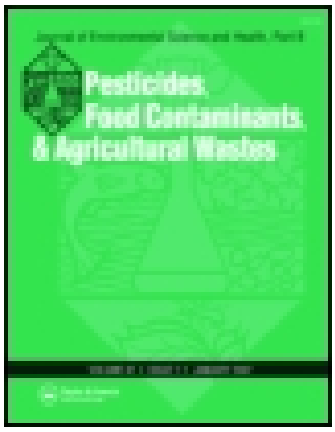

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Mercaptobenzothiazole-on-gold organic phase biosensor systems: 1. Enhanced organosphosphate pesticide determination

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This paper reports the construction of the gold/mercaptobenzothiazole/polyaniline/acetylcholinesterase/polyvinylacetate (Au/MBT/PANI/AChE/PVAc) thick-film biosensor for the determination of certain organophosphate pesticide solutions in selected aqueous organic solvent solutions. The Au/MBT/PANI/AChE/PVAc electrocatalytic biosensor device was constructed by encapsulating acetylcholinesterase (AChE) enzyme in the PANI polymer composite, followed by the coating of poly(vinyl acetate) (PVAc) on top to secure the biosensor film from disintegration in the organic solvents evaluated. The electroactive substrate called acetylthiocholine (ATCh) was employed to provide the movement of electrons in the amperometric biosensor. The voltammetric results have shown that the current shifts more anodically as the Au/MBT/PANI/AChE/PVAc biosensor responded to successive acetylthiocholine (ATCh) substrate addition under anaerobic conditions in 0.1 M phosphate buffer, KCl (pH 7.2) solution and aqueous organic solvent solutions. For the Au/MBT/PANI/AChE/PVAc biosensor, various performance and stability parameters were evaluated. These factors include the optimal enzyme loading, effect of pH, long-term stability of the biosensor, temperature stability of the biosensor, the effect of polar organic solvents, and the effect of non-polar organic solvents on the amperometric behavior of the biosensor. The biosensor was then applied to detect a series of 5 organophosphorous pesticides in aqueous organic solvents and the pesticides studied were parathion-methyl, malathion and chlorpyrifos. The results obtained have shown that the detection limit values for the individual pesticides were 1.332 nM (parathion-methyl), 0.189 nM (malathion), 0.018 nM (chlorpyrifos).

Keywords: Organic phase biosensor; polyaniline; mercaptobenzothiazole; acetylcholinesterase; organophosphorous pesticides; parathion-methyl; malathion; chlorpyrifos.

Introduction

The agricultural industry has seen decades of use of organophospohorous pesticides (OPs) despite the toxic and hazardous nature of these compounds and the fact that these compounds have been used as chemical warfare agents in military applications. The toxic effects of OPs occurs due to their ability to irreversibly modify the catalytic serine residue in acetylcholinesterases (AChE) and subsequent inhibition of the AChE effectively prevents nerve transmission by blocking breakdown of the transmitter choline.[1–3]

The effect of OPs on the environment can be quite severe. Among all the different types of environmental pollutants monitored to date, pesticides are of relevant concern since they have a high toxicity and are still frequently used. Organophosphorous pesticides are widely used for agricultural and domestic purposes, but despite their low persistence in the environment, they have a high acute toxicity to human health and ecosystems. In agriculture these insecticides are used to control amongst others insect and arthropod pests on crops of grains, nuts, cottons and fruits. The point source discharges of OPs have been found to be responsible for aquatic life toxicity. The detection of these pesticides has therefore become a priority since the presence of OPs in natural waters and in foodstuffs is a major-concern for public health.[1,4]

Several standard methods have been traditionally used for pesticide detection and they include gas chromatography (GC) with different detectors (e.g. mass spectrometry in GC-MS), high performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry (HPLC-MS) or UV detection (HPLC-UV).[1,4–6]

The standard methods have certain disadvantages that include the following. They require long assay time and may

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be restricted to a limited number of pesticides. The methods are also known to be expensive and quite laborious requiring labor-intensive preparation of samples and skilled personnel. An alternative to the standard techniques is the use of biosensors as an analytical tool for pesticide determination since it has good selectivity, fast response, miniature size sensors for on-site analysis, low cost, requires no sample pre-treatment, and delivers reproducible results.[1,4–6]

Some basic principles apply to the functioning of an AChE biosensor. The AChE enzyme is first immobilized on the working electrode surface, using either direct or indirect immobilization techniques. In direct immobilization techniques, the enzyme can be incorporated in the carbon paste used for the working electrode surface.[7] Using an indirect approach, physical entrapment of the enzyme is done by incorporating the enzyme in a polymer matrix such as polyaniline (PANI) that will act as an electron shuttling mediator between the electrode surface and the enzyme.[8] The next step involves the use of the appropriate substrate such as acetylthiocholine (ATCh) that forms an electroactive product called thiocholine (TCh) in its interaction with AChE. Thiocholine produces an irreversible oxidation peak that can be monitored in the biosensor's inhibition by OPs, since the inhibition produces a decline in the oxidation current of TCh.[2,4,6,9]

In this paper, we describe the application of a mercaptobenzothiazole-on-gold biosensor system for organophosphate pesticide compounds. The aim of this work was to improve the detection limit of organophosphorous insecticides with an AChE biosensor, applied to various water miscible organic solvents. The activity of the AChE immobilized in the biosensor construction was measured by amperometry based on the detection of thiocholine produced in the enzymatic hydrolysis of acetylthiocholine as substrate. The biosensor study was carried out in aqueous organic media to ascertain the role of organic phase on the reactivity of the enzyme and the performance of the biosensor.

Materials and methods

Reagents and materials

The reagents aniline (99%), potassium dihydrogen phosphate (99+%), disodium hydrogen phosphate (98+%) and diethyl ether (99.9%) were obtained from Aldrich, Germany. The acetylthiocholine chloride (99%) was obtained from Sigma, Germany. The mercaptobenzothiazole (MBT), acetylcholinesterase (AChE, from Electrophorus electricus, EC 3.1.1.7; ~ 850 U/mg), acetylcholine chloride (99%) and acetone (>99.8%, pestanal) were obtained from Fluka, Germany. The hydrogen peroxide (30%) and the organic solvents ethanol (99.9%, absolute grade), acetonitrile (99.9%, pestanal grade) were purchased from Riedel-de Haën, Germany. The potassium chloride, sulphuric acid (95%), and hydrochloric acid (32%) were obtained from Merck, South Africa. Organophosphorous pesticides used in this study include fenthion, diazinon, chlorpyrifos, malathion and parathion-methyl. These pesticide standards were purchased from Riedel-de Haën, Germany.

Platinum (Pt) wires as counter electrodes were obtained from Sigma-Aldrich, South Africa. Alumina micropolish and polishing pads that were used for the polishing of the working electrode were obtained from Buehler, IL, USA.[10]

Instrumentation

All electrochemical protocols were performed and recorded with a computer interfaced to a BAS-50/W electrochemical analyzer with BAS-50/W software (Bioanalytical Systems, Lafayette, IN, USA), using either cyclic voltammetry (CV), Oysteryoung square wave voltammetry (OSWV), differential pulse voltammetry (DPV) or time-based amperometric modes. A conventional three-electrode system was employed. The working electrode was a gold disc electrode (diameter: 1.6 mm; area: 2.01×10^{-2} cm^2; Bioanalytical Systems, Lafayette, IN, USA). Silver/silver chloride (Ag/AgCl – 3 M NaCl type) was used as the reference electrode and a platinum wire was used as auxiliary electrode.[11]

Electrode surface preparation

Prior to use, gold electrodes were first polished on aqueous slurries of 1 μm, 0.3 μm and 0.05 μm alumina powder. After thorough rinsing in deionized water followed by acetone, the electrodes were etched for about 5 minutes in a hot 'Piranha' solution {1:3 (v/v) 30% H_2O_2 and concentrated H_2SO_4} and rinsed again with copious amounts of deionized water. The polished electrodes were then cleaned electrochemically by cycling the potential scan between -200 and + 1500 mV (vs. Ag/AgCl) in 0.05 M H_2SO_4 at the scan rate of 40 mV.s^{-1} for 10 min or until the CV characteristics for a clean Au electrode were obtained.

The platinum (Pt) counter electrode was regularly cleaned before and after synthesis and in between synthesis and analysis. This involved flaming the Pt electrode in a Bunsen burner until it was white hot, followed by rinsing with copious quantities of deionized water.[11–13]

Preparation of mercaptobenzothiazole self-assembled monolayer on gold electrode

A self-assembled monolayer (SAM) of mercaptobenzothiazole (MBT) was formed by immersing the cleaned Au electrode into an ethanol solution containing 10 mM of MBT for 2 hours. After deposition the SAM electrode was rinsed extensively with ethanol and water and kept in 0.1 M phosphate buffer (pH 7.2) for later use. This electrode was then referred to as Au/MBT.[14–17]

Electropolymerization of polyaniline (PANI) films onto an Au/MBT electrode

A three-electrode arrangement was set up in a sealed 10 mL electrochemical cell. Polyaniline (PANI) films were prepared by electropolymerisation from a 0.2 M aniline solution dissolved in 1 M hydrochloric acid (HCl) onto the previously prepared Au/MBT-modified electrode. The aniline/HCl solution was first degassed by passing argon (Ar) through the solution for approximately ten minutes and keeping the Ar blanket during electropolymerization. Initial optimization of the potential window for electropolymerization was performed. During electropolymerization the potential was scanned from an initial potential (E_i) of −200 mV to a switch potential (E_λ) of +1200 mV, at a scan rate of 40 mV/s vs. Ag/AgCl as a reference. The polymerization process was stopped after 10 voltammetric cycles, to ensure a smooth and relatively thin polymer film surface was obtained. The Au/MBT-polyaniline modified electrode was then rinsed with deionized water and used as the working electrode in subsequent studies. The electrode will be referred to as Au/MBT/PANI for the gold-MBT-PANI modified.[13,15,18,19]

Preparation of Au/MBT/PANI modified enzyme electrode

Following the electropolymerisation of a fresh PANI polymer film on an Au/MBT electrode, the Au/MBT/PANI electrode was transferred to a batch cell, containing 1 mL argon degassed 0.1 M phosphate buffer (pH 7.2) solution. The PANI polymer film was then reduced at a potential of –500 mV (vs. Ag/AgCl) until a steady current was achieved, which took approximately thirty minutes.

Electrochemical incorporation of the enzyme acetylcholinesterase (AChE) onto the PANI film was carried out next. This involved the addition of 60 μL of AChE to the 0.1 M phosphate buffer (pH 7.2) solution. After the enzyme solution was argon degassed, enzyme immobilization was achieved by oxidation of the PANI film in the presence of AChE at a potential of +400 mV (vs. Ag/AgCl) until a steady current was achieved, which took approximately forty minutes.

During the oxidation step, the enzyme AChE was electrostatically attached to the polymer film via an ion-exchange process. The biosensor was then rinsed with deionised water to remove any unbound enzyme and stored in the working 0.1 M phosphate buffer (pH 7.2) solution at 4°C. The resulting biosensor will be referred to as Au/MBT/PANI/AChE biosensor.

For the Au/MBT/PANI/AChE bioelectrode, after enzyme incorporation the bioelectrode was arranged vertically and then coated with a 2 μL drop of poly(vinyl acetate) (PVAc) solution (0.3 M) prepared in acetone and left to dry for 1 min. The resulting biosensor will be referred to as Au/MBT/PANI/AChE/PVAc biosensor.[10,11,18,19]

Electrochemical measurements using AChE-based biosensors in the presence of acetylthiocholine as substrate

The electrochemical cell used for the electrocatalytic oxidation of acetylthiocholine (ATCh) consisted of Au/MBT/PANI/AChE/PVAc bioelectrode, platinum wire and silver/silver chloride (Ag/AgCl) as the working, counter and reference electrode, respectively. A 1 mL test solution containing 0.1 M phosphate (0.1 M KCl, pH 7.2) solution was degassed with argon before any substrate was added and after each addition of small aliquots of 0.01 M acetylthiocholine (ATCh). Cyclic, square wave and differential pulse voltammetry were used to measure the responses of the AChE-based biosensor towards ATCh as substrate.[13,20–22]

Cyclic voltammetry (CV) was performed at a slow scan rate of 10 mV.s^{-1} to study the catalytic oxidation of ATCh by applying a linear potential scan between −400 mV and +1800 mV (vs. Ag/AgCl). The cyclic voltammogram was first obtained in the absence of the substrate ATCh, followed by analysis in the presence of ATCh as substrate. Sequential 20 mL aliquots of 0.01 M acetylthiocholine (ATCh) were then added to the 1 mL of 0.1 M phosphate buffer (0.1 M KCl, pH 7.2) solution, degassed with argon and a blanket of gas was kept for the duration of the experiment. The phosphate buffer solution was stirred after each addition of ATCh. This was done to ensure homogeneity of the solution before measurements were taken.[11,18,19]

Osteryoung-type square wave voltammetry (OSWV) was performed immediately after cyclic voltammetric analysis with the AChE-based biosensor in 1 mL of 0.1 M phosphate buffer (0.1 M KCl, pH 7.2) solution, containing different concentrations of ATCh as the substrate under anaerobic conditions (system kept under an argon blanket). The anodic difference square wave voltammogram (SWV) was collected in an oxidation direction only by applying a linear potential scan between −400 mV and +1800 mV (vs. Ag/AgCl), at a step potential of 4 mV, a frequency of 5 Hz, and a square wave amplitude of 50 mV. The SWV was first obtained in the absence of the substrate ATCh, followed by analysis in the presence of ATCh as substrate.[11,23]

Differential pulse voltammetry (DPV) immediately followed SWV analysis with the AChE-based biosensor in 1 mL of 0.1 M phosphate buffer (0.1 M KCl, pH 7.2) solution, containing different concentrations of ATCh as the substrate under anaerobic conditions (system kept under an argon blanket). The anodic difference differential pulse voltammogram (DPV) was collected in an oxidation direction only by applying a linear potential scan between −400 mV and +1800 mV (vs. Ag/AgCl), at a scan rate of 10 mV.s^{-1} and a pulse amplitude of 50 mV. The sample width, pulse width and pulse period were 17 ms, 50 ms and 200 ms, respectively. The DPV was first obtained in the absence of the substrate ATCh, followed by analysis in the presence of ATCh as substrate.[11,23]

Inhibitory studies of AChE-based biosensors in the presence of pesticide inhibitors

A new Au/MBT/PANI/AChE/PVAc biosensor was prepared each time a new organophosphorous pesticide was studied. A new biosensor was also prepared for each of the six concentrations of the organophosphorous pesticides studied. The electrochemical cell consisted of Au/MBT/PANI/AChE/PVAc bioelectrode, platinum wire and Ag/AgCl as the working, counter and reference electrode, respectively. A 1 mL test solution containing 0.1 M phosphate (0.1 M KCl, pH 7.2) solution was degassed with argon before any substrate was added and after each addition of small aliquots of 0.01 M acetylthiocholine (ATCh).

Inhibition plots for each of the organophosphorous pesticides detected were obtained using the percentage inhibition method. The following procedure was used. The biosensor was first placed in a stirred 1 mL of 0.1 M phosphate (0.1 M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution was added until a stable current and a maximum concentration of 2.4 mM were obtained. This steady state current is related to the activity of the enzyme in the biosensor when no inhibitor was present. This was followed by incubating the biosensor in anaerobic conditions for 20 min with a standard pesticide phosphate buffer-organic solvent mixture. This was followed by multiple additions of a standard ATCh substrate solution (anaerobic conditions), to a fresh 1 mL of 0.1 M phosphate (0.1 M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution was again added, until a stable current was obtained. The maximum concentration of acetylthiocholine (ATCh) was again 2.4 mM. The percentage inhibition was then calculated using the formula:[24–27]

$$I\% = \frac{(I_1 - I_2)}{I_1} \times 100 \qquad (1)$$

where I% is the degree of inhibition, I_1 is the steady-state current obtained in buffer solution, I_2 is the steady-state current obtained after the biosensor was incubated for 20 min in phosphate buffer-organic solvent mixture.

Cyclic, square wave and differential pulse voltammetric measurements were performed after each addition of ATCh up to a maximum concentration of 2.4 mM. Cyclic voltammetry (CV) was performed at a scan rate of 10 mV.s^{-1} by applying a linear potential scan between −400 mV and +1800 mV (vs. Ag/AgCl). For some experimental runs the anodic difference square wave voltammogram (SWV) was collected in an oxidation direction only by applying a linear potential scan between – 400 mV and + 1800 mV (vs. Ag/AgCl), at a step potential of 4 mV, a frequency of 5 Hz, and a square amplitude of 50 mV.

The anodic difference differential pulse voltammogram (DPV) was collected in an oxidation direction only by applying a linear potential scan between – 400 mV and + 1800 mV (vs. Ag/AgCl), at a scan rate of 10 mV.s^{-1} and a pulse amplitude of 50 mV. The sample width, pulse width and pulse period were 17 ms, 50 ms and 200 ms, respectively.[11,23]

Optimisation of acetylcholinesterase (AChE) enzyme loading

The operation of the Au/MBT/PANI/AChE/PVAc biosensor was evaluated at different amounts of AChE enzyme incorporated into the biosensor. To achieve this, 0.1 M phosphate buffer, 0.1 M KCl (pH 7.2) solutions were prepared and used. Following the electropolymerisation of a fresh PANI polymer film on an Au/MBT electrode, the Au/MBT/PANI electrode was transferred to a batch cell, containing 1 mL argon degassed 0.1 M phosphate buffer (pH 7.2) solution. The PANI polymer film was then reduced at a potential of −500 mV (vs. Ag/AgCl) until a steady current was achieved, which took approximately thirty minutes.

Electrochemical incorporation of the enzyme acetylcholinesterase (AChE) onto the PANI film was carried out next. This involved the addition of 40 μL of AChE to the 0.1 M phosphate buffer (pH 7.2) solution. After the enzyme solution was argon degassed, enzyme immobilization was achieved by oxidation of the PANI film in the presence of AChE at a potential of +400 mV (vs. Ag/AgCl) until a steady current was achieved, which took approximately forty minutes. The Au/MBT/PANI bioelectrode was arranged vertically and then coated with a 2 μL drop of poly(vinyl acetate) (PVAc) solution (0.3 M) prepared in acetone and left to dry for 1 min.. The same procedure was followed to incorporate 60 and 80 μL of AChE enzyme into the PANI polymer surface.

Voltammetric characterization was performed at a slow scan rate of 10 mV.s^{-1} to study the catalytic oxidation of ATCh by applying a linear potential scan between −400 mV and +1800 mV (vs. Ag/AgCl). The voltammograms were first obtained in the absence of the substrate ATCh, followed by analysis in the presence of ATCh as substrate. Sequential 20 mL aliquots of 0.01 M acetylthiocholine (ATCh) were then added to the 1 mL of 0.1 M phosphate buffer (0.1 M KCl, pH 7.2) solution, degassed with argon and a blanket of gas was kept for the duration of the experiment. The phosphate buffer solution was stirred after each addition of ATCh. This was done to ensure homogeneity of the solution before measurements were taken.[27,28]

pH Optimization for acetylcholinesterase (AChE) immobilized in Au/MBT/PANI/AChE biosensor

The operation of the Au/MBT/PANI/AChE/PVAc biosensor was evaluated at different pH values. To achieve this, 0.1 M phosphate buffer, 0.1 M KCl solutions were prepared at different pH values of 6.0; 6.5; 7.2; 7.5 and 8.0. A 1 mL test solution containing 0.1 M phosphate buffer,

0.1 M KCl solution was degassed with argon before any substrate was added. The Au/MBT/PANI/AChE/PVAc biosensor was then evaluated in the 1 mL test solution with small aliquots of the substrate consisting of 0.01 M acetylthiocholine (ATCh) being added to the test solution, followed by degassing. The maximum current response of the biosensor was then obtained at the different pH values after 2 mM of the ATCh substrate was added to the Au/MBT/PANI/AChE/PVAc biosensor.[22,24,29]

Long-term stability investigation of Au/MBT/PANI/AChE biosensor

The operation of the Au/MBT/PANI/AChE/PVAc biosensor was evaluated at different time intervals of 7 day periods for a total of 30 days, using one specific biosensor. A 1 mL test solution containing 0.1 M phosphate buffer, 0.1 M KCl solution was degassed with argon before any substrate was added. The Au/MBT/PANI/AChE/PVAc biosensor was then evaluated in the 1 mL test solution with small aliquots of the substrate consisting of 0.01 M acetylthiocholine (ATCh) added to the test solution, followed by degassing. The maximum current response of the biosensor was then obtained after 2 mM of the ATCh substrate was added to the Au/MBT/PANI/AChE/PVAc biosensor. This procedure was performed on 0, 7, 14, 21 and 28 days using one specific Au/MBT/PANI/AChE/PVAc biosensor.[24]

Temperature stability investigation of Au/MBT/PANI/AChE biosensor

The temperature stability of the Au/MBT/PANI/AChE/PVAc biosensor was evaluated at different temperature values. To achieve this, the optimum temperature for AChE activity in the constructed biosensor was determined by assaying the biosensor at various temperatures of 10, 15, 20, 25, 30, and 35°C.

A 1 mL test solution containing 0.1 M phosphate buffer, 0.1 M KCl solution was degassed with argon before any substrate was added, and incubated in a small water bath for approximately 10 minutes at a specific temperature. The Au/MBT/PANI/AChE/PVAc biosensor was then evaluated in the 1 mL test solution with small aliquots of the substrate consisting of 0.01 M acetylthiocholine (ATCh) added to the test solution, followed by degassing. The maximum current response of the biosensor was then obtained after 2 mM of the ATCh substrate was added to the Au/MBT/PANI/AChE/PVAc biosensor. This procedure was performed at 10, 15, 20, 25, 30, and 35°C using different Au/MBT/PANI/AChE/PVAc biosensors.[30,31]

Determination of the Limit of Detection (LOD)

A 1 mL test solution containing 0.1 M phosphate buffer, 0.1 M KCl solution was degassed with argon before any substrate was added. The AChE-biosensor was then evaluated in the 1 mL test solution by performing 10 replicate measurements on the 0.1 M phosphate buffer, 0.1 M KCl solution, or on any one of the analyte (standard pesticide) solutions at the lowest working concentration. A calibration graph of current (A) versus saline phosphate buffer or analyte concentration was then constructed for which the slope and the linear range was then determined. The limit of detection (LOD) was then calculated with the following equation:

$$LOD = \frac{3 \cdot \sigma_{n-1}}{m} = \frac{3 \cdot s}{m} \quad (2)$$

where s is the standard deviation of the 10 replicate measurements on the 0.1 M phosphate buffer, 0.1 M KCl solution, or on any one of the analyte (standard pesticide) solutions at the lowest working concentration. The variable m represents the slope of the calibration graph in the linear range that is also equal to the sensitivity of the measurements performed.[3]

Results and discussion

Design of an Au/MBT/PANI/AChE/PVAc biosensor for pesticide detection

In this work it is demonstrated that a gold disc electrode can be coated with a mercaptobenzothiazole (MBT) self-assembled monolayer (SAM) prior to polyaniline electropolymerization, followed by AChE immobilization and poly (vinyl acetate) coating to create a thick film electrode for sensitive organophosphorous pesticide detection. The dual role of polyaniline, which shows electrocatalytic activity towards thiocholine and serves as an immobilization matrix for the AChE as an enzyme and that of poly (vinyl acetate) (PVAc) as a binder in this thick-film electrode is demonstrated.

Different technologies exist to develop thick-film biosensors for pesticide detection.[20,24,32] These different technologies can be divided into three categories: (i) multiple-layer deposition with biological deposition by hand or electrochemically, (ii) using screen-printing techniques of composite inks or pastes in two or more steps with biological deposition done by screen-printing, (iii) using a one-step deposition layer also called the biocomposite strategy. One of the main aims of this work was to develop an electrode which can be exposed to organic solutions containing potential inhibitors without having the polymer layer after separating from the electrode surface after use thereby using poly(vinyl acetate) as the binder to circumvent this problem. Cellulose acetate is known to be used as a synthetic resin in screen-printing inks to improve printing qualities or as a selective membrane over platinum anodes to reduce interferences.[33,34]

The detection of pesticides in non-aqueous environments has been reported but few publications refer to the use of immobilized AChE biosensors in non-aqueous media. Organophosphorous and carbamate pesticides are characterized by a low solubility in water and a higher solubility in organic solvents. It is for this fact that the extraction and concentration of pesticides from fruits, vegetables, etc. are carried out in organic solvents. It is known that some enzymes, e.g. glucose oxidase, work well in both water and organic solvents, while other enzymes require a minimum amount of water to retain catalytic activity. To circumvent the problem of hydrophilic solvents stripping the enzymes of essential water of hydration necessary for enzymatic activity, it is recommended that 1–10% water be added to the organic solvent for sufficient hydration of the active site of the enzyme.[26,35–38]

Polyaniline (PANI) was used as a mediator in this biosensor construction to harvest its dual role as immobilization matrix for AChE and use its electrocatalytic activity towards thiocholine (TCh) for amperometric sensing. With that in mind, the following amperometric sensor design and mechanism is proposed and explained.

In Figure 1 the schematic representation for the biosensor reaction taking place is presented. In Figure 1 it can be seen that as acetylthiocholine (ATCh) is catalyzed by acetylcholinesterase (AChE), it forms thiocholine (TCh) and acetic acid. Thiocholine is electroactive and is oxidized in the reaction. In return the conducting PANI polymer reacts with thiocholine and also accepts an electron from mercaptobenzothiazole as it is oxidized through interaction with the gold electrode.[3]

Substrate evaluation of AChE-polyaniline biosensor in 0.1 M phosphate buffer (pH 7.2) saline solution

In Figure 2 the DPV responses of the Au/MBT/PANI/AChE/PVAc biosensor for the successive addition of ATCh substrate to a 0.1 M phosphate buffer, KCl (pH 7.2) solution is shown.

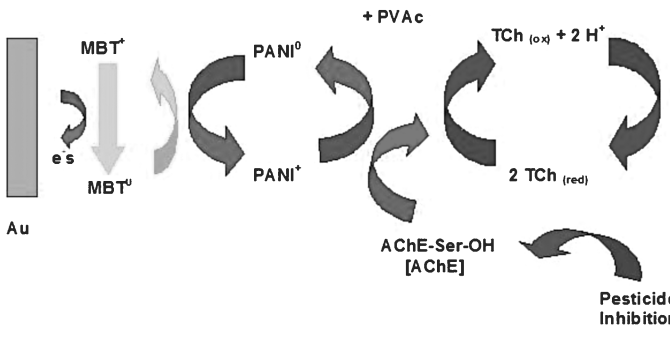

Fig. 1. The schematic representation of the Au/MBT/PANI/AChE/PVAc biosensor reaction occurring at the gold SAM modified electrode.[3,27]

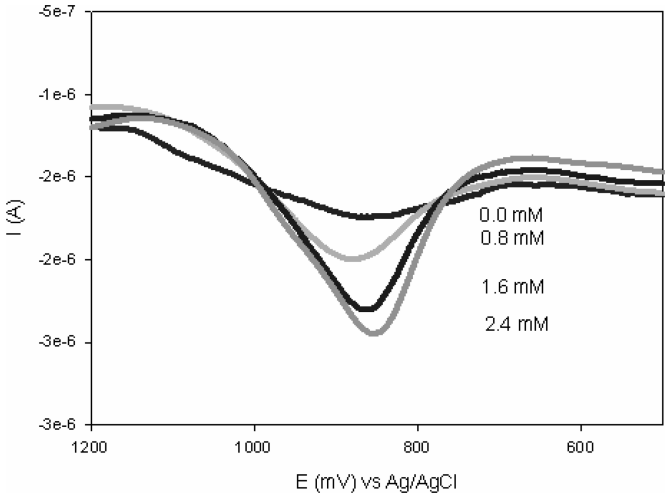

Fig. 2. DPV responses for successive ATCh substrate addition to the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution at a scan rate of 10 $mV.s^{-1}$, and in a potential window of +500 to +1200 mV.

The DPV results in Figure 2 are shown in a shorter potential window to highlight the observed increase in anodic peak current. The results show the voltammetric responses for the electrocatalytic oxidation of acetylthiocholine at the Au/MBT/PANI/AChE/PVAc biosensor. The DPV responses shows an increase in peak current heights upon the successive additions of ATCh as substrate, with the results more pronounced around a specific potential.

The peak current, I_{peak}, for specific concentrations of the ATCh substrate added in Figure 2 was -1.737×10^{-6} A for 0.0 mM of ATCh added, followed by -1.992×10^{-6} A for 0.8 mM of ATCh, then -2.314×10^{-6} A was obtained for 1.6 mM of ATCh added, while it was -2.448×10^{-6} A for the highest concentration of 2.4 mM of ATCh added. These results clearly indicated that a biosensor was established and that AChE as enzyme was hydrolyzing the substrate ATCh, with the increase in oxidative current as the preferred route of reaction.[3]

Optimal acetylcholinesterase (AChE) enzyme loading of Au/MBT/PANI/AChE/PVAc biosensor

The amount of enzyme incorporated during the biosensor construction is an important element during construction and it affects the sensor's limit of detection. If the aim of the biosensor is to achieve the lowest possible detection, only a minimum amount of enzyme must be used.[39–41]

In Figure 3 the results for the amperometric responses of the Au/MBT/PANI/AChE/PVAc biosensor to different amounts of the enzyme AChE incorporated into the biosensor is shown.

Since the enzyme AChE was incorporated into the PANI polymer matrix by adsorption at fixed potential, different amounts of the enzyme was dissolved in a 1 mL of 0.1 M

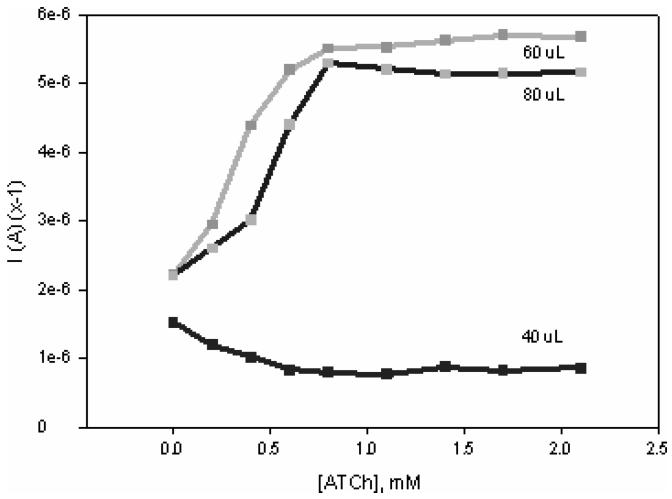

Fig. 3. The amperometric responses of the AChE biosensor to different amounts of enzyme incorporated into the biosensor. These responses were measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution at 25°C.

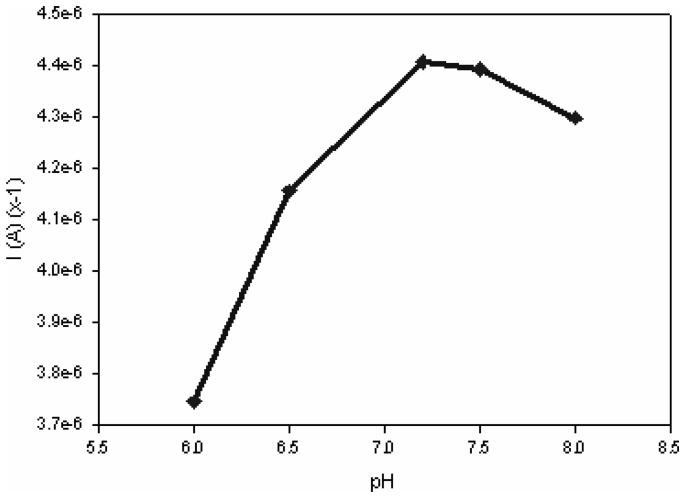

Fig. 4. Graph displaying the effect of pH on the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution with 2 mM of ATCh added.

phosphate buffer (pH 7.2) solution. From the results in Figure 3 it can be seen that the biggest increase in current for the successive addition of ATCh substrate, was experienced when the biosensor had 60 μL of AChE dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.2) solution. The results obtained when 80 μL of AChE was used, does not show a very big difference in the current response when compared to the use of 60 μL of AChE. In both these cases it is observed that the biosensor response to ATCh substrate addition starts to level off after 1.0 mM of the substrate has been added. When the results for the use of 60 and 80 μL of AChE is compared to that of the 40 μL of AChE, a big difference in the amperometric response was observed, with the current increasing in several magnitudes. When a small amount of enzyme is incorporated into the biosensor, a very small response in anodic current was thus observed. Therefore, optimal enzyme loading is important and should be optimized as it has an effect on the detection limit of the constructed biosensor.[3]

pH effect on the immobilized AChE in Au/MBT/PANI/AChE/PVAc biosensor

The pH value of the working solution is usually regarded as the most important factor in determining the performance of a biosensor and its sensitivity towards inhibitors. It is thus a rule that the pH maximum of the enzyme activity is evaluated as most appropriate for the substrate and inhibitor determination. And there is agreement that the pH-dependence of the observed inhibiting effect often corresponds to that of the response of a biosensor.[40,42]

It is for this reason that the operation of the biosensor was evaluated at different pH values. Other researchers[37,43,44] have shown that the optimal working pH for the cholinesterases is near 7.5 but it depends on the polymer matrix used for enzyme immobilization, although working pH values between 8 and 9 have also been reported.[24] In Figure 4 the results for the investigation into the effect of different pH values on the working of the Au/MBT/PANI/AChE/PVAc biosensor is displayed.

The Au/MBT/PANI/AChE/PVAc biosensor was evaluated at pH values of 6.0; 6.5; 7.2; 7.5 and 8.0 in a 1 mL of 0.1 M phosphate buffer (pH 7.2) solution, to which a total of 2 mM of ATCh substrate was added. The results obtained are shown in Figure 4 indicating that the highest anodic current was obtained at pH = 7.2, while the result for pH = 7.5 is nor far off. The response profile thus indicate that an optimum pH can be obtained between 7.0 and 7.5, which falls within the range reported in literature[45,46] for the optimum pH of the free enzyme activity in solution. The results further illustrate that enzyme incorporated in the Au/MBT/PANI/AChE/PVAc biosensor was easily accessed by the buffer solution and the acetylthiocholine used as substrate in the buffer solution.[3]

Long-term stability determination of Au/MBT/PANI/AChE/PVAc biosensor

The Au/MBT/PANI/AChE/PVAc biosensor that was prepared under optimum conditions was tested at 25°C to determine the long-term stability of the biosensor. The biosensor was stored at 4°C for a length of approximately 30 days and the biosensor was tested every 7 days by adding the substrate ATCh to a 1 mL of 0.1 M phosphate buffer, KCl (pH 7.2) solution, containing the biosensor, and measuring the current at every addition. The results obtained for the successive addition of the substrate to the biosensor on 0, 7, 14, 21 and 28 days are displayed in Figure 5.

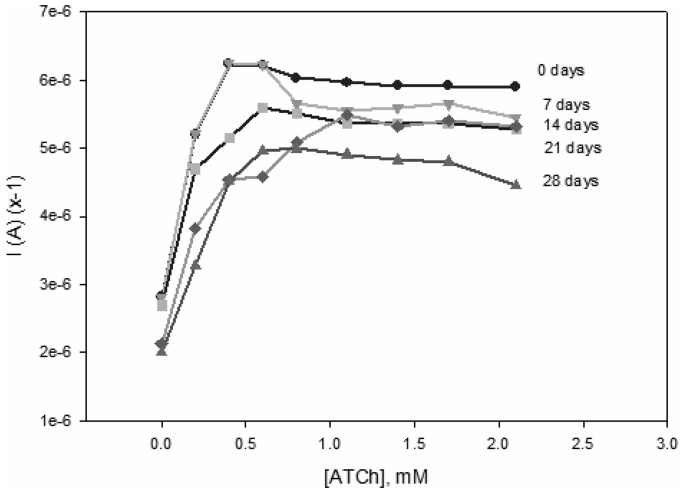

Fig. 5. Graph displaying the results for the long-term stability of the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution at 25°C, for successive additions of the ATCh substrate.

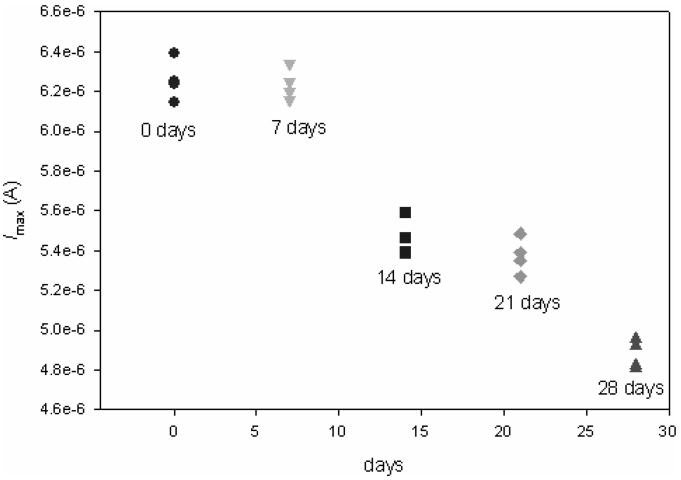

Fig. 6. Graph of variation in maximum current (I_{max}) vs. days in long-term stability determination ($n = 4$) of the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution at 25°C, for 2.0 mM of ATCh added.

The results in Figure 5 show that the biosensor responses reach a maximum current within the first 0.4 to 1.1 mM of substrate added to the biosensor. After 1.1 mM of substrate added it reaches a plateau and minimum changes in the current is observed. The results further indicate a gradual decrease in the first 21 days, with a bigger difference in current observed on the 28th day of biosensor response measured. On the first day of analysis, the maximum current (I_{max}) for the biosensor was measured at -6.234×10^{-6} A, while on the 28th day after construction the biosensor response at I_{max} was -4.957×10^{-6} A. The difference in current response on the 28th day was 20% of the initial value. In the work done by Sen *et al.*[45] a polyvinylferrocenium modified Pt electrode was constructed and the amperometric response of the biosensor to choline and acetylcholinesterase was measured, indicating that the enzyme electrode responses gradually decreased in the first 25 days.

During the long-term stability studies of the Au/MBT/PANI/AChE/PVAc biosensor, a total of four measurements were made with the biosensor on a specific day to determine any variation in the readings obtained. This was further done to determine the operational stability of the biosensor during the long-term stability testing. The results obtained for the maximum current (I_{max}) of the four readings taken on a specific day, are displayed in Figure 6.[3]

The results shown in Figure 6 indicate first that there was some variation in the readings obtained on a specific day, but the deviation is relatively small indicating that the biosensor was stable during the period of investigation. The results in the graph also shows that during the first 7 days of construction, the values of I_{max} obtained are relatively close at the same maximum current, while there is a drop in the current at day 14, which on the other hand was relatively close to the maximum current obtained after 21 days of construction. After 28 days there was a drop in the maximum current of the biosensor during analyses, which agree with the results shown in Figure 5.[3]

Temperature stability investigation of Au/MBT/PANI/AChE/PVAc biosensor

Another critical factor in the determination of the activity and stability of a biosensor, is the temperature at which measurements are performed.[42,45,47] The response of the Au/MBT/PANI/AChE/PVAc biosensor to successive additions of the substrate ATCh in a 1 mL of 0.1 M phosphate buffer, KCl (pH 7.2) solution, was determined at different temperatures varying from 10 to 35°C. In Figure 7 the

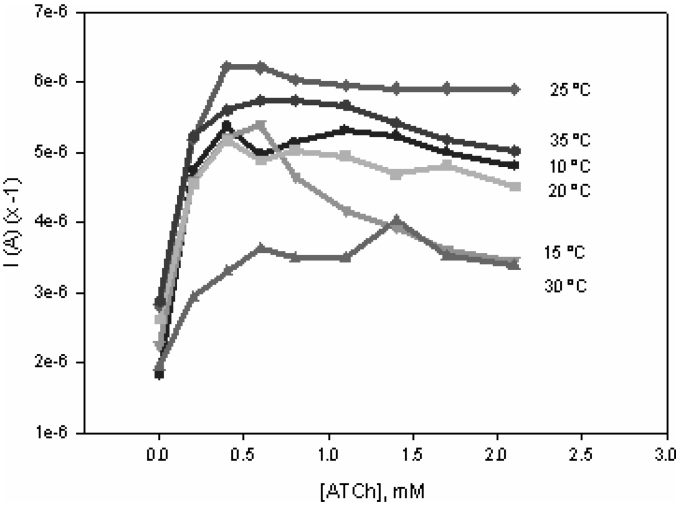

Fig. 7. Graph displaying the effects of temperature on the activity of the enzyme in the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution.

results obtained for the response of the enzyme AChE at different temperatures are shown.

The results in Figure 7 show that for the six temperatures investigated, maximum current (I_{max}) was reached within 0.6 to 1.0 mM of ATCh substrate added, indicating that the enzyme AChE responded favorably to most temperatures evaluated. The activity of the immobilized enzyme reached a maximum at 25°C, with the highest current obtained at this temperature. At the low temperatures of 10, 15 and 20°C, it was observed that the activity of the enzyme was relatively similar during the first 0.5 mM of ATCh substrate added. On the other hand, the higher temperatures of 30 and 35°C gave mixed results with the activity of the enzyme high at 35°C, while the lowest activity was obtained for 30°C. At 15°C, it was observed that after maximum current was reached at 0.6 mM of substrate added, there was a big decrease in the activity of the enzyme, which can be attributed to denaturation of the enzyme. At none of the other temperatures investigated, the same effect was observed.[3]

The effect of polar organic solvents on the amperometric behavior of the biosensor

It is known that organic solvents can induce extensive changes in the activity and specificity of an enzyme. This is because the enzyme's structure and reactivity depends on several non-covalent interactions in the biocatalyst, which includes hydrogen bonding, ionic, hydrophobic, and van der Waals interactions. Enzymes have further evolved to maintain their structural stability in aqueous medium, but organic solvents are known to disrupt the abovementioned forces of interaction in the enzyme, causing changes in the kinetic and thermodynamic behavior of the enzyme.[48] Any changes that occur in solvent hydrophobicity, dielectric constant and water content of the reaction medium, affect the ability of enzymes to use their free energy of binding with a substrate, leading to changes in substrate specificity and reactivity.[48,49] The solvents media that can be used for biosensing can be classified into two groups, i.e. anhydrous organic media and water-containing media. In the case of anhydrous organic media, it refers to pure solvents or a mixture of pure organic solvents that may be polar or non-polar in nature. On the other hand, water-containing organic media consist of micro-aqueous systems, water-organic solvent mixtures, water and immiscible organic solvent biphasic systems and reverse micellar solutions.[48,50] The term micro-aqueous reaction media are associated with the non-polar organic solvents that are immiscible with water. Since it is known that enzymes generally require essential water of hydration for activity, it is essential that non-polar solvents be saturated with water before they are used as reaction media for biosensing. These systems will contain a water content that is very insignificant when compared to the water content of organic solvents, and it depends on the ability of the solvent to absorb water. Since the so-called anhydrous aqueous systems require a minimum amount of water for enzyme activity, it is more suitable to refer to such a system as a micro-aqueous solution.[48,51] In the case of polar organic solvents, they can be used as systems that contain some amount of water. The hydration of polar solvents ensures that the flexibility, structure and local dielectric constant of the enzyme redox site environment, stay as much as possible, unaltered. If the effect of increased solvent polarity occurs, it will weaken the electrostatic forces in the enzyme, which will lead to water partitioning out of the enzyme into the bulk solvent. Therefore, biosensors exhibit much greater reactivity in the presence of polar organic solvents that contain some amount of water.[48]

The effect of organic solvents on the activity of AChE in the constructed Au/MBT/PANI/AChE/PVAc biosensor has been studied in the presence of polar organic solvents containing a 0–10% aqueous solution of water. The polar organic solvents used in this study includes acetonitrile, acetone and ethanol. The response of the Au/MBT/PANI/AChE/PVAc biosensor was first measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution, in the presence of a fixed concentration of ATCh. The biosensor was thereafter incubated for 20 minutes in an aqueous-solvent mixture or the pure organic solvent. The response of the Au/MBT/PANI/AChE/PVAc biosensor was then again measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution, in the presence of a fixed concentration of ATCh. The results of the two respective measurements were then used to calculate the percentage inhibition using the formula.[3]

$$I\% = \frac{(I_1 - I_2)}{I_1} \times 100 \tag{3}$$

where $I\%$ is the degree of inhibition, I_1 is the steady-state current obtained in buffer solution after the addition of ATCh substrate, and I_2 is the steady-state current obtained after the AChE biosensor was incubated for 20 minutes in the organic solvent. In Figure 8 the results obtained for the inhibition of AChE in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in (a) 10% water-organic solvent mixture, (b) 5% water-organic solvent mixture, and pure organic solvent are shown.[3]

From the results in Figure 8 it can be seen that for the three different 10% water-organic solvent mixtures investigated, the lowest decrease in catalytic activity of AChE was observed in acetone, compared to acetonitrile and ethanol. For the 5% water-organic solvent mixtures, ethanol had the lowest decrease on the catalytic activity of AChE, while in the pure polar organic solvent it was observed that ethanol had again the lowest decrease on the catalytic activity of AChE.[3]

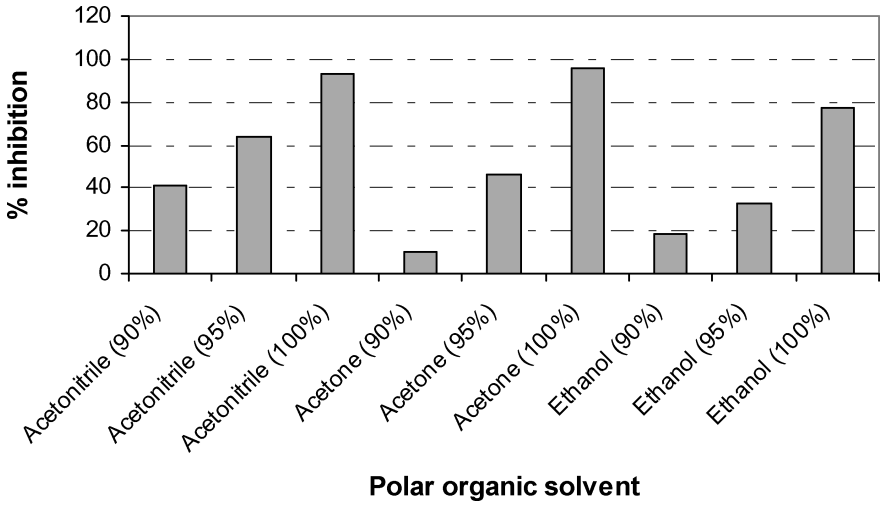

Fig. 8. Results obtained for the inhibition of AChE in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in (a) 10% water-organic solvent mixture, (b) 5% water-organic solvent mixture, and pure organic solvent. The ATCh concentration was 2.0 mM.

Results for the water-organic solvent mixture investigations

The results for the 10% water-organic solvent mixture in Figure 8 also show that the highest decrease in the catalytic activity of AChE was observed when the biosensor was exposed to acetonitrile, while the lowest decrease was observed in acetone. If one looks at the use of organic solvents in biocatalysis, research have shown that enzymes have a high activity in hydrophobic solvents that have a log *P* value greater than 4. On the other hand, enzyme activity will be low in hydrophilic solvents with a log *P* value less than 2, where *P* is the octanol/water partition coefficient of a specific organic solvent.[19] The log *P* values of the polar solvents used in this study are −0.33, −0.23 and −0.24 for acetonitrile, acetone and ethanol respectively.[52] Since the log *P* values of the above three solvents are less than 2, it was expected that the AChE activity will not be high since the solvents are hydrophilic. The effect of adding 10% water to keep the active center of the AChE enzyme hydrated during the biosensor studies, help to show that only 10% inhibition of the AChE catalytic activity was observed in acetone.[3]

The results for the 5% water-organic solvent mixture show that the highest inhibition experienced by AChE as enzyme in the Au/MBT/PANI/AChE/PVAc biosensor was in acetonitrile as solvent, while the lowest inhibition was experienced in ethanol. Since the enzyme was incubated in 5% water-organic solvent mixtures, a higher degree of inhibition was seen from the results when compared to that of the 10% water-organic solvent mixtures. This can be attributed to the fact that less water was used in the water-organic solvent mixtures under discussion, as compared to the first results reported. When 5% water-organic solvent mixtures were used, the lowest inhibition observed was 10% for acetone, while for the 10% water-organic solvent mixtures, the lowest inhibition observed were 33% for ethanol.[3]

The results obtained for the exposure of the enzyme to pure organic solvents showed higher degrees of inhibition. When these results were compared to that of the water-organic solvent mixtures, it is observed that the highest inhibiting effect on the enzyme AChE, was experienced when the enzyme was exposed to the pure organic solvents. The individual inhibition results obtained are 93% in acetonitrile, 96% in acetone and 77% in ethanol. The lowest percentage inhibition of the enzyme AChE was experienced in ethanol, therefore suggesting that the assay of the pesticides in pure ethanol solvent will deliver good results. It further suggests that ethanol will be a good extraction organic solvent, with the added effect of good solubility of ATCh and pesticides in the solvent, eliminating the need to avoid the inactivation effect of the solvent on biosensor response.[3,26]

A summary of the results obtained for the inhibition of AChE as enzyme in the Au/MBT/PANI/AChE/PVAc biosensor, is shown in Table 1.

The results in Table 1 indicate that a 90% aqueous-solvent mixture of acetone and water gave the best results and the smallest degree of inhibition of the electrocatalytic effect of AChE in the Au/MBT/PANI/AChE/PVAc biosensor investigated. The results obtained for the 95% aqueous-solvent mixtures and the pure polar organic solvents clearly indicate the solvent hydrating effect of the three solvent investigated increases as the amount of water is decreased. These results are in line with the investigations reported by Evtugyn *et al.*[40] on the presence of water and in the enzyme's active centre.[3]

Organophosphorous pesticide detection with Au/MBT/PANI/AChE/PVAc biosensor

A new biosensor was prepared each time a new organophosphorous pesticide was studied since the pesticides inhibit

Table 1. Summary of the results obtained for the percentage inhibition of the enzyme acetylcholinesterase (AChE) in the different polar aqueous-solvent mixtures investigated.

		% Inhibition of enzyme, AChE, in different solvent mixtures		
Polar organic solvent	*Log P*	*90% aqueous-solvent mixture*	*95% aqueous-solvent mixture*	*100% pure solvent*
acetonitrile	−0.33	41	63	93
acetone	−0.23	10	47	96
ethanol	−0.24	18	33	77

the enzyme AChE irreversibly (Albareda-Sirvent *et al.* 2001[24]).

Voltammetric results of Au/MBT/PANI/AChE/PVAc biosensor analysis of organophosphates

In Figure 9 the DPV responses for the calibration of the Au/MBT/PANI/AChE/PVAc biosensor to 2.4 mM of the substrate ATCh before incubation and the respective responses obtained after incubation in different parathion-methyl pesticide concentrations are shown.

The results in Figure 9 shows the voltammetric results obtained after 2.4 mM of the substrate ATCh was added to the AChE biosensor, as well as for the highest and lowest standard pesticide concentrations used in the biosensor inhibition studies. The results show that after ATCh addition, an anodic peak was obtained at approximately +1018.9 mV (vs. Ag/AgCl). After incubation studies in different standard pesticide concentrations, the anodic peak potentials had shifted to approximately +910.3 mV (vs. Ag/AgCl). Furthermore, it was observed that there was a relatively high decrease in anodic current after incubation in a 0.6 ppb parathion-methyl standard pesticide solution. This decrease was relatively smaller when the standard pesticide concentration was increased to 10.0 ppb.[3]

In Figure 10 the DPV responses for the calibration of the Au/MBT/PANI/AChE/PVAc biosensor to 2.4 mM of the substrate ATCh before incubation and the respective responses obtained after incubation in different malathion pesticide concentrations are shown.

The results in Figure 10 indicate that an anodic peak was obtained at approximately +1378.6 mV (vs. Ag/AgCl) after 2.4 mM of the substrate ATCh was added to the AChE biosensor. A shift in the peak potential values occurred after the biosensor was incubated in the different malathion standard pesticide concentrations and was subjected to ATCh substrate addition afterwards. For malathion as pesticide a relatively bigger decrease in anodic current was observed between the different pesticide concentrations evaluated.[3]

In Figure 11 the DPV responses for the calibration of the Au/MBT/PANI/AChE/PVAc biosensor to 2.4 mM of the substrate ATCh before incubation and the respective responses obtained after incubation in different chlorpyrifos pesticide concentrations are shown.

Figure 11 shows the voltammetric results obtained after 2.4 mM of the substrate ATCh was added to the AChE biosensor, as well as for the highest and lowest standard pesticide concentrations used in the biosensor inhibition studies. The voltammetric results further show anodic peak data around an average peak potential of +875.9 mV (vs. Ag/AgCl). After incubation studies in different standard

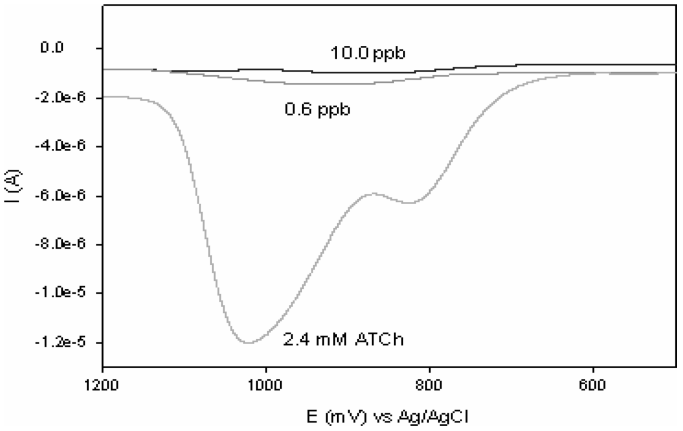

Fig. 9. Results for the DPV responses obtained after parathion-methyl pesticide inhibition studies at different concentrations.

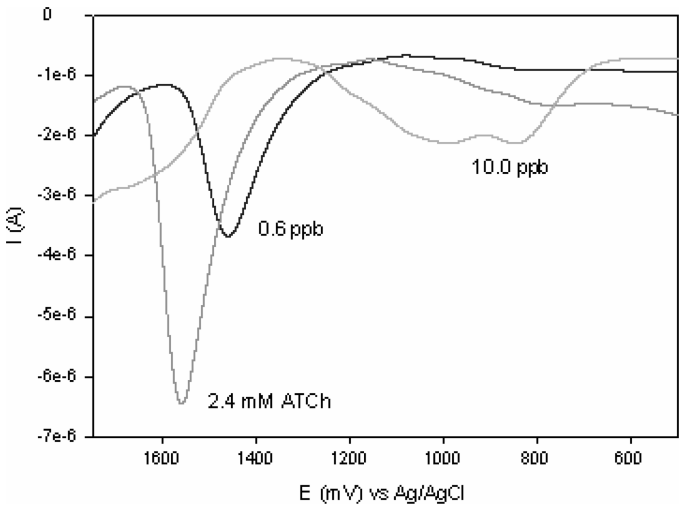

Fig. 10. Results for the DPV responses obtained after malathion pesticide inhibition studies at different concentrations.

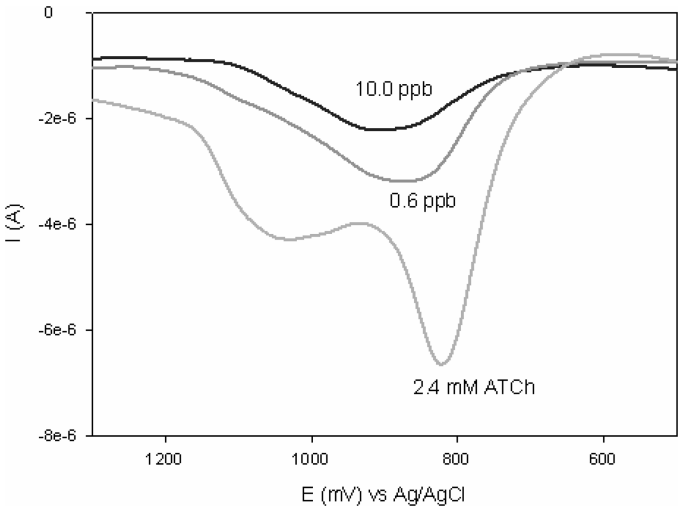

Fig. 11. Results for the DPV responses obtained after chlorpyrifos pesticide inhibition studies at different concentrations.

pesticide concentrations, the anodic peak current decreased for 0.6 ppb of chlorpyrifos added, with a further decrease in anodic current when the chlorpyrifos concentration was 10.0 ppb. The voltammetric results obtained for the analysis of the organophosphorous standard pesticide solutions with the Au/MBT/PANI/AChE/PVAc biosensor, indicate that for the three different pesticides evaluated, the electrocatalytic activity of the AChE enzyme is reduced as the pesticide concentration is increased.[3]

Inhibition plots for standard samples evaluated

Inhibition plots for each of the organophosphorous pesticides detected were obtained using the percentage inhibition method. This involved first placing the biosensor being under a stirred 1 mL of 0.1 M phosphate (0.1M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution were added until a stable current and a maximum concentration of 2.4 mM were obtained. This steady state current is related to the activity of the enzyme in the biosensor when no inhibitor was present. This was followed by incubating the biosensor in anaerobic conditions for 20 min with a standard pesticide saline acetone-phosphate buffer mixture. This was followed by multiple additions of a standard ATCh substrate solution (anaerobic conditions), to a fresh 1mL of 0.1 M phosphate (0.1M KCl, pH 7.2) solution (anaerobic conditions) and multiple additions of a standard acetylthiocholine (ATCh) substrate solution was again added, until a stable current was obtained. The maximum concentration of acetylthiocholine (ATCh) was again 2.4 mM. The percentage inhibition was then calculated.[3,24–26]

In Table 2 the results calculated for the percentage inhibition of the AChE enzyme after incubation in pesticide solutions are shown.

Table 2. Table of results for the percentage inhibition values obtained at the six different organophosphorous pesticide concentrations investigated with the Au/MBT/PANI/AChE/PVAc biosensor.

$-\log[pesticide]$	$\%\, I_{parathion-methyl}$	$\%\, I_{malathion}$	$\%\, I_{chlorpyrifos}$
0.222	14.40	31.96	43.42
0.000	22.18	41.55	51.71
−0.301	34.10	59.10	60.00
−0.699	55.93	67.43	67.49
−0.845	67.21	71.65	72.05
−1.000	81.64	75.59	78.61

From the results in Table 2 it can be seen that for the three different organophosphorous pesticides investigated with the Au/MBT/PANI/AChE/PVAc biosensor, an increase in the percentage inhibition was observed as the concentration of the standard pesticide solutions were increased. At a pesticide concentration of 0.6 ppb used, the lowest percentage inhibition was obtained with parathion-methyl as pesticide, while with the same concentration of pesticide used, the highest percentage inhibition was also obtained with chlorpyrifos as pesticide. When 10.0 ppb of pesticide concentration was used, the lowest percentage inhibition was obtained with malathion as pesticide, compared to the highest percentage inhibition obtained with parathion-methyl as pesticide. The above results shown in Table 2 were then used to construct graphs of percentage inhibition vs. – log [pesticide] that is shown in Figure 12.[3]

The results in Figure 12 show the combined plot for the percentage inhibition vs. – log [pesticide] results for the six different organophosphorous standard pesticide solutions investigated. The graph in (a) represents the results for parathion-methyl, in (b) malathion and in (c) chlorpyrifos.

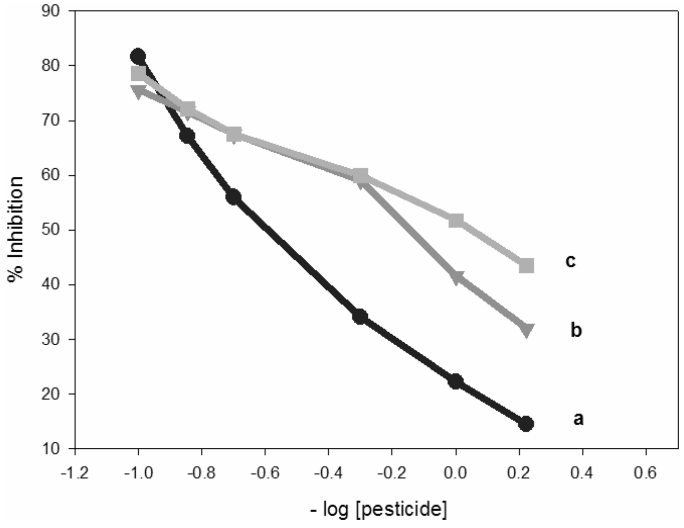

Fig. 12. Graph of percentage inhibition vs. – log [pesticide] for the three different organophosphorous pesticides investigated with the Au/MBT/PANI/AChE/PVAc biosensor.

Table 3. Results for the different parameters calculated from the inhibition plots of the Au/MBT/PANI/AChE/PVAc biosensor detection of standard organophosphorous pesticide solutions ($n = 2$).

	Organophosphorous pesticides				
Pesticide	*Sensitivity (% I/decade)*	*Detection limit (ppb)*	*Detection limit (ppt)*	*Detection limit (nM)*	*Regression coefficient*
Parathion-methyl	−53.66	0.388	388.0	1.332	0.9766
Malathion	−35.24	0.0623	62.3	0.189	0.9679
Chlorpyrifos	−26.68	0.0127	12.7	0.018	0.9875

The results further indicate that the inhibition effect of the pesticides called malathion and chlorpyrifos on the AChE biosensor response are relatively similar for four of the concentrations investigated, since the results for the inhibition plots of these two pesticides are relatively close. Further analyses of the inhibition plots were done and the results are shown in Table 3.[3]

The results in Table 3 show the parameters calculated from the inhibition plots of the Au/MBT/PANI/AChE/PVAc biosensor for the detection of standard organophosphorous pesticide solutions. These parameters include the sensitivity and the detection limit estimated from the inhibition plots. The lowest sensitivity was obtained for chlorpyrifos as pesticide, while the highest sensitivity was obtained for parathion-methyl as pesticide. Chlorpyrifos represents a more powerful organophosphate than the other two pesticides studied (due to the three chlorine atoms substituted in its pyridine ring structure) and with the constructed Au/MBT/PANI/AChE/PVAc biosensor, a very good sensitivity was obtained. For parathion-methyl as pesticide the highest sensitivity was obtained for this study, which is also higher than the 48% sensitivity reported by Lin *et al.* (2004[53]) obtained with a disposable carbon nanotube modified screen-printed biosensor.[3]

Conclusion

This paper has successfully demonstrated the construction and use of an Au/MBT/PANI/AChE/PVAc thick-film biosensor for the detection of organophosphate pesticides in polar organic solvents. It has further shown that self-assembled monolayers can be applied in thick-film biosensor construction and that the poly(vinyl acetate) film does not interfere with the PANI-AChE electrocatalytic activity towards thiocholine. The results obtained show very good detection limits for the standard samples of parathion-methyl, malathion and chlorpyrifos investigated with the Au/MBT/PANI/AChE/PVAc biosensor. When the results for the detection limits of 1.332 nM for parathion-methyl, 0.189 nM for malathion, and 0.018 nM for chlorpyrifosare viewed, several interesting findings were concluded. These include the lowest nanomolar (nM) detection limit for chlorpyrifos of 0.018 nM and a relatively low level of 1.332 nM for parathion-methyl.

Acknowledgments

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References

[1] Valdés-Ramírez, G.; Fournier, D.; Ramírez-Silva, M.T.; Marty, J-L. Sensitive amperometric biosensor for dichlorovos quantification: Application to detection of residues on apple skin. Talanta **2008**, *74*, 741–746.
[2] Dua, D.; Huanga, X.; Cai, J.; Zhang, A. Amperometric detection of triazophos pesticide using acetylcholinesterase biosensor based on multiwall carbon nanotube–chitosan matrix. Sensors and Actuators B **2007**, *127*, 531–535.
[3] Somerset, V.S. Mercaptobenzothiazole-on-gold biosensor systems for organophosphate and carbamate pesticide sompounds. Unpublished PhD Thesis. University of the Western Cape, Bellville, South Africa, **2007**; 1–531.
[4] Kuswandi, B.; Fikriyah, C.I.; Gani, A.A. An optical fiber biosensor for chlorpyrifos using a single sol–gel film containing acetylcholinesterase and bromothymol blue. Talanta **2008**, *74*, 613–618.
[5] Vidal, J.C.; Esteban, S.; Gil, J.; Castillo, J.R. A comparative study of immobilization methods of a tyrosinase enzyme on electrodes and their application to the detection of dichlorvos organophosphorus insecticide. Talanta **2006**, *68*, 791–799.
[6] Schulze, H.; Schmid, R.D.; Bachmann, T.T. Rapid detection of neurotoxic insecticides in food using disposable acetylcholinesterase-biosensors and simple solvent extraction. Anal. Bioanal. Chem. **2002**, *372*, 268–272.
[7] Bonnet, C.; Andreescu, S.; Marty, J-L. Adsorption: an easy and efficient immobilisation of acetylcholinesterase on screen-printed electrodes. Anal. Chim. Acta **2003**, *481*, 209–211.
[8] Cosnier, S. Biomolecule immobilization on electrode surface by entrapment or attachment to chemically polymerised films. A review. Biosens. Bioelectron. **1999**, *14*, 443–456.
[9] Snejdarkova, M.; Svobodova, L.; Evtugyn, G.; Budnikov, H.; Karyakin, A.; Nikolelis, D.P.; Hianik, T. Acetylcholinesterase sensors based on gold electrodes modified with dendrimer and polyaniline A comparative research. Anal. Chim. Acta **2004**, *514*, 79–88.
[10] Somerset, V.S.; Klink, M.J.; Sekota, M.M.C.; Baker, P.G.L.; Iwuoha, E.I. Polyaniline-Mercaptobenzothiazole Biosensor for Organophosphate and Carbamate Pesticides. Anal. Letters **2006**, *39*, 1683–1698.

[11] Morrin, A.; Moutloali, R.M.; Killard, A.J.; Smyth, M.R.; Darkwa, J.; Iwuoha, E.I. Electrocatalytic sensor devices: (I) cyclopentadienylnickel(II) thiolato Schiff base monolayer self-assembled on gold. Talanta **2004**, *64*, 30–38.

[12] Raj, C.R.; Ohsaka, T. Voltammetric detection of uric acid in the presence of ascorbic acid at a gold electrode modified with a self-assembled monolayer of heteroaromatic thiol. Jnl. Electroanal. Chem. **2003**, *540*, 69–77.

[13] Michira, I.; Akinyeye, R.; Somerset, V.; Klink, M.J.; Sekota, M.; Al-Ahmed, A.; Baker, P.G.L.; Iwuoha, E. Synthesis, Characterisation of Novel Polyaniline Nanomaterials and Application in Amperometric Biosensors. Macromol. Symp. **2007**, *255*, 57–69.

[14] Mazur, M.; Krysinski, P.; Jackowska, K. Electrochemical deposition of poly(o-anisidine) and polypyrrole at octadecanethiol coated gold electrodes. Thin Solid Films **1998**, *330*, 167–172.

[15] Mazur, M.; Krysiński, P. Bulk- and surface-initiated chemical in situ polymerization of 2,5-dimethoxyaniline and 2-methoxyaniline on thiol-coated gold electrodes. Electrochimica Acta **2001**, *46*, 3963–3971.

[16] Mazur, M.; Krysinski, P.; Palys, B. Preparation of ultrathin films of polyaniline and its derivatives by electrochemical deposition on thiol modified gold. Jnl. Electroanal. Chem. **2002**, *533*, 145–152.

[17] Mazur, M.; Tagowska, M.; Pays, B.; Jackowska, K. Template synthesis of polyaniline and poly(2-methoxyaniline) nanotubes: comparison of the formation mechanisms. Electrochem. Commun. **2003**, *5*, 403–407.

[18] Mathebe, N.G.R.; Morrin, A.; Iwuoha, E.I. Electrochemistry and scanning electron microscopy of polyaniline/peroxidase-based biosensor. Talanta **2004**, *64*, 115–120.

[19] Iwuoha, E.I.; De Villaverde, D.S.; Garcia, N.P.; Smyth, M.R.; Pingarron, J.M. Reactivities of organic phase biosensors. 2. The amperometric behaviour of horseradish peroxidase immobilised on a platinum electrode modified with an electrosynthetic polyaniline film. Biosens. Bioelectron. **1997**, *12*, 749–761.

[20] Joshi, K.A.; Tang, J.; Haddon, R.; Wang, J.; Chen, W.; Mulchandania, A. (2005). A Disposable Biosensor for Organophosphorus Nerve Agents Based on Carbon Nanotubes Modified Thick Film Strip Electrode. Electroanal. **2005,** *17*, 54–58.

[21] Sotiropoulou, S.; Fournier, D.; Chaniotakisa, N.A.; Genetically engineered acetylcholinesterase-based biosensor for attomolar detection of dichlorvos. Short Communication. Biosens. Bioelectron. **2005**, *20*, 2347–2352.

[22] Pritchard, J.; Law, K.; Vakurov, A.; Millner, P.; Higson, S.P.J. Sonochemically fabricated enzyme microelectrode arrays for the environmental monitoring of pesticides. Biosens. Bioelectron. **2004**, *20*, 765–772.

[23] Iwuoha, E.I.; Smyth, M.R. Reactivities of organic phase biosensors: 6. Square-wave and differential pulse studies of genetically engineered cytochrome P450cam (CYP101) bioelectrodes in selected solvents. Biosens. Bioelectron. **2003**, *18*, 237–244.

[24] Albareda-Sirvent, M.; Merkoći, A.; Alegret, S. Pesticide determination in tap water and juice samples using disposable amperometric biosensors made using thick-film technology. Anal. Chim. Acta **2001**, *442*, 35–44.

[25] Sotiropoulou, S.; Chaniotakis, N.A. Lowering the detection limit of the acetylcholinesterase biosensor using a nanoporous carbon matrix. Anal. Chim. Acta **2005**, *530*, 199–204.

[26] Wilkins, E.; Carter, M.; Voss, J.; Ivnitski, D. A quantitative determination of organophosphate pesticides in organic solvents. Electrochem. Commun. **2000**, *2*, 786–790.

[27] Somerset, V.S.; Klink, M.J.; Baker, P.G.L.; Iwuoha, E.I. Acetylcholinesterase-polyaniline biosensor investigation of organophosphate pesticides in selected organic solvents. Jnl. Environ. Sci. Health **2007**, *B42*, 297–304.

[28] Nunes, G.S.; Barceló, D.; Grabaric, B.S.; Diaz-Cruz, J.M.; Ribeiro, M.L. Evaluation of a highly sensitive amperometric biosensor with low cholinesterase charge immobilized on a chemically modified carbon paste electrode for trace determination of carbamates in fruit, vegetable and water samples. Anal. Chim. Acta **199**, *399*, 37–49.

[29] Bucur, B.; Danet, A.F.; Marty, J-L. Cholinesterase immobilisation on the surface of screen-printed electrodes based on concanavalin A affinity. Anal. Chim. Acta **2005**, *530*, 1–6.

[30] Ricci, F.; Amine, A.; Palleschi, G.; Moscone, D. Prussian Blue based screen printed biosensors with improved characteristics of long-term lifetime and pH stability. Biosens. Bioelectron. **2003**, *18*, 165–174.

[31] Kuralay, F.; Özyörük, H.; Yildiz, A. Potentiometric enzyme electrode for urea determination using immobilized urease in poly(vinylferrocenium) film. Sens. Actuat. B **2005**, *109*, 194–199.

[32] Albareda-Sirvent, M.; Merkoći, A.; Alegret, S. Configurations used in the design of screen-printed enzymatic biosensors. A review. Sens. Actuat. B **2000**, *69*, 153–163.

[33] McGovern, S.T.; Spinks, G.M.; Wallace, G.G. Micro-humidity sensors based on a processable polyaniline blend. Sens. Actuat. B **2005**, *107*, 657–665.

[34] Hart, A.L.; Matthews, C.; Collier, W.A. Estimation of lactate in meat extracts by screen-printed sensors. Anal. Chim. Acta **1999**, *386*, 7–12.

[35] Klibanov, A.M. Asymmetric enzymatic oxidoreductions in organic solvents. Curr. Opin. Biotechnol. **2003**, *14*, 427–431.

[36] Andreescu, S.; Noguer, T.; Magearu, V.; Marty, J-L. Screen-printed electrode based on AChE for the detecton of pesticides in presensce of organic solvents. Talanta **2002**, *57*, 169–176.

[37] Palchetti, I.; Cagninia, A.; Del Carlo, M.; Coppi, C.; Mascini, M.; Tumer, A.P.F. Determination of anticholinesterase pesticides in real samples using a disposable biosensor. Anal. Chim. Acta **1997**, *337*, 31–321.

[38] Iwuoha, E.I.; Adeyoju, O.; Dempsey, E.; Smyth, M.R.; Liu, J.; Wang, J. Investigation of the effects of polar organic solvents on the activity of tyrosinase entrapped in a poly(ester-sulphonic acid) polymer. Biosens. Bioelectron. **1995**, *10*, 661–667.

[39] Sotiropoulou, S.; Fournier, D.; Chaniotakisa, N.A. Genetically engineered acetylcholinesterase-based biosensor for attomolar detection of dichlorvos. Short Communication. Biosens. Bioelectron. **2005**, *20*, 2347–2352.

[40] Evtugyn, G.A.; Budnikov, H.C.; Nikolskaya, E.B. Sensitivity and selectivity of electrochemical sensors for inhibitor determination. Talanta **1998**, *46*, 465–484.

[41] Bucur, B.; Danet, A.F.; Marty, J-L. Versatile method of cholinesterase immobilisation via affinity bonds using concanavalin A applied to the construction of a screen-printed biosensor. Biosens. Bioelectron. **2004**, *20*, 217–225.

[42] Yang, M.; Yang, Y.; Yang, Y.; Shen, G.; Yu, R. Microbiosensor for acetylcholine and choline based on electropolymerization/sol–gel derived composite membrane. Anal. Chim. Acta **2005**, *530*, 205–211.

[43] Hart, A.L.; Collier, W.A.; Janssen, D. The response of screen-printed enzyme electrodes containing cholinesterases to organo-phosphates in solution and from commercial formulations. Biosens. Bioelectron. **1997**, *12*, 645–654.

[44] Cagnini, A.; Palchetti, I.; Lionti, I.; Mascini, M.; Turner, A.P.F. Disposable ruthenized screen-printed biosensors for pesticides monitoring. Sens. Actuat. B **1995**, *24–25*, 85–89.

[45] Sen, S.; Gülce, A.; Gülce, H. Polyvinylferrocenium modified Pt electrode for the design of amperometric choline and acetylcholine enzyme electrodes. Biosens. Bioelectron. **2004**, *19*, 1261–1268.

[46] Arkhypova, V.N.; Dzyadevych, S.V.; Soldatkin, A.P.; Elúkaya, A.V.; Martelet, C.; Jaffrezic-Renault, N. Development and optimisation of biosensors based on pH-sensitive field effect transistors and cholinesterases for sensitive detection of solanaceous glycoalkaloids. Biosens. Bioelectron. **2003**, *18*, 1047–1053.

[47] Timur, S.; Pazarlioglu, N.; Pilloton, R.; Telefoncu, A. Thick film sensors based on laccases from different sources immobilized in polyaniline matrix. Sens. Actuat. B **2004**, *97*, 132–136.
[48] Iwuoha, E.I.; Smyth, M.R.; Lyons, M.E.G. Organic phase enzyme electrodes: kinetics and analytical applications. Biosens. Bioelectron. **1997b**, *12*, 53–75.
[49] Dordick, J.S. Designing Enzymes for Use in Organic Solvents. Biotechnol. Progr. **1992**, *8*, 259–267.
[50] Chatterjee, S.; Russell, A.J. Determination of equilibrium and individual rate constants for subtilisin-catalyzed transesterification in anhydrous environments. Biotechnol. Bioengineer. **1992**, *40*(9), 1069–1077.
[51] Borzeix, F.; Monot, F.; Vandecasteele, J-P. Strategies for enzymatic esterification in organic solvents: Comparison of microaqueous, biphasic, and micellar systems. Enzyme Microb. Technol. **1992**, *14*(10), 791–797.
[52] Konash, A.; Magner, E. Characterization of an organic phase peroxide biosensor based on horseradish peroxidase immobilized in Eastman AQ. Biosens. Bioelectron. **2006**, *22*, 116–123.
[53] Lin, Y.; Lu, F.; Wang, J. Disposable Carbon Nanotube Modified Screen-Printed Biosensor for Amperometric Detection of Organophosphorous Pesticides and Nerve Agents. Electroanal. **2004**, *16*(1–2), 145–149.