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A disposable amperometric biosensor for rapid screening of anticholinesterase activity in soil extracts

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A disposable amperometric biosensor for the determination of anticholinesterase activity in soil extracts is described. The sensitive membrane was obtained by co-crosslinking acetylcholinesterase and choline oxidase with bovine serum albumin using glutaraldehyde. The anticholinesterase activity of the soil extract was measured using chronoamperometry at 650 mV *vs.* Ag/AgCl to monitor the biocatalytically produced H_2O_2 before and after the inhibition step. An inhibition percentage of 38 ± 4% was recorded for soil extracts spiked with 10 ppb of ethyl parathion. The device has the potential to be used as a gross sensor for the assessment of anticholinesterase activity in soil extracts.

Introduction

There is an urgent need for the development of rapid and low-cost screening procedures to assess the increasing impact of pesticides in the environment originating from industrial manufacturing processes and intensive agricultural practice. Organophosphorous (*e.g.* malathion, fenthion, paraoxon and parathion) and carbamate (*e.g.*, carbaryl and aldicarb) insecticides show low environmental persistence, but are of high concern due to their acute toxicity. In fact they cause irreversible inhibition of cholinesterase enzymes involved in nerve impulse transmission. The generally accepted inhibition mechanism of acetylcholinesterase (AChE) by organophosphate esters has been described by Aldridge.[1]

Gas chromatography (GC), high performance liquid chromatography (HPLC) and GC coupled with mass spectrometry (GC-MS) are the most commonly employed analytical techniques for pesticides detection.[2] These procedures discriminate between different compounds which belong to the same class, but, despite their sensitivity, require expensive instrumentation and skilled personnel. Consequently, these techniques can hardly be used in the field and have analysis costs that may be prohibitively high for screening purposes. Therefore, there is an increasing interest in gross sensors, *i.e.* sensors capable of providing information about a gross parameter (*e.g.* sample toxicity) that, in the specific case, can be represented by the anticholinesterase activity of the analysed sample. The anticholinesterase activity (*i.e.* the degree of AChE inhibition) can be measured by a kinetic measurement, performed using different electrochemical transducers such as differential pH-meters,[3] ISFETs,[4] light addressable potentiometric sensors (LAPS),[5] amperometric carbon modified electrodes[6] or, as described in this paper, by a H_2O_2-detecting amperometric biosensor based on co-immobilised choline oxidase (ChO) and AChE.[7–9]

The degree of inhibition depends, among other parameters, on pesticide concentration and exposure time, and can be calculated (at a defined pH value and at constant inhibitor concentration in excess with respect to AChE) by the following expression:[10,11]

$$-2.3\log\left(\frac{A_t}{A_0}\right) = k_i[\mathrm{PX}]t \quad (1)$$

where A_0 and A_t are the enzyme activity before and after the inhibition step, respectively, [PX] the concentration of the inhibitor (pesticide), t the inhibition time and k_i is the 'bimolecular reaction constant'[12] also known as the inhibition constant. In the case of an immobilised enzyme, eqn. (1) should be rewritten in order to account for the partition coefficient of the inhibitor between the solution and the membrane. Assuming no concentration polarisation of PX in the membrane eqn. (1) can be written:

$$-2.3\log\left(\frac{A_t}{A_0}\right) = k_i k_p[\mathrm{PX}]t = k_i'[\mathrm{PX}]t \quad (2)$$

where k_p is the partition coefficient (usually < 1) and k'_i can be considered as an apparent inhibition constant. Eqn. (2) permits an easy way to estimate k'_i for a given inhibitor. The A_t/A_0 ratio can be reasonably replaced by i_i/i_0, where i_0 and i_i are the steady-state current signals relevant to the detection of H_2O_2 produced by choline oxidase, before and after the pesticide inhibition, respectively.

The inhibition of AChE is essentially an irreversible process; once exposed to the inhibitor the enzyme is inactivated and the sensor can be reused only adopting a suitable procedure for the enzyme reactivation.[13] This characteristic implies that analysis by low-cost, disposable biosensors with immobilized ChO/AChE enzymes should represent an ideal assay format for the screening of the anticholinesterase activity. The fabrication of such a device and its application to soil extracts analysis is described in the following sections.

Experimental

Materials

Choline chloride, acetylcholine chloride, choline oxidase (ChO, EC 1.1.3.17 from *Alcaligenes* species, 14.6 U mg^{-1} of solid), acetyl cholinesterase (AChE, EC 3.1.1.7, type VI-S, from electric eel, 225 U mg^{-1} of solid), bovine serum albumin (BSA, fraction V) and glutaraldehyde (GLU, grade II, 25% aqueous solution) were obtained from Sigma (Sigma, St. Louis, MO, USA) and used without further purification. Choline chloride was dried under vacuum over P_2O_5 for at least 3 days and stored in a vacuum dessiccator. All other chemicals were of analytical-reagent grade. Choline and acetylcholine standards were prepared in phosphate buffer (pH 7.0, ionic strength = 0.1) solution (PBS) and stored in the dark at 4 °C. More diluted solutions were prepared just before use. Soil samples of different origin, were air dried at 35 °C and sieved through a 1

mm sieve. Ethyl parathion was purchased from LabService Analytica (Anzola Emilia, Italy) and used as received. Pesticide stock solutions were prepared in hexane, and subsequently stored at 4 °C in the dark. Aqueous diluted solutions were prepared by pipetting a known amount of stock solution, drying it carefully under a low flow of nitrogen, and adding the required amount of PBS.

Apparatus

Electrochemical experiments were carried out using an EC 400 electrochemical detector (EG & G, Princeton Applied Research, Princeton, NJ, USA), connected to a Y-t strip chart recorder (Kipp & Zoonen Model B112). The screen-printed electrodes were purchased by Ing. Krejci Engineering (Tisnov, Czech Republic), together with the connectors, and used as received (for a scheme of this device, see ref. 14). The platinum working electrode (active area of 0.43 ± 0.03 mm^2) is surrounded by a quasi-reference electrode, made with a Ag/AgCl paste. The Pt counter electrode (approximate area: 4 mm^2) is the outermost ring of the device. All measurements were performed in PBS containing 10 mM Cl^-. In this way, a stable potential of the reference electrode was obtained. The working electrode potential was maintained at + 0.65 V.

Preparation of enzyme electrode

A 300 μl volume of PBS containing 16 mg of BSA, 1 mg of ChO and 1 mg of AChE were carefully mixed with 30 μl of 2.5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1 + 9 with PBS). 1 μl of the resulting solution was pipetted onto the Pt working electrode surface (avoiding air-bubble formation), carefully spread-out to form a complete surface covering, and air-dried at room temperature. After preparation, the sensors were soaked in a stirred supporting electrolyte to remove any weakly bound and adsorbed enzyme and to swell the enzyme layer itself; usually, about 15 min were necessary to obtain a stable and steady-state response to substrate addition. When not in use, sensors were stored in PBS at 4 °C in the dark.

Sample preparation

Soil samples were treated according to a well-established procedure[15] consisting essentially of a solvent extraction step (acetone–CH_2Cl_2, 1 + 1 v/v) followed by purification on a BioBeads SX3 gel permeation column (GPC). The GPC eluate was reduced to dryness and the residue reconstituted in a 2 ml volume of an acetone–hexane mixture (1 + 1 v/v). The entire procedure provided a ten-fold preconcentration factor. A 1 ml aliquot of this extract was reduced to dryness under a gentle stream of N_2 and reconstituted in 100 μl of phosphate buffer; a 50 μl aliquot was applied onto the sensor substrate and left for a given time (inhibition step). In some cases a 1 ml aliquot of the extract was spiked with ethyl parathion at 10 ppb level (corresponding to 1 μg Kg^{-1} of dry soil) and treated as before.

Measurement of the anticholinesterase activity

The percentage of inhibition (*I*%) was quantified by the equation:

$$I\% = \frac{i_0 - i_i}{i_0} 100 \qquad (3)$$

where i_0 and i_i have been previously defined.

The first measurement step was made by recording the steady-state oxidation current (i_0) obtained by dropping 50 μl of 2 mM acetylcholine onto the sensitive part of the device and applying 650 mV *vs.* the quasi reference electrode. The disposable biosensor was then thoroughly washed and 50 μl of the aqueous solution obtained from the reconstitution of the soil extract was dropped onto its surface. At the end of the incubation step, the enzymatic activity was recorded again (i_i), allowing the calculation of *I*% by eqn. (3).

Results and discussion

The immobilization procedure as well as the influence of various parameters (pH, GLU, BSA, ChO, AChE concentrations, AChE/ChO loading ratio and enzyme-layer thickness) have already been described in a previous paper.[7]

The particular configuration of the device permits chrono-amperometric determinations in a small sample volume dropped directly onto the device surface (maintained in a horizontal position) and covering the three-electrode system. At a fixed time after the drop deposition, the potential is applied and current–time responses such as those displayed in Fig. 1 can be obtained. As can be seen, after a transient decay a steady state current is achieved in a reasonably short time (the shape of the current transient is discussed in ref. 14). Experiments with the electrode dipped into the solution gave the same results, demonstrating that the diffusion layer was thinner than the thickness of the solution layer dropped onto the device.

Calibration curves for Ch and ACh have been quickly obtained using this procedure, dropping a small volume of choline standard solution onto the device, measuring the steady-state current and repeating this operation, after a washing step of the device with PBS.

Among other parameters, the ACh concentration used in the measurement steps is particularly important. The calibration curve for ACh (Fig. 2) presents a linear region and, after 'saturation', the response starts to decrease with increasing ACh concentration due to substrate inhibition of AChE.[16] A 2 mM concentration value (that is in the 'saturation' zone) was chosen in order to obtain maximum sensitivity and to ensure an amperometric response which was independent of fluctuations in the acetylcholine concentration.

The inhibition time plays an important role in the definition of the sensitivity of the method, and has to be selected on the

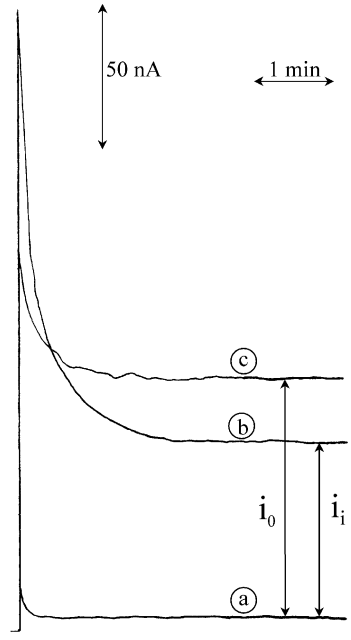

Fig. 1 Chronoamperometric responses of a Pt/BSA-AChE-ChO disposable sensor obtained with 50 μl of 2 mM choline solution directly dropped onto the sensitive part of the device before (**c**) and after (**b**) incubation with the extract of a contaminated soil sample. Curve **a** is the response obtained with a phosphate buffer solution.

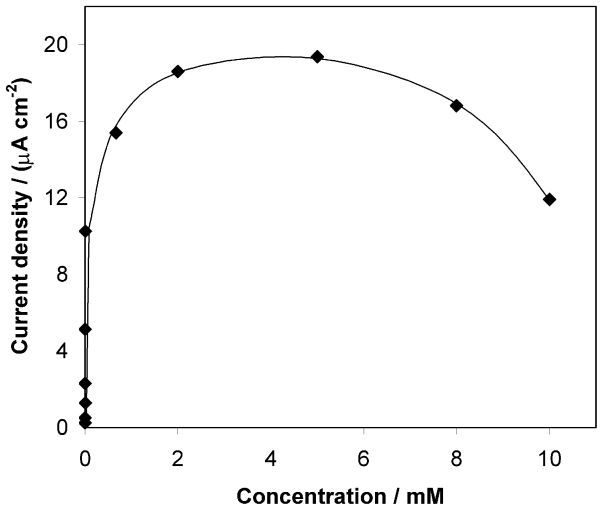

Fig. 2 Calibration curve for ACh on a Pt/BSA-AChE-ChO disposable sensor. Experimental conditions as in Fig. 1.

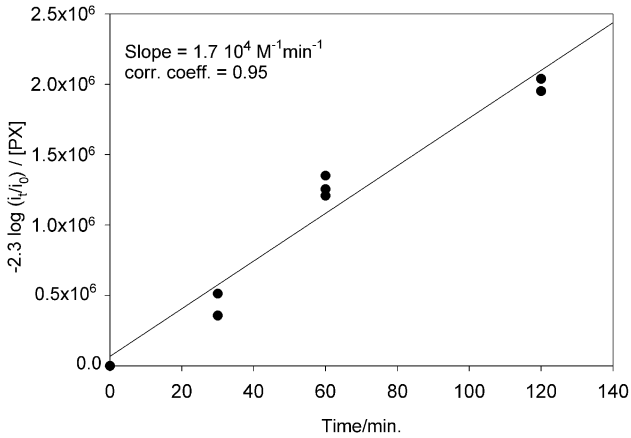

Fig. 3 Enzyme inhibition as a function of the incubation time. Experimental conditions: [ACh] = 2 mM in PBS (ionic strength = 0.1, pH = 7.0) added with 10 mM Cl^-. Applied potential: 0.65 V *vs.* Ag/AgCl quasi reference electrode. Ethyl parathion concentration: 100 ppb.

basis of expected concentration levels of pesticide in the sample. At a fixed inhibitor concentration the degree of inhibition shows a logarithmic dependence on the inhibition time [see eqn. (2)], allowing the estimation of k'_i. The relative decrease in current before and after the inhibition step is presented in Fig. 1. The apparent inhibition constant, k'_i, (calculated at pH 7.0) was 1.7×10^4 M^{-1} min^{-1} which appears reasonable considering that for ethyl paraoxon (the metabolite of ethyl parathion considered more toxic than the parent compound) a k_i value of 2.9×10^5 M^{-1} min^{-1} has been reported.[17]

A one-hour inhibition time at ambient temperature was used as a default value in the analysis of different soil extracts. One, out of the 50 analysed samples, showed a *I*% of 29 ± 3; preliminary GC analysis of that sample gave evidence of the presence of traces of methyl parathion. All the other samples gave a *I*% of always less than 5%, which is the minimum appreciable value.

When uncontaminated soil extracts were spiked with 10 ppb of ethyl parathion and re-applied on the same sensor that had been used for the analysis of the unspiked extract, the *I*% (38.3 ± 1.5) expected from data in Fig. 3 was indeed observed (see Table 1). Since after the first incubation step with pesticide-free extracts, the sensor performance remained practically unchanged, this experiment demonstrates also the robustness of the biosensor towards potentially adverse matrix effects.

Note that for screening purposes no biosensor calibration is strictly necessary, since the sensor is designed as an "on-off switch" that has only to discriminate between samples needing or not needing further analytical work. In this respect it is interesting to observe that a clear "on" signal can be obtained at a concentration level (1 μg Kg^{-1} of dry soil) which is about one order of magnitude lower than the detection limits offered by GC-MS.[18] Finally, even if the absolute sensitivity of different sensors towards acetylcholine suffers from a certain degree of variability (RSD% = 16.5% on ten different devices), the *I*% value showed a very good between-sensors reproducibility (see Table 1) indicating the possibility of mass production. Other organophosphates (*e.g.* fenthion, malathion, dimethoate) and some metabolites like paraoxon and malaoxon give an inhibited response; this finding reinforces the possibility of using the described device as a 'gross sensor'.

Table 1 Percentage inhibition ± *s* (on three different sensors) for ethyl parathion spiked and unspiked soil extracts. *nd* = inhibition not detected (*i.e. I*% < 5)

Sample extract	1	2	3	4
Unspiked[a]	nd	nd	nd	nd
Spiked at 10 ppb	38 ± 3	40 ± 3	39 ± 2	37 ± 4

[a] A total of 50 samples analyzed

Work is in progress aimed at a deeper characterisation of the sensor behaviour and at a comparison of sensor response with GC-MS response. The possibility of using 'raw' soil extracts (*i.e.* extracts not purified by gel permeation) is being also investigated.

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