pesticide inhibits the reaction of the enzyme, tyrosinase has been immobilized by covalent or non-specific interactions on a variety of electrode surfaces to construct amperometric biosensors, namely, electropolymerization of aniline onto a polyacrylonitrile-coated Pt electrode [20], onto electropolymerized poly(3,4-ethylenedioxythiophene) [21], in the form of Tyr-colloidal gold onto a carbon-paste electrode [22], onto rigid composite matrices of teflon–graphite, reticulated vitreous carbon-epoxy resin and graphite-ethylene/propylene/diene terpolymer [17], onto screen-printed carbon electrodes [23], by cross-linking with glutaric dialdehyde [24], by grafting onto an electropolymerized poly(dicarbazole) film [19], by cross-linking onto self-assembled monolayers [17], using silica sol–gel composite films [25] or with hydrogels and poly(vinyl alcohol) matrices [26].

Several substrates for Tyr with different affinities for the active site of the enzyme have also been used for amperometric inhibition biosensor designs for pesticides and, consequently, the sensitivity range varies considerably depending on the characteristics of the substrate. The most frequently used substrate is catechol [27,28] but other substrates employed include L-DOPA, dopamine, epinephrine and 3,4-dihydroxyphenylacetic acid [29].

In a previous paper, it was shown that the redox 1,2-naphthoquinone-4-sulfonate (NQS) mediator in solution, in its reduced form, may be an active substrate of Tyr and has been used for determining the inhibition produced by organophosphorous pesticides. The authors created an enzymatic on/off switch mechanism by electroanalytically reducing the NQS to the enzymatically active substrate (*o*-diol) molecule ($H_2$NQS) at the working electrode [30]. However, the mediator was used in the dissolved sample as a diffusing agent and was added for every analysis carried out. In this paper, the use of a similar mediated system is described, for which different Tyr immobilization procedures were designed and compared. Unlike the previous study, co-immobilization of the NQS mediator enzyme directly onto the electrode was carried out in most designs. In this way, a reagentless biosensor was constructed for the direct determination of the pesticide without having to add the substrate to every sample solution.

The organophosphorus dichlorvos pesticide was used as a model. Dichlorvos is a less persistent pesticide when it enters the environment (when dissolved in water it takes about 24–36 h for half of the chemical to be broken down with microorganisms) and does not appear to accumulate in plants, fish or animals [31]. It also evaporates easily into the air where it is broken down into less harmful chemicals. The operational and analytical characteristics of all the biosensors for the dichlorvos pesticide were studied and their analytical performances compared.

## 2. Experimental

### 2.1. Apparatus and reagents

The electroanalytical experiments were performed in an Autolab PGSTAT 1212 from Eco Chemie (Utrecht, Netherlands), with a BAS C3 voltammetric cell-stand (Bioanalytical Systems, West Lafayette, USA). The potentiostatic system consisted of a three-electrode system with a Pt-wire auxiliary electrode and an Ag/AgCl (3 M NaCl) reference electrode. Au-disks (1.6 mm diameter), glassy-carbon (3 mm diameter), carbon-paste (hole of 1.6 mm diameter, 3 mm depth) and Pt-disk (1.6 mm diameter) working electrodes were from BAS (Bioanalytical Systems, BAS, Lafayette, USA). Au wires (0.5 mm diameter, 5 cm length), also used as working electrodes, were purchased from GoodFellow (Cambridge, UK).

Tyrosinase (Tyr) enzyme from mushroom (EC 1.14.18.1, activity 2,250 units per mg solid), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), 1,2-naphthoquinone-4-sulfonic acid (NQS, sodium salt, 95% approximately), polyacrilic acid (mw 450,000) (PAA), 6.8-thioctic acid (1,2-dithiolane-3-pentanoic acid), *o*-phenylenediamine dihydrochloride (*o*PD) (99%), glutaric dialdehyde (25 wt.% solution in water) and nafion perfluorinated ion-exchange resin (5 wt.% solution in a mixture of aliphatic alcohols and water) were from Sigma–Aldrich (St. Louis, USA). Graphite powder (>99.9%, <100 μm particles diameter), dichlorvos and diazinon were from Fluka (Riedel-de-Hain, Switzerland). All other reagents were from Merck (Darmstadt, Germany). NQS solutions were prepared immediately before use. Py was distilled in a nitrogen atmosphere before being used as monomers in electropolymerization and was stored in the dark.

### 2.2. Preparation of the enzymatic biosensors

#### 2.2.1. Immobilization of Tyr and NQS in electropolymerized films of PPy and oPPD

The glassy-carbon working electrode (GC) was polished with $Al_2O_3$ (0.05 μm particle diameter) on a soft cloth to remove adsorbed organic matter and thoroughly washed with water. Five microlitres of a solution of freshly prepared NQS 50 μM were added and allowed to dry for 30 min. Five microlitres of Tyr (20 mg $mL^{-1}$) and 5 μL of glutaraldehyde 0.25% (w/v) were left to react and allowed to dry, and at last electropolymerization was then carried out using a solution containing the Py monomer 0.40 M in a 0.05 M phosphate buffer and KCl 0.10 M applying a potential of +0.80 V for 10 s. If, instead of PPy, the outer layer is poly(*o*-phenylenediamine) (*o*PPD), the procedure is the same using a $5 \times 10^{-3}$ M *o*PD monomer solution in a 0.05 M buffer solution with application of the same potentiual for 10 s. Worse results were obtained with greater polymer thicknesses with longer electrolysis times. The biosensors were stored for 24 h at 4 °C before use in a previously deoxygenized 0.05 M phosphate buffer solution.

#### 2.2.2. Covalent attachment to a thioctic acid monolayer

The polycristalline Au-wire electrode was cleaned with a *piranha* solution for 5 min (75/25 $H_2SO_4/H_2O_2$, v/v) to remove all traces of organic material, washed thoroughly with water and polished with 1 μm diamond powder on a nylon disk surface and then with 0.05 μm $Al_2O_3$ powder on a soft cloth. The electrode was then sonicated for 5 min and washed with ethanol. The Au surface was amalgamated by immersion in liquid Hg for 1 min and left to react, followed by dissolution of the amalgam layer in 12 mM nitric acid for 10 min.

The surface of the Au-wire electrode was immersed for 2 h in a 20 mM. 40% (v/v) thioctic acid solution in ethanol and washed with ethanol/water (75/25, v/v) to remove all traces of mercaptane. Tyr was then covalently attached to the carboxylic groups of the SAM monolayer by immersion of the modified electrode in a 20 mg $mL^{-1}$ Tyr solution in the presence of 5 mM EDC in 0.01 M phosphate buffer (pH 7) for 14 h at 4 °C. The biosensor was finally washed with 0.05 M (pH 7.4) phosphate buffer and stored for 24 h at 4 °C in another 0.05 M phosphate buffer solution prior to use. After each use the biosensor was stored at 4 °C and in the same buffer solution. An NQS diffusing mediator in solution (50 μM) was used with this biosensor for analysing the pesticide.

#### 2.2.3. Carbon-paste composites

The carbon paste was prepared by mixing 50 mg of graphite with 10 μL of 15 μM NQS, 10 μL of Tyr 20 mg/mL and 5 μL of glutaraldehyde 0.25% (w/v), which were left to react for 30 min. Ten microlitres of silicon oil were finally added for bonding purposes. The paste was packed in the hole of the carbon-paste working electrode.

Another similar biosensor was prepared but with two adjacent layers of the carbon paste: one formed by mixing 20 mg of graphite, 10 μL of 50 μM NQS and 3 μL of silicon oil (approximately 1 mm depth) in the internal layer and the other formed by mixing 30 mg of graphite, 15 μL of Tyr 20 mg/mL and 5 μL of 0.20% glutaraldeyde (thickness of approximately 2 mm) in the outer layer, which was left to react for 30 min after which 5 μL of Si-oil were added for bonding. The tip of

the electrode was gently rubbed on fine paper to make a flat surface. The biosensor was stored at 4 °C 24 h prior to use.

#### 2.2.4. Copolymerization with glutaraldehyde and nafion

Ten microlitres of 50 μM NQS were injected onto the glassy-carbon electrode surface and allowed to dry in the $N_2$ current. Ten microlitres of Tyr (20 mg/mL) and 5 μL of 0.25% (w/v) glutaraldehyde were then added and left to react for 20 min. The electrode was thoroughly washed with water and then 10 μL of 0.5% nafion (w/v) were added for casting onto the electrode surface to form a protective outer layer. The resulting membrane was finally allowed to dry in the air at room temperature. The biosensor was stored at 4 °C for 24 h prior to use.

### 2.3. Chronocoulometric determination of dichlorvos

Chronocoulometric measurements were performed in an initial volume of 15 mL of a solution containing 0.05 M PB to which between 5 and 300 μL of the pesticide sample were added until the required concentration was obtained. With every addition of the pesticide the solution was allowed to stabilize for 90 s (at a conditioning potential of $E = +0.10$ V) and the potential for current measurement ($E = -0.150$ V) was then applied for 10 s and the charge variation in function of time recorded. After register of the chronoamperogram the potential corresponding to a third stage of biosensor regeneration ($E = +0.10$ V) was then applied for 30 s.

When the potential is applied in the measurement step the charge measured during the first seconds is predominantly non-faradaic and was therefore not taken into consideration. For this reason, the total integrated charge during the last 5 s of the measurement step was used for calibration of the biosensors. The inhibition percentage was calculated in function of the initial charge obtained in the absence of the pesticide. In some cases, the total charge was measured between 5 and 15 s (the total time of the second measurement stage in these cases was 15 s) to check the reproducibility of the signal.

## 3. Results and discussion

### 3.1. Optimization of the electrode modifications and biosensor behaviour

The aim of the present paper is to compare the analytical properties of different amperometric inhibition biosensors with immobilized enzyme tyrosinase (Tyr) for the determination of dichlorvos [31], which was taken as an OP pesticide model. NQS and Tyr were co-immobilized using different physical and chemical immobilization procedures, which, in most cases, had been used previously in our laboratory with other enzymes [32–35]. A diagram showing the arrangement of the layers of all the biosensors can be seen in Fig. 1 together with the nomenclature used for each. The procedures used basically consist of: (i) entrapment of the enzyme and mediator within the electropolymerized polymers (GC-NQS-Tyr-PPy, GC-NQS-Tyr-*o*PPD), (ii) covalent attachment of the enzyme on SAM monolayers (Au-SAM-Tyr), (iii) crosslinking with glutaraldehyde (and protection with an external layer of nafion) (GC-NQS-Tyr-Nf) and (iv) dispersion in a carbon-paste electrode (CP-NQS-Tyr).

Tyr is a copper-containing (Type-III) monooxygenase catalysing the *o*-hydroxilation of monophenols to the corresponding catechols (monophenolase or cresolase activity), and the oxidation of catechols to the corresponding *o*-quinones (diphenolase or catecholase activity). In it resting state, the *met* form of Tyr predominates, which is unable to act on monophenols, but this form oxidizes catechols to the corresponding *o*-quinones with concomitant reduction to a *deoxy* form, this latter converted by molecular oxygen to the *oxy* form, still capable of oxidizing the diphenol while turning again to the *met* state

The activity of the immobilized Tyr enzyme was modulated with the potential applied to the electrode. At first the NQS adsorbed onto the electrode (or diffusing from the solution in the type ii) Au-SAM-Tyr biosensor) is in its *o*-quinone form, but with a cathodic potential pulse applied to the working electrode (−150 mV) is converted into its

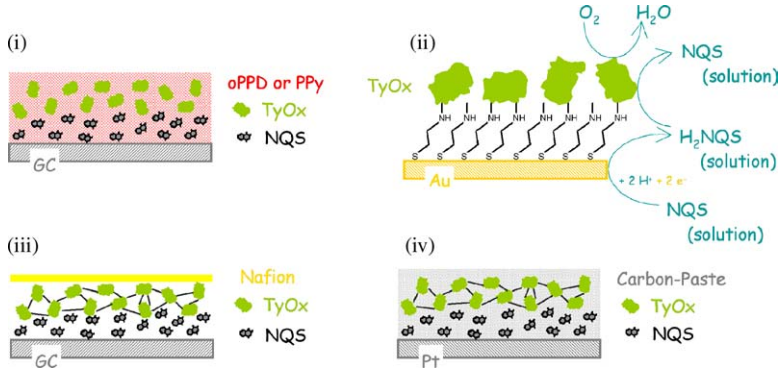


Fig. 1. Biosensor designs and nomenclature used: (i) NQS adsorption and enzyme entrapment in electropolymerized poly(*o*-phenylene-diamine) (GC-NQS-Tyr-*o*PPD), or poly(pyrrole) (GC-NQS-Tyr-PPy); (ii) Au-SAM-Tyr: covalent attachment of Tyr (carbodiimide procedure) to a self-assembled monolayer of thioctic acid; (iii) GC-NQS-Tyr-Nf: adsorption of NQS, crosslinking of Tyr with glutaraldehyde and retention with an outer layer of nafion; (iv) CP-NQS-Tyr: dispersion of NQS and Tyr (cross-linked with glutaraldehyde) in a graphite carbon-paste electrode.

*o*-diol (catechol) molecule, which is converted into a reactive substrate of the enzyme. Application of an anodic potential (+100 mV) to the electrode transforms the remaining $H_2NQS$ into its oxidized *o*-quinone form, which terminates the enzymatic reaction.

The reactions scheme produced in the biosensors is the following:

$$NQS + 2H^+ + 2e^- \rightarrow H_2NQS\,(E = -150\,mV)$$

$$H_2NQS + met\text{-Tyr}\,[Cu_2{}^{+4}] - 2e^- \rightarrow NQS + deoxy\text{-Tyr}[Cu_2{}^{+2}] + 2H^+$$

$$O_2 + deoxy\text{-Tyr}[Cu_2{}^{+2}] \rightarrow oxy\text{-Tyr}[Cu_2{}^{+4}/O_2{}^{-2}]$$

$$H_2NQS + oxy\text{-Tyr}[Cu_2{}^{+4}/O_2{}^{-2}] + 2H^+ - 2e^- \rightarrow NQS + met\text{-Tyr}[Cu_2{}^{+4}] + 2H_2O$$

being the overall enzymatic reaction the catalytic transfer of four electrons from two molecules of substrate $H_2NQS$ to one molecule of oxygen for catecholate process.

Dichlorvos causes reversible competitive inhibition on the mediator side of Tyr in the presence of the substrate $H_2NQS$, as was yet reported in a previous paper [30], which permits the analytical determination of the pesticide. In this work, similar biosensors were designed, but with immobilizing of the mediator. The relative positions of the co-immobilized NQS and enzyme in each biosensor were verified by preliminary tests in the first studies to determine their functioning according to the mechanism described, making sure that the decrease in the steady-state catalytic charge was produced by the inhibiting action of dichlorvos and depended on its concentration, so that the immobilization procedure and relative concentrations of the (bio)reagents on the electrode design could be subsequently optimized.

The presence of adsorbed NQS on the working electrode was tested by cyclic voltammetry to determine its possible loss on the electrode surface, which would result in a loss of the generating capacity of the biosensor signal. When the cyclic voltammogram (from +0.20 to −0.20 V, scan-rate ($v$) from 2 to 20 mV $s^{-1}$) of a GC electrode with NQS adsorbed on its surface (addition of 5 μL of 20–100 μM NQS on a GC electrode) was made, the behaviour observed was typical of an electroactive species adsorbed onto the electrode, $i_p$ (the maximum value of the current of the two anodic and cathodic peaks) being proportional to $v$ (scan rate of potentials) and the symmetrical peaks proportional to the maximum current value (voltammograms not given). The maximum potential values of the current peaks appear at a potential of around −0.033 V (cathodic) and +0.009 V (anodic) (scan-rate 2 mV $s^{-1}$). However, the $i_p$ decreased at uncovered electrodes, indicating a gradual loss of the adsorbed NQS in the solution, which means that it should be retained to avoid its loss in the biosensors in which it is immobilized. Frequent voltammograms of the biosensors revealed in some cases the gradual leaking of the mediator, which had a direct influence on biosensor lifetime and so in each case an attempt was made to ensure that the loss was minimal. When NQS is in solution, typically irreversible and diffusive behaviour appears with bare-GC working electrodes ($i_p/c$ is proportional to $v^{1/2}$), with maximum of the voltammetric peaks at approximately −0.014 V (cathodic) and +0.031 V (anodic) at a slow scan-rate of 2 mV $s^{-1}$.

The permeability of several layers (nafion, PPy and *o*PPD) was also initially assayed with cyclic voltammograms of solutions of NQS (50 μM) to obtain preliminary information about the retaining capacity of the different aforementioned polymer and influence of the thicknesses for the mediator. With Au-SAM-Tyr, NQS cannot be adsorbed onto the electrode as the presence of adsorbed NQS would impede the correct formation of the SAM monolayer because mercaptane should be joined directly to the clean metal surface (Au). PPy and *o*PPD (with a short electropolymerization time of about 1 min) have low permeability compared with NQS while nafion (casting with 10 μL of 1%, w/v, nafion) impedes the cyclic voltammogram of NQS in solution on GC, making the layer completely impermeable to this molecule.

All the biosensors were tested (preliminary tests) for variations in the $O_2$ concentration in the solution and their influence on the signal obtained by the enzyme, observing the response of the enzyme in $N_2$, air and $O_2$ saturated solutions. The enzymatic reaction only takes place in the presence of $O_2$ and so the differences observed in the 3 cases indicate that the enzymatic reaction takes place (the $H_2NQS$ is enzymatically re-oxidized in presence of oxygen) and is what generates the measured catalytic current. This dependence was also verified by the variation in signal with the addition of different concentrations of the inhibitor in preliminary studies.

The inhibitory effects of pesticides on enzyme activity were monitored by chronocoulometry (integration of the chronoamperometric current decay) during the reductive potential pulse ($E = -150$ mV, second potential step), in a similar way to that previously described [30]. The oxidative pulse ($E = +100$ mV) at the electrode terminates the enzymatic reaction switching-off the enzyme reaction due to the lack of substrate. Non-faradaic charging current predominates at first of the applied potential pulse ($E = -150$ mV), so the current during the first 5 s. was not integrated for enzymatic data analysis, most of the non-faradaic current (capacitative) being eliminated. Logically, the maximum value of the faradaic current (due to the electrochemical reduction of NQS) at the beginning of the pulse decreases but the sensitivity obtained was always sufficient with improved reproducibility owing to the fact that only the steady-state current produced by the enzymatic reaction was measured.

In all designs, an attempt was made to ensure that the formation rate of the activating substrate $H_2NQS$ was the limiting concentration rather than the concentration of available oxygen in the enzyme. A typical EC′ mechanism was observed in the catalytic regeneration of the electroactive species (C′ refers to the generated concentration of

$H_2$NQS), indicating a relationship between the inhibition percentage (%Inh) and the dichlorvos concentration. The enzyme turnover depends on the relative concentrations of oxygen (approximately 1 mM in air-saturated conditions) and of $H_2$NQS concentration (the formation rate of $H_2$NQS depends on the amount of NQS immobilized (or in solution) and the potential applied to the electrode). The ideal limiting conditions were therefore selected empirically and so the dependence of the current caused by the mediator could be monitored and related to the concentration of the pesticide. $O_2$ depletion in the reaction layer of the biosensor during measurement of the catalytic current (during the application of the current pulse ($E = -150$ mV) in the measurement step) was avoided, which probably accounts for the fact that the results were more reproducible when the currents were measured during the last 5 s of the measurement stage instead of during the last 10 s.

Increasing concentrations of dichlorvos result in competitive inhibition with the mediator for the enzyme, leading to a decrease in the cathodic reduction current of NQS due to its slower enzymatic regeneration rate in the presence of $O_2$. Calibration was carried out by calculating the inhibition percent (%Inh) produced by pesticide on the enzyme, measuring the total charge in the presence of the pesticide ($Q_{inh}$) and the total initial charge without the pesticide ($Q_{no\_inh}$) using the following mathematical expression: $\%Inh = 100[(Q_{no\_inh} - Q_{inh})/Q_{no\_inh}]$.

Fig. 2 shows a typical decrease in the chronoamperometric current during application of the potential pulse of −150 mV, with and without the addition of the pesticide and Fig. 3 a typical plot of the %Inh variation of the enzyme versus the concentration of dichlorvos for GC-NQS-Tyr-*o*PPD. The plot in Fig. 3 shows a maximum inhibition value for high concentrations of pesticide and a variation of the linear signal for low concentrations where calibration was carried out in all cases. The inhibition effects of the pesticide on the current decay were calculated in 5 s ($Q_1$) in some cases and also in 10 s ($Q_1 + Q_2$) in others (see Fig. 2) in order to study their influence. Better reproducibility was generally obtained by measuring $Q_1$ and this measurement was then used in the calibration calculations. Long applications of the potential pulse during the measurement may lead to depletion of $O_2$, accounting for the irreproducibility of catalytic measured current. Once the functioning of all the biosensors had been verified, the immobilization conditions were optimized for each biosensor in order to obtain the best analytical properties. The optimized conditions are those described in the section of procedures. In the synthesis of electropolymers, which were formed under mildly oxidative conditions from aqueous media, values of thicknesses exceeding the optimum (studied electropolymerization times were up to 15 min with *o*PD and up to 10 min with Py) tended to decrease sensitivity significantly in the determination of dichlorvos. The permeability of *o*PPD towards the analite is also better than that produced by PPy and so sensitivity is also better with the former.

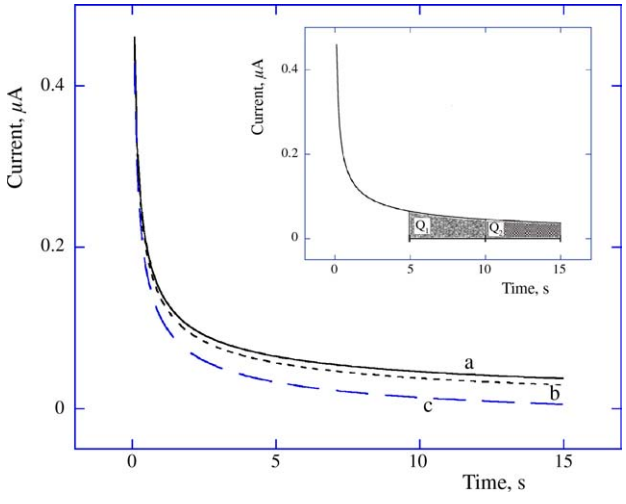


Fig. 2. A typical chronoamperometric current decay plot with a GC-NQS-Tyr-*o*PPD biosensor in a solution of: (a) phosphate buffer 0.05 M (15 mL); (b) phosphate buffer 0.05 M with addition of 20 μL of dichlorvos 1.5 mM; (c) same solution of (b) with the addition of another 20 μL of dichlorvos 6 mM. Inhibition effects was calculated in 5 s ($Q_1$) and in 10 s ($Q_1 + Q_2$) after the first 5 s of application of the potential pulse ($E = -150$ mV) (inset).

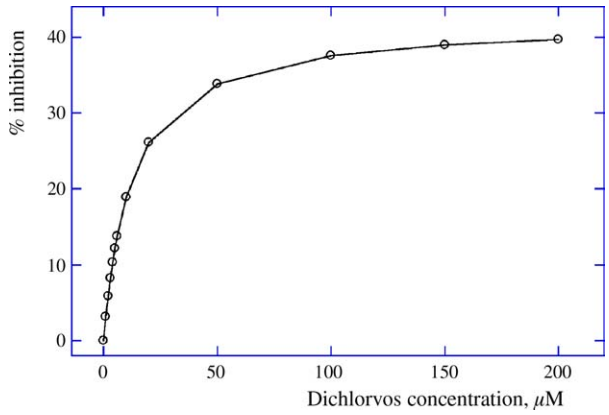


Fig. 3. Typical calibration plot for a GC-NQS-Tyr-*o*PPD biosensor. Percent inhibition represents: $\%Inh = 100[(Q_{no\_inh} - Q_{inh})/Q_{no\_inh}]$. $Q_{inh}$ is the charge measured at each dichlorvos concentration. $Q_{no\_inh}$ is the total-charge measured without the pesticide in the solution. Supporting electrolyte: phosphate buffer solution 0.05 M.

For Au-SAM-Tyr different types of working electrodes were tested (disk electrodes and Au wire) as well as different mercaptanes (cystamine and thioctic acid), different SAM formation times (from 5 min to 12 h) and the addition of varying concentrations of NQS to the measurement solution (20–100 μM). The optimized results are gathered in the Procedures Section. With the gold wire electrode the greater volume/area ratio makes it more ideal for improving the supply of enzyme to the immobilized enzyme by diffusion, which affects both sensitivity and response time [32]. Prior amalgamation of the Au wire electrode before formation of the SAM led to a loss of atomic planarity of the Au surface and the crystalline SAM formed was therefore less oriented [32]. In addition, small mercury impurities remained after careful cleaning of the mercury, producing pinholes in the SAM,

which together with the greater roughness of the electrode surface favoured the charge-transfer, which improved the signal if compared with electrodes which were modified without prior amalgamation of the Au wire. Better results were also obtained with thioctic acid than with cystamine because of looser packing of the alkylic chains due to the structures of the two molecules, also favouring the charge-transfer and diffusion of NQS from the solution to the working electrode.

The worst analytical results were obtained with the electrode integrated into carbon-paste matrices. The formation of two graphite paste layers for filling the hole of the electrode was also assayed, the inner layer being a mixture of graphite + binder + NQS and the outer of graphite + binder + Tyr. No enzymatic biosensor response was obtained in this case, probably due to the distance of the mediator from the active site of the enzyme in these conditions. In the first procedure described with the hole filled with a uniform graphite paste containing the enzyme and mediator, enzyme response to the inhibitor was observed, although the analytical properties were worse than with the other designs. Apart from that, the electrode was prone to significant mediator leakage, resulting in poor long-term stability.

With the GC-NQS-Tyr-Nf biosensor, the presence of nafion improved biosensor lifetime because NQS and the immobilized enzyme were retained and acted as an exclusion layer protecting the modified electrode from interfering (e.g. electroactive) or fouling species. Higher concentrations than the optimized concentration (addition of 10 μL of nafion 0.5%, w/v) led to a rapid loss of biosensor sensitivity due to less permeability and the difficulty of analyte diffusion to the inner enzymatic layer. Preliminary tests using voltammetry in a 50 μM NQS solution also showed that a bare-GC-electrode coated with casted-nafion (addition of 10 μL 2%, w/v) does not produce voltammetric peaks, indicating that Nf is an important layer/barrier preventing mediator leaking from the electrode.

### 3.2. Analytical properties of the dichlorvos biosensors

The analytical properties obtained for each biosensor design in the determination of dichlorvos are given in Table 1. With the covalent attachment of the enzyme to the SAM monolayer the NQS mediator should be added to each measurement solution, as previously indicated, because the mediator is not immobilized on the electrode. However, the mechanism of the signal generation is similar to that of the immobilized mediator, the maximum measured current being equally limited by the catalytic regeneration rate of the diffusing mediator. In spite of this difference, the sensitivity data and the analytical properties in general are comparable to those of the other biosensors, although obviously biosensor lifetime in GC-SAM-Tyr is influenced by the duration of the enzyme rather than by an additional loss of the immobilized mediator.

The best results with regard to sensitivity, detection limit and biosensor lifetime (12 days) in the determination of dichlorvos were obtained with GC-NQS-Tyr-*o*PPD biosensor, which was designed by adsorption of NQS directly onto the electrode and retention of Tyr by the electropolymerized layer of *o*PPD. This design probably functions best because of the greater proximity of the electrode, NQS and Tyr if compared with the other configurations formed by covalent attachment or diffusion in the carbon paste, which facilitates the approach of $H_2$NQS to the active site of the enzyme. Moreover, the greater diffusionability of dichlorvos through *o*PPD and a more suitable environment for enzyme stability may lead to better analytical properties, if compared with biosensor designs based on the PPy polymer or with the carbon-paste electrode. The detection limit for dichlorvos with GC-NQS-Tyr-*o*PPD was at 60 nM and the sensitivity in the calibration linear area was 1.89 %Inh $\mu M^{-1}$, as can be seen in Table 1.

Table 1
Analytical performance of the biosensors

| | PPy[a] | *o*PPD[b] | SAM[c,f] | Nafion[d] | CPE[e] |
|---|---|---|---|---|---|
| Linear range (up to, μM)[g] | 5 | 8 | 5 | 4 | 3 |
| Sensitivity (%Inh $\mu M^{-1}$)[h] | 0.09 | 1.89 | 1.25 | 1.44 | 0.03 |
| Reproducibility (%CV)[i,j] | 11.6[i] | 6.4[i] | 7.8[i] | 7.2[j] | 11.7[j] |
| Limit of detection (μM)[k] | 0.33 | 0.06 | 0.21 | 0.44 | 1.12 |
| Lifetime (days)[l] | 4 | 12 | 10 | 4 | 2 |
| $\%Inh_{max}$ (%)[m] | 18 | 42 | 38 | 25.7 | 16 |

[a] GC-NQS-Tyr-PPy.
[b] GC-NQS-Tyr-*o*PPD.
[c] Au-SAM-Tyr.
[d] GC-NQS-Tyr-Nf.
[e] CP-NQS-Tyr.
[f] For a concentration in solution of NQS of 50 μM.
[g] For $r > 0.9950$.
[h] Linear concentrations range.
[i] Coefficient of variation (%CV) for a 4 μM dichlorvos solution ($n = 5$).
[j] Coefficient of variation (%CV) for a 2 μM dichlorvos solution ($n = 5$).
[k] Calculated as $\%Inh = 3SD_{blank.}$
[l] 20 measurements of dichlorvos per day.
[m] For 300 μM dichlorvos.

The lifetime of all the biosensors was calculated for a basis of 20 determinations of the pesticide per day and the data in the Table 1 refer to the total number of days when sensitivity decreased more than 20% compared with the initial sensitivity. However, unusually high signals were observed in some cases during the first 2–4 determinations of the pesticide with a recently prepared biosensor and so analytical properties were considered when sensitivity reached a stable value after the first determinations.

### 3.3. Determination of dichlorvos in an artificial river sample

Although the biosensors described were studied with dichlorvos, other organophosphorus pesticides could be

Table 2
Results on the determination of dichlorvos in spiked river water samples by the standard addition method

| | Dichlorvos obtained ($\mu g\,L^{-1}$) | Relative error (%) | Reproducibility[a] (%) |
|---|---|---|---|
| Sample 1[b] | 174.1 | +8.8 | 8.4 |
| Sample 2[c] | 345.9 | +8.1 | 7.9 |
| Sample 3[d] | 664.6 | +7.2 | 8.2 |

[a]Reproducibility is expressed as the coeficient of variation for $n = 4$ measurements on the same sample. Solids were filtered from the samples through a 50 μm filter before analysis. Assigned dichlorvos concentrations were: [b]160 $\mu g\,L^{-1}$, [c]320 $\mu g\,L^{-1}$, and [d]620 $\mu g\,L^{-1}$.

determined with good analytical properties, if they produce competitive inhibition of Tyr with respect to the enzyme substrate [8]. Preliminary studies were also carried out in our laboratory with diazinon pesticide, which gives a response with similar (or even better) sensitivity than dichlorvos, despite the fact that the diazinon solutions were prepared in absolute alcohol as diazonin is not very soluble in water. The presence of small amounts of ethanol in the measurement solution, due to the addition of diazinon solutions, did not modify the enzymatic activity of Tyr in the tests carried out in our laboratory, in agreement with the behaviour reported by other authors [30].

Artificial samples were prepared by adding dichlorvos to a natural river water samples (River Ebro) for the statistical study of the determination of the pesticide with the GC-NQS-Tyr-*o*PPD biosensor design. Samples were spiked with a known amount of dichlorvos and recovery measurements made. For unspiked samples, the amount of inhibition was below 3%, and results were corrected treating unspiked samples as blanks. Prior to this, the river sample was filtered using a pore size of 50 μm to remove dispersed solid material. Standard addition was used with triple addition of a standard dichlorvos solution, and four consecutive independent measurements for each sample was made.

The results obtained are given in Table 2. The concentrations of dichlorvos obtained are expressed by $\bar{x} + k_{\bar{x}}s$, $\bar{x}$ being the mean value for $n = 4$ consecutive independent determinations, $s$ the standard deviation of the mean and $k_{\bar{x}}$ its confidence factor ($k_{\bar{x}} = t/n^{1/2}$, with $t$ the Student's statistical value for the number of degrees of freedom of the system $n = n - 1$, for a significance level of $P = 0.05$). The determinations were carried out satisfactorily, and in all cases the relative error was below +9%, which shows good agreement between the determined value and the assigned dichlorvos concentration at the significance level considered.

## 4. Conclusions

Four enzymatic biosensor designs were constructed for determining dichlorvos, based on the reversible inhibition produced by the pesticide on immobilized Tyr enzyme. The enzymatic activity of the Tyr can be modulated by electrogenerating the active catechol substrate ($H_2$NQS) from the oxidized form of the mediator NQS (*o*-quinone molecule). The pesticide inhibits the enzymatic re-oxidation of $H_2$NQS to NQS in the presence of oxygen, then decrease in the catalytic current of the reduction of NQS was related to the dichlorvos concentration. In all cases, the amount of $H_2$NQS electrogenerated was intended to be the limiting concentration rather than the oxygen co-substrate concentration to obtain a pseudo-first-order enzymatic reaction kinetics. The dichlorvos inhibition was reversible and the enzymatic activity was recovered for a new cycle after removal of the pesticide sample and application of the anodic potential ($E = +100$ mV) to the working electrode.

The immobilization procedures used were: (i) entrapment by electropolymerization (GC-NQS-Tyr-*o*PPD and GC-NQS-Tyr-PPy), (ii) covalent attachment on SAM monolayers (Au-SAM-Tyr), (iii) crosslinking with glutaraldehyde (GC-NQS-Tyr-Nf) and nafion covering and (iv) dispersion within a graphite-paste electrode (CP-NQS-Tyr). In all designs (excluded (ii) with SAM) NQS was immobilized together with the enzyme, which permitted the mechanism of a reagentless biosensor.

The best analytical properties were achieved using Tyr and NQS entrapment within an *o*PPD electropolymerized polymer, obtaining the GC-NQS-Tyr-PPy biosensor, which had a detection limit of a dichlorvos concentration of 0.06 μM. With the (ii) Au-SAM-Tyr biosensor the NQS mediator was added to the measurement solution (50 μM) instead of being immobilized on the electrode, because of the formation of the SAM layer directly on the surface of the Au bare electrode.

The immobilization procedures of Tyr and NQS described could be used in the future to design new biosensors for other analytes provided they produce similar reversible and competitive inhibition on the Tyr bioelectrocatalysis, as e.g. with other organochlorinated or carbamate derivative pesticides. Nevertheless, slight variations should be optimized in these cases, for example, the permeability of the electropolymerized polymers towards the analyte and the concentration of the immobilized mediator. The generation rate of the *o*-diol according to the degree of inhibition produced should also be studied. Other soluble and insoluble *o*-quinonic molecules could also be studied in order to improve the analytical properties of the biosensors with these mediators once it has been determined that the reduced form of these molecules behave as active substrates of the enzyme.

## Acknowledgements

This work was financially supported by the Spanish Government of Aragon (Education and Science Department) with Project P134/2001, and by the Spanish Ministry of Science and Technology, in the framework of the "*Plan Nacional de Investigación científica, desarrollo e innovación tecnológica I + D + I*", under Project BQU2002/02240.

## References


[1] F.S. He, Neurotoxicology 21 (2000) 829.
[2] S. Varfolomeyev, I. Kurochkin, A. Eremenko, E. Efremenko, Pure Appl. Chem. 74 (2002) 2311.
[3] S. Sole, S. Alegret, Environ. Sci. Pollut. Res. 8 (2001) 256.
[4] P.D. Patel, Trends Anal. Chem. 21 (2002) 96.
[5] M. Del Carlo, M. Mascini, A. Pepe, G. Diletti, D. Compagnone, Food Chem. 84 (2004) 651.
[6] C. Aprea, C. Colosio, T. Mammone, C. Minoia, M. Maroni, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 769 (2002) 191.
[7] J. Parellada, A. Narvaez, M.A. Lopez, E. Dominguez, J.J. Fernandez, V. Pavlov, I. Katakis, Anal. Chim. Acta 362 (1998) 47.
[8] C. Nistor, J. Emneus, Waste Manage. 19 (1999) 147.
[9] N.G. Karousos, S. Aouabdi, A.S. Way, S.M. Reddy, Anal. Chim. Acta 469 (2002) 189.
[10] W. Wei, R.H. Wang, L.H. Nie, S.Z. Yao, Instrum. Sci. Technol. 25 (1997) 157.
[11] T.M. Anh, S.V. Dzyadevych, M.C. Van, N.J. Renault, C.N. Duc, J.M. Chovelon, Talanta 63 (2004) 365.
[12] E.V. Gogol, G.A. Evtugyn, J.L. Marty, H.C. Budnikov, V.G. Winter, Talanta 53 (2000) 379.
[13] G. Turdean, I. Popescu, L. Oniciu, Can. J. Chem. 80 (2002) 315.
[14] M. Trojanowicz, Electroanalysis 14 (2002) 1311.
[15] F. Mazzei, F. Botrè, C. Botrè, Anal. Chim. Acta 336 (1996) 67.
[16] S.Q. Liu, J.H. Yu, H.X. Ju, J. Electroanal. Chem. 540 (2003) 61.
[17] B. Serra, S. Jiménez, M.L. Mena, A.J. Reviejo, J.M. Pingarrón, Biosens. Bioelectron. 17 (2002) 217.
[18] S. Cosnier, J.J. Fombon, P. Labbe, D. Limosin, Sens. Actuators B Chem. 59 (1999) 134.
[19] S. Cosnier, S. Szunerits, R.S. Marks, J.P. Lellouche, K. Perie, J. Biochem. Biophys. Methods 50 (2001) 65.
[20] H.G. Xue, Z.Q. Shen, Talanta 57 (2002) 289.
[21] C. Vedrine, S. Fabiano, C. Tran-Minh, Talanta 59 (2003) 535.
[22] S.Q. Liu, J.H. Yu, H.X. Ju, J. Electroanal. Chem. 540 (2003) 61.
[23] E.A. Cummings, S. Linquette-Mailley, P. Mailley, S. Cosnier, B.R. Eggins, E.T. McAdams, Talanta 55 (2001) 1015.
[24] R. Chuanmin, L. Yanbin, Talanta 54 (2001) 1095.
[25] S. Campuzano, B. Serra, M. Pedrero, F.J.M. de Villena, J.M. Pingarron, Anal. Chim. Acta 494 (2003) 187.
[26] S.C. Chang, K. Rawson, C.J. McNeil, Biosens. Bioelectron. 17 (2002) 1015.
[27] C. Kranz, H. Wohlschläger, H.L. Schmidt, W. Schuhmann, Electroanalysis 10 (1998) 546.
[28] J. Wang, V.B. Nascimento, S.A. Kane, K. Rogers, M.R. Smyth, L. Angnes, Talanta 43 (1996) 1903.
[29] J.L. Besombes, S. Cosnier, P. Labbé, G. Reverdy, Anal. Chim. Acta 311 (1995) 255.
[30] W.R. Everett, G.A. Rechnitz, Anal. Chem. 70 (1998) 807.
[31] Agency for Toxic Substances and Disease Registry (ATSDR), Toxicological profile for dichlorvos, U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA, 1997.
[32] J.C. Vidal, J. Espuelas, J.R. Castillo, Anal. Biochem. 333 (2004) 88.
[33] J.C. Vidal, E. García, J.R. Castillo, Microchim. Acta 143 (2003) 93.
[34] J.C. Vidal, E. García, S. Méndez, P. Yarnoz, J.R. Castillo, Analyst 124 (1999) 319.
[35] J.C. Vidal, S. Méndez, J.R. Castillo, Anal. Chim. Acta 385 (1999) 203.