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Mercaptobenzothiazole-on-Gold Organic Phase Biosensor Systems: 3. Thick-Film Biosensors for Organophosphate and Carbamate Pesticide Determination

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1. Introduction

The last few decades has seen an increase in the use of pesticides in order to increase crop yields. This has resulted in the increased use of organophosphorous (OP) and carbamate (CM) pesticide compounds since they result in much lower bioaccumulation and higher biodegradability, therefore they have replaced organochlorine as the most popular pesticides. However, as with the overuse of many pesticides, the OP and CM compounds leave residues in the soil, crops and surface water, which in turn exert a great threat to the environment and human health. The OP and CM compounds enter organisms and then inhibit the activity of acetyl cholinesterase (AChE) by irreversibly binding to the active site of this enzyme, which is important for the transmission of nerve impulses (Wu et al., 2009; Somerset et al., 2009; García de Llasera et al., 2009; Mavrikou et al., 2008; Liu et al., 2008; Boon et al., 2008; Pinheiro & De Andrade, 2009).

A broad range of adverse effects can result from AChE inhibition and it includes abdominal pain and cramps, glandular secretions, skeletal muscle twitching, flaccid paralysis, tiredness, nausea, blurred vision, drowsiness, eye pain, convulsions, respiratory failure and untimely death. Furthermore, OP and CM compounds are now also known to have mutagenic, carcinogenic and teratogenic effects and have been included in the list of known endocrine disruptor compounds (Luo & Zhang, 2009; Wu et al., 2009; Liu et al., 2008; Fu et al., 2009; Caetano & Machado, 2008).

Due to the increasing toxicity and adverse effects of pesticides, many countries are now monitoring environmental and food samples for pesticides and have established maximum residue levels (MRLs) for various pesticides in food products (Hildebrandt *et al.*, 2008). Some of the conventional methods used for chemical analysis of pesticides include spectrophotometry, infrared spectrometry, flow-injection chemiluminescence, fluorimetry, fluorescence spectrometry, mass spectrometry, but mainly chromatographic techniques, such as gas chromatography-mass spectrometry (GC-MS) and high-performance liquid

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chromatography (HPLC) (Wu et al., 2009; García de Llasera et al., 2009; Mavrikou et al., 2008; Liu et al., 2008; Caetano & Machado, 2008).

There is no doubt that these methods are highly efficient and allow discrimination among different types of OP and CM compounds, but they require tedious sample pre-treatments, highly qualified technicians and sophisticated instruments. Furthermore, these methods are also known to be time consuming and not suitable for field analysis of multiple samples (Liu et al., 2008; Hildebrandt et al., 2008).

For this reason several rapid, relatively inexpensive, sensitive screening analytical techniques that need little sample pre-treatment are constantly being developed for the identification and quantification of OP and CM compounds (Liu *et al.*, 2008). Biosensors have filled the gap in this regard and these analytical devices are based on the intimate contact between a bio-recognition element that interacts with the analyte of interest and a transducer element that converts the bio-recognition event into a measurable signal. Among the different types of biosensors, the electrochemical sensors are of special interest due to the high sensitivity inherent to the electrochemical detection and the possibility to miniaturise the required instrumentation, thereby making the construction of compact and portable analysis devices possible (Campàs et al., 2009; Mavrikou et al., 2008).

In this paper, we describe the application of a mercaptobenzothiazole-on-gold biosensor system for the analysis of OP and CM pesticide compounds. The aim of this work was to improve the detection limit of these 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 for detecting both OP and CM pesticide compounds.

2. Materials and methods

2.1 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 chlorpyrifos, malathion and parathion-methyl. Carbamate pesticides include carbaryl, carbofuran and methomyl. 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 (Somerset et al., 2007; Somerset et al., 2009).

2.2 Instrumentation

All electrochemical protocols were performed and recorded with a computer interfaced to a BAS-50/W electrochemical analyser 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} cm²; 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 (Morrin et al., 2004 ; Somerset et al., 2006).

2.3 Electrode surface preparation

Prior to use, gold electrodes were first polished on aqueous slurries of 1 μ m, 0.3 μ m and 0.05 μ m alumina powder. After thorough rinsing in deionised water followed by acetone, the electrodes were etched for about 5 minutes in a hot 'Piranha' solution {1:3 (v/v) 30 % H₂O₂ and concentrated H₂SO₄} and rinsed again with copious amounts of deionised 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 H₂SO₄ 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 deionised water (Michira et al., 2007; Somerset et al., 2007).

2.4 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 (Mazur et al. 2003; Somerset et al., 2007).

2.5 Electropolymerisation 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 electropolymerisation. Initial optimisation of the potential window for electropolymerisation was performed. During electropolymerisation 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 polymerisation 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 deionised 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 electrode (Somerset et al., 2007; Somerset et al., 2009).

2.6 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 μ L of AChE to the 0.1 M phosphate buffer (pH 7.2) solution. After the enzyme solution was argon degassed, enzyme immobilisation 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 μ L 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 (Somerset et al., 2006; Somerset et al., 2009).

2.7 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 Ag/AgCl as the working, counter and reference electrode, respectively. A 1 ml test solution containing 0.1 M phosphate (0.1M 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. 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 μ L 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.

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.

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 (Pritchard et al. 2004; Joshi et al. 2005; Sotiropoulou et al. 2005; Somerset et al., 2007; Somerset et al., 2009).

2.8 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 or carbamate pesticide was studied. A new biosensor was also prepared for each of the six concentrations of the OP and CM 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 OP and CM 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 1ml 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 (Albareda-Sirvent et al., 2001; Sotiropoulou and Chaniotakis 2005; Wilkins et al., 2000; Somerset et al., 2009):

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

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 (ADPV) 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 (Somerset et al., 2007; Somerset et al., 2009).

2.9 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 μ L of AChE to the 0.1 M phosphate buffer (pH 7.2) solution. After the enzyme solution was argon degassed, enzyme immobilisation 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 μ L 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 μ L of AChE enzyme into the PANI polymer surface. Voltammetric characterisation 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 (Nunes et al. 1999; Somerset et al., 2007; Somerset et al., 2009).

2.10 pH Optimisation for acetylcholinesterase (AChE) immobilised 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 (Albareda-Sirvent et al., 2001; Pritchard et al., 2004; Bucur et al., 2005; Somerset et al., 2009).

2.11 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) being 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 (Albareda-Sirvent et al., 2001; Somerset et al., 2009).

2.12 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) being 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 (Ricci et al., 2003; Kuralay et al., 2005; Somerset et al., 2009).

2.13 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 (Somerset et al., 2007; Somerset et al., 2009).

3. Results and discussion

3.1 Biosensor design for pesticide detection

Different technologies have been developed over the years for the manufacturing of thick-film biosensors for pesticide detection. The major technologies can be divided into three categories of (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. This work has seen the development of an electrode that can be exposed to organic solutions containing potential inhibitors without having the polymer layer separating from the electrode surface after use. Therefore the use of poly(vinyl acetate) as the binder was employed 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 (Hart et al. 1999; Albareda-Sirvent et al. 2000; Albareda-Sirvent et al. 2001; Joshi et al. 2005; McGovern et al. 2005).

The detection of pesticides in non-aqueous environments has been reported but few publications refer to the use of immobilised AChE biosensors in non-aqueous media. Organophosphorous and carbamate pesticides are characterised 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 (Somerset et al., 2007; Somerset et al., 2009).

In the amperometric sensor design, we have used polyaniline (PANI) as a mediator in the biosensor construction to harvest its dual role as immobilisation matrix for AChE and use its electrocatalytic activity towards thiocholine (TCh) for amperometric sensing. The biosensor mechanism for the Au/MBT/PANI/AChE/PVAc biosensor is shown in Figure 1.

Figure 1 displays the schematic representation for the Au/MBT/PANI/AChE/PVAc biosensor mechanism. It further shows that as acetylthiocholine (ATCh) is catalysed by acetylcholinesterase (AChE), it forms thiocholine (TCh) and acetic acid. Thiocholine is electroactive and is oxidised in the reaction. In return the conducting PANI polymer reacts with thiocholine and also accepts an electron from mercaptobenzothiazole as it is oxidised through interaction with the gold electrode (Somerset et al., 2007; Somerset et al., 2009).

3.2 Successive substrate addition to Au/MBT/PANI/AChE/PVAc biosensor

The functioning of the biosensor was established with the successive addition of acetylthiocholine (ATCh) aliquots as substrate to the Au/MBT/PANI/AChE/PVAc biosensor. Cyclic voltammetric (CVs) results were collected by applying sequential linear potential scan between - 400 to + 1800 mV (vs. Ag/AgCl), at a scan rate of 10 mV.s⁻¹. The CVs were performed at this scan rate to ensure that the fast enzyme kinetics could be monitored. The three CVs for successive 0.01 M ATCh substrate additions to Au/MBT/PANI/AChE/PVAc biosensor in 1 ml of 0.1 M phosphate buffer, KCl (pH 7.2) solution are shown in Figure 2 (Somerset et al., 2007; Somerset et al., 2009).

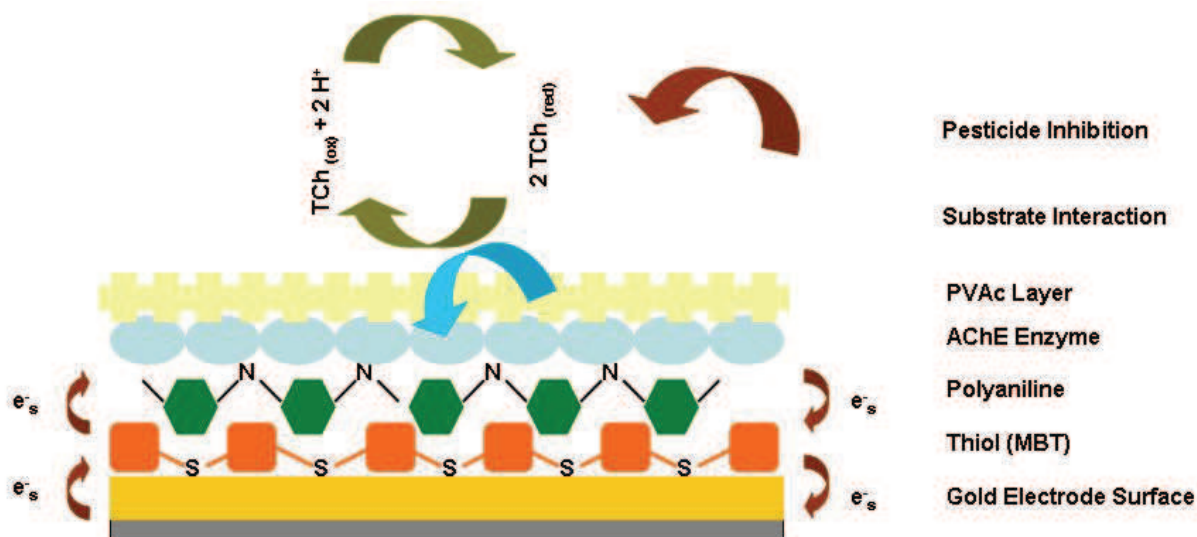


Fig. 1. The schematic representation of the Au/MBT/PANI/AChE/PVAc biosensor reaction occurring at the gold SAM modified electrode.

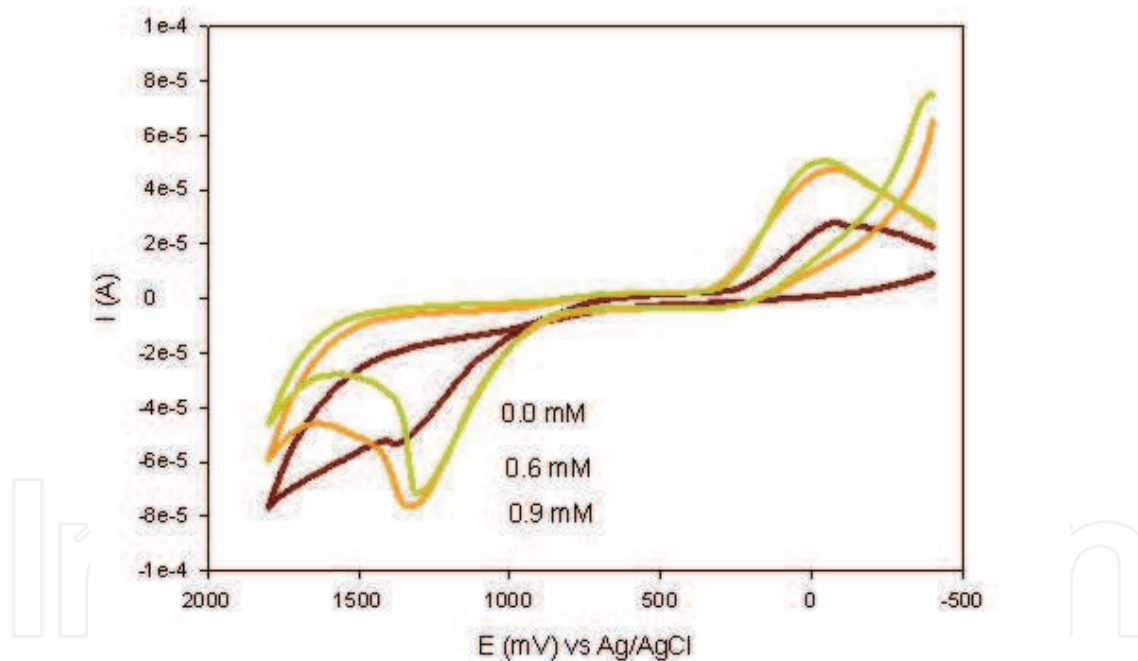


Fig. 2. CV response of successive ATCh substrate addition to Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution at a scan rate of 10 mV.s⁻¹.

A clear shift in peak current (I_p) was observed as the concentration of the substrate, ATCh, was increased indicating the electrocatalytic functioning of the biosensor. The results in Figure 2 further illustrate that in increase in the reductive current is also observed, but the magnitude is smaller when compared to the increases in oxidative current. This clearly illustrates that the oxidative response of the biosensor to ATCh addition is preferred (Somerset et al., 2007; Somerset et al., 2009).

The cyclic voltammetric (CV) results of the Au/MBT/PANI/AChE/PVAc biosensor were substantiated with the collection of differential pulse voltammetric (DPV) results. The DPV results obtained for the biosensor in a 1 ml of 0.1 M phosphate buffer, KCl (pH 7.2) solution are shown in Figure 3.

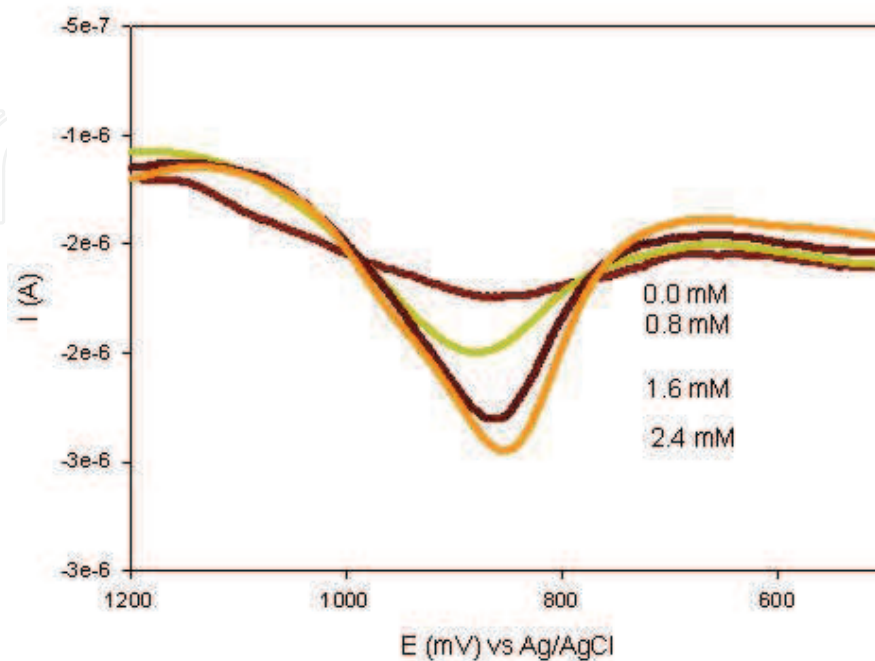


Fig. 3. DPV response of successive ATCh substrate addition to Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution at a scan rate of $10 \text{ mV}\cdot\text{s}^{-1}$, and in a potential window of + 500 to + 1200 mV.

The DPV results in Figure 3 were collected 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 potentials as compared with those observed in the CV responses in Figure 2 (Somerset et al., 2007; Somerset et al., 2009).

3.3 Optimum enzyme loading investigation

One of the variables optimised for the constructed biosensor, was the amount of enzyme incorporated during the biosensor development. The results obtained for 3 of the different amounts of the enzyme AChE incorporated into the biosensor are shown in Figure 4.

The results in Figure 4 show that the biggest increase in current for the successive addition of ATCh substrate, was experienced when the biosensor had $60 \mu\text{L}$ of AChE dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.2) solution. The results obtained when $80 \mu\text{L}$ of AChE was used, does not show a very big difference in the current response when compared to the use of $60 \mu\text{L}$ 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 $80 \mu\text{L}$ of AChE is compared to that of the $40 \mu\text{L}$ of

AChE, a big difference in the amperometric response was observed. It was then decided to use 60 μL of AChE in the biosensor construction (Somerset et al., 2007; Somerset et al., 2009).

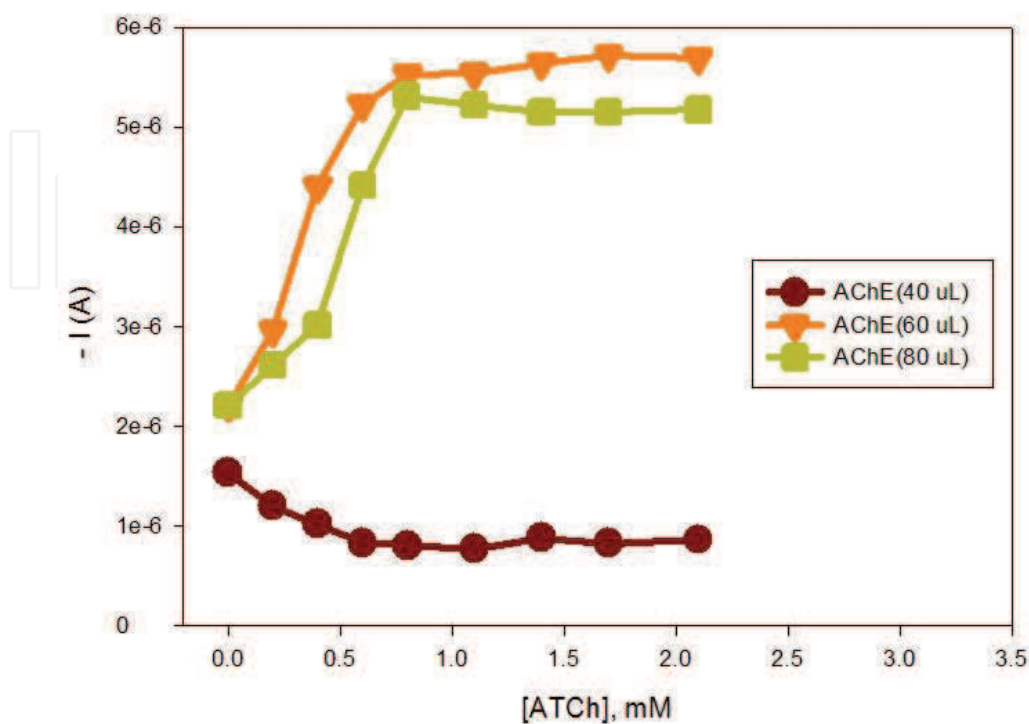


Fig. 4. The amperometric response of the AChE biosensor to different amounts of enzyme incorporated into the biosensor. These responses were measured in a 0.1 M phosphate buffer, KCl (pH 7.2) solution at 25 °C.

3.4 Optimisation of various biosensor parameters

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 (Yang et al. 2005).

For this reason the operation of the biosensor was evaluated at different pH values. In Figure 5 the results for the investigation into the effect of different pH values on the working of the Au/MBT/PANI/AChE/PVAc biosensor can be seen.

The results in Figure 5 indicate that the highest anodic current was obtained at pH = 7.2, while the result for pH = 7.5 show a small difference. 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 for the optimum pH of the free enzyme activity in solution (Arkhypova et al. 2003; Sen et al. 2004; Somerset et al., 2007; Somerset et al., 2009).

The parameters for long-term stability and increasing temperature on the functioning of the biosensor were also investigated. To determine the long-term stability of the biosensor, it 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. This was followed by investigating 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, at different temperatures varying from 10 to 35 °C (Somerset et al., 2007; Somerset et al., 2009).

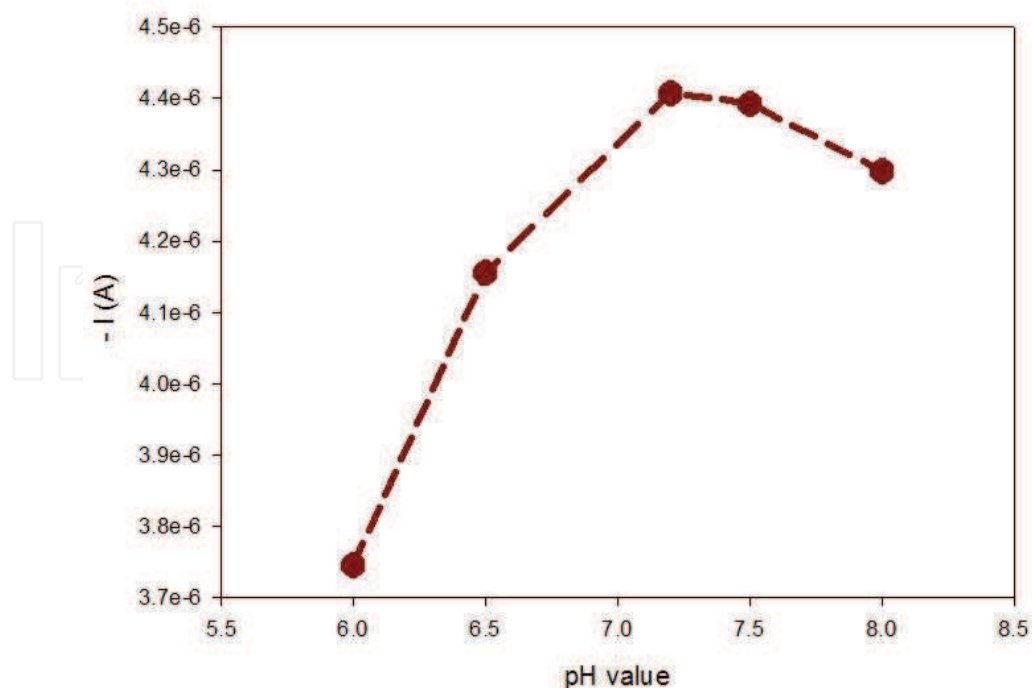


Fig. 5. Graph displaying the effect of pH on the Au/MBT/PANI/AChE/PVAc biosensor in 0.1 M phosphate buffer, KCl (pH 7.2) solution with 2 mM of ATCh added.

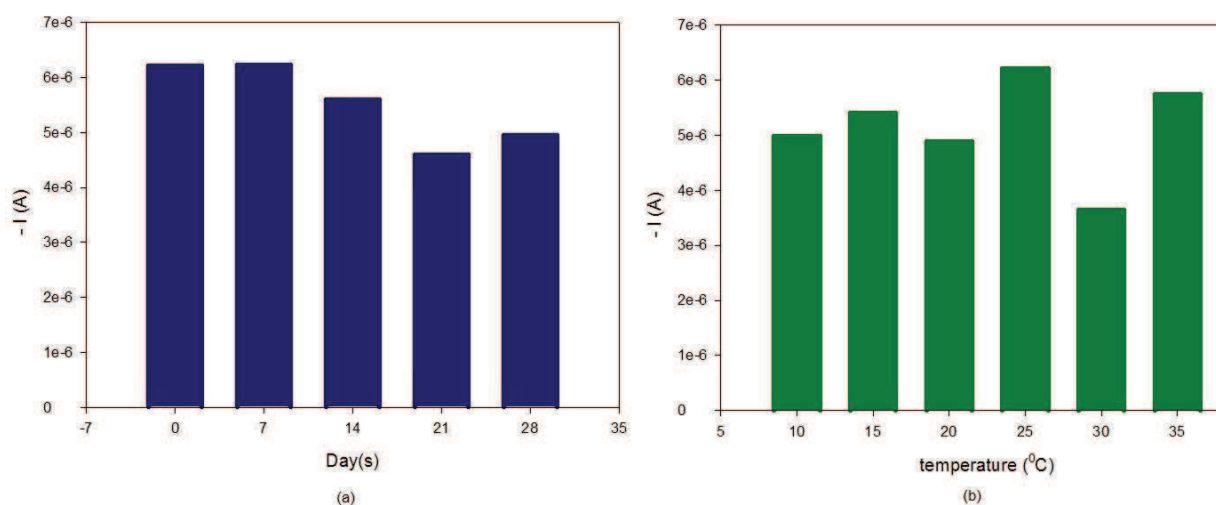


Fig. 6. Graph displaying the results for the long-term (a) and temperature (b) stability of the Au/MBT/PANI/AChE/PVAc biosensor in a 0.1 M phosphate buffer, KCl (pH 7.2) solution for successive additions of the ATCh substrate.

The results in Figure 6 (a) have shown that the biosensor responses reach a maximum current (I_{max}) within 0.6 mM of substrate added to the 0.1 M phosphate buffer, KCl (pH 7.2) solution. Not shown here is the fact that after 0.6 mM of substrate added, the biosensor response reaches a plateau and minimum changes in the current was observed. The results further indicate that at a substrate concentration of 0.6 mM, the maximum current (I_{max}) response show relatively minimum changes with one order magnitude difference between the initial current response, compared to the results obtained after 28 days.

The results for the temperature stability investigation in Figure 6 (b) have shown that for the six temperatures investigated, maximum current (I_{max}) was also reached within 0.6 mM of ATCh substrate added. These results indicate that the enzyme AChE responded favourably to most temperatures evaluated, ranging from 10 to 35 °C (Somerset et al., 2007; Somerset et al., 2009).

3.5 Biosensor behaviour in organic solvents

The influence of organic solvents on the activity of the enzyme 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 water solution. The polar organic solvents investigated in this study include 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

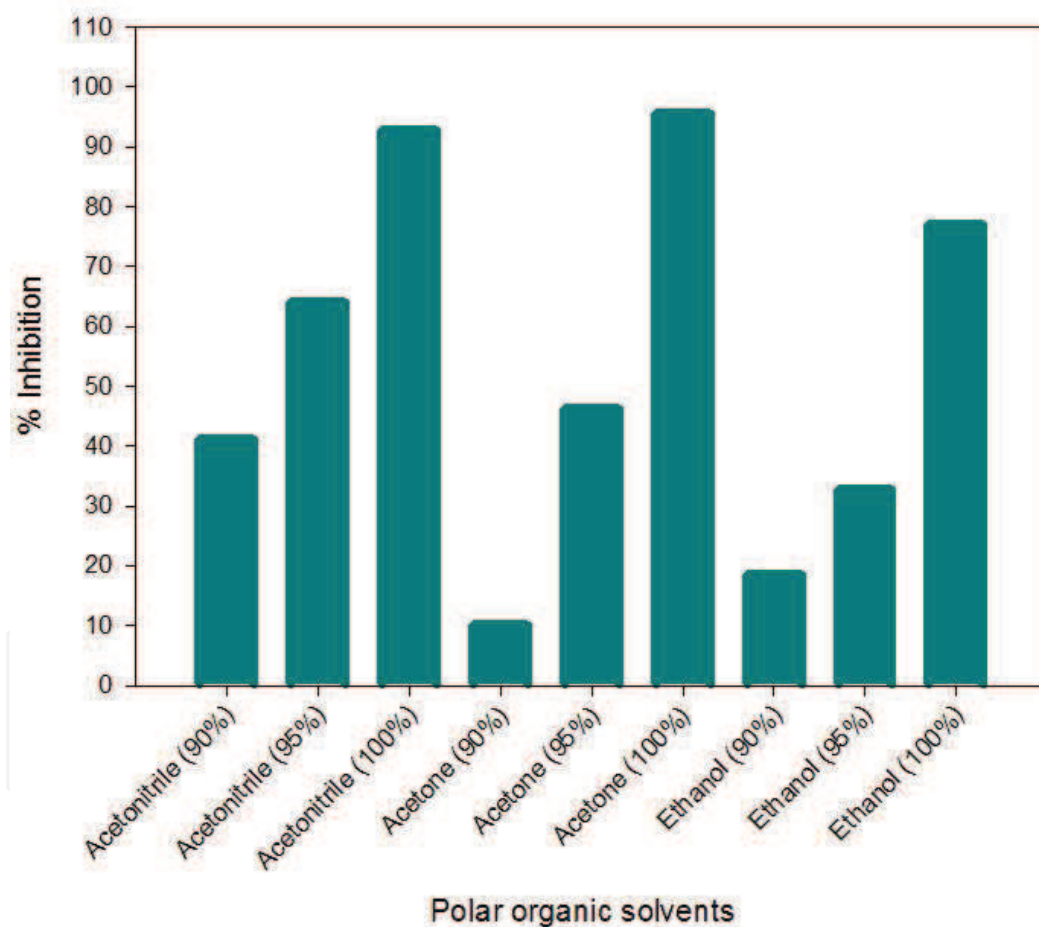


Fig. 7. Results obtained for the inhibition of AChE in the Au/MBT/PANI/AChE/PVAc biosensor after 20 minutes of incubation in (a) 10% water-organic solvent mixture, (b) 5% water-organic solvent mixture, and pure organic solvent. The ATCh concentration was 2.0 mM.

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 in equation (1) (Somerset et al., 2007; Somerset et al., 2009).

The results obtained in Figure 7 shows that for the three different 10% water-organic solvent mixtures investigated, the lowest decrease in catalytic activity of the enzyme AChE was observed in acetone, compared to acetonitrile and ethanol. For the 5% water-organic solvent mixtures, ethanol had the lowest decrease in the catalytic activity of AChE, while in the pure polar organic solvent it was again observed that ethanol had the lowest decrease in the catalytic activity of AChE (Somerset et al., 2007; Somerset et al., 2009).

3.6 Inhibition studies of standard organophosphorous pesticide samples

Inhibition plots for each of the three organophosphorous pesticides investigated were constructed using the percentage inhibition method. The method for the inhibition studies is described in section 2.8. Graphs of percentage inhibition vs. $-\log$ [pesticide] concentration were constructed and the results are shown in Figure 8.

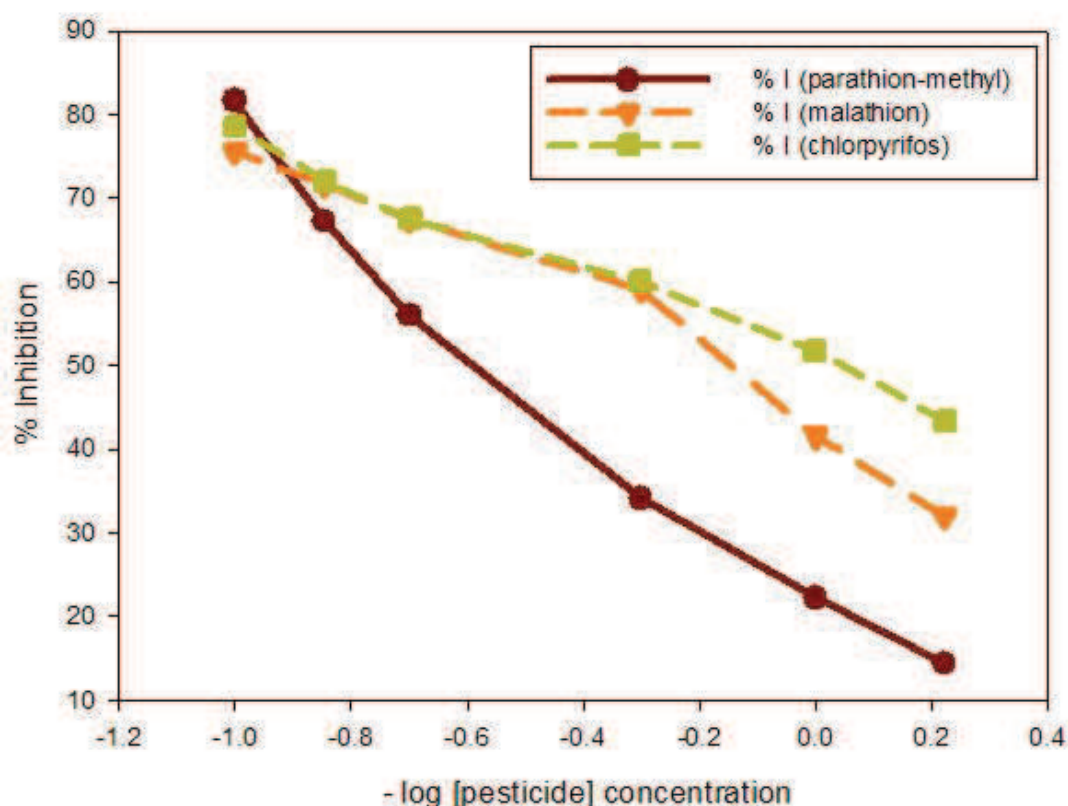


Fig. 8. Graph of percentage inhibition vs. $-\log$ [pesticide] concentration for three different organophosphorous pesticides investigated with the Au/MBT/PANI/AChE/PVAc biosensor.

The results shown in Figure 8 are that for the combined plot of the percentage inhibition vs. $-\log$ [pesticide] concentration results for the three different organophosphorous standard pesticide solutions investigated. The inhibition results for the pesticides called malathion and chlorpyrifos on the AChE biosensor response are relatively similar, for 4 of the concentrations investigated. It was also observed that the percentage inhibition results for malathion and chlorpyrifos, are higher compared to that obtained for parathion-methyl for most of the concentrations investigated. Further analyses of the inhibition plots and pesticide data were done and the results for the sensitivity, detection limits and regression coefficients are shown in Table 1 (Somerset et al., 2007; Somerset et al., 2009).

Organophosphorous pesticides			
Pesticide	Sensitivity (%I/decade)	Detection limit (nM)	Regression coefficient
parathion-methyl	-53.66	1.332	0.9766
Malathion	-35.24	0.189	0.9679
Chlorpyrifos	-26.68	0.018	0.9875

Table 1. 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).

The results in Table 1 shows the parameters for the sensitivity and detection limit estimated from the inhibition plots in Figure 8. The highest sensitivity was obtained for chlorpyrifos as pesticide, while the lowest sensitivity was obtained for parathion-methyl as pesticide. Chlorpyrifos represents a more powerful organophosphate than the rest of the three 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. The best detection limit of 0.018 nM was also obtained for chlorpyrifos as pesticide (Somerset et al., 2007; Somerset et al., 2009).

3.7 Inhibition studies of standard carbamate pesticide samples

Similarly, inhibition plots for each of the three carbamate pesticides detected were obtained using the percentage inhibition method. Graphs of percentage inhibition vs. $-\log$ [pesticide] concentration were constructed and the results are shown in Figure 9.

The results for the combined plot of the percentage inhibition vs. $-\log$ [pesticide] concentration for the three different carbamate standard pesticide solutions investigated are shown in Figure 9. Analysis of the results shows that carbaryl had the lowest inhibition results for most of the concentrations investigated, while carbofuran had the best inhibition responses. Further analyses of the inhibition plots and pesticide data were done and the results for the sensitivity, detection limits and regression coefficients are shown in Table 2 (Somerset et al., 2007; Somerset et al., 2009).

Table 2 shows the results for the sensitivity and detection limit estimated from the inhibition plots shown in Figure 9. The highest sensitivity results were obtained for methomyl and carbaryl, while the results for carbofuran are the lowest. The difference between the sensitivity results for methomyl and carbaryl, showed also relatively small differences. The best detection limit of 0.111 nM was also obtained for methomyl as pesticide (Somerset et al., 2007; Somerset et al., 2009).

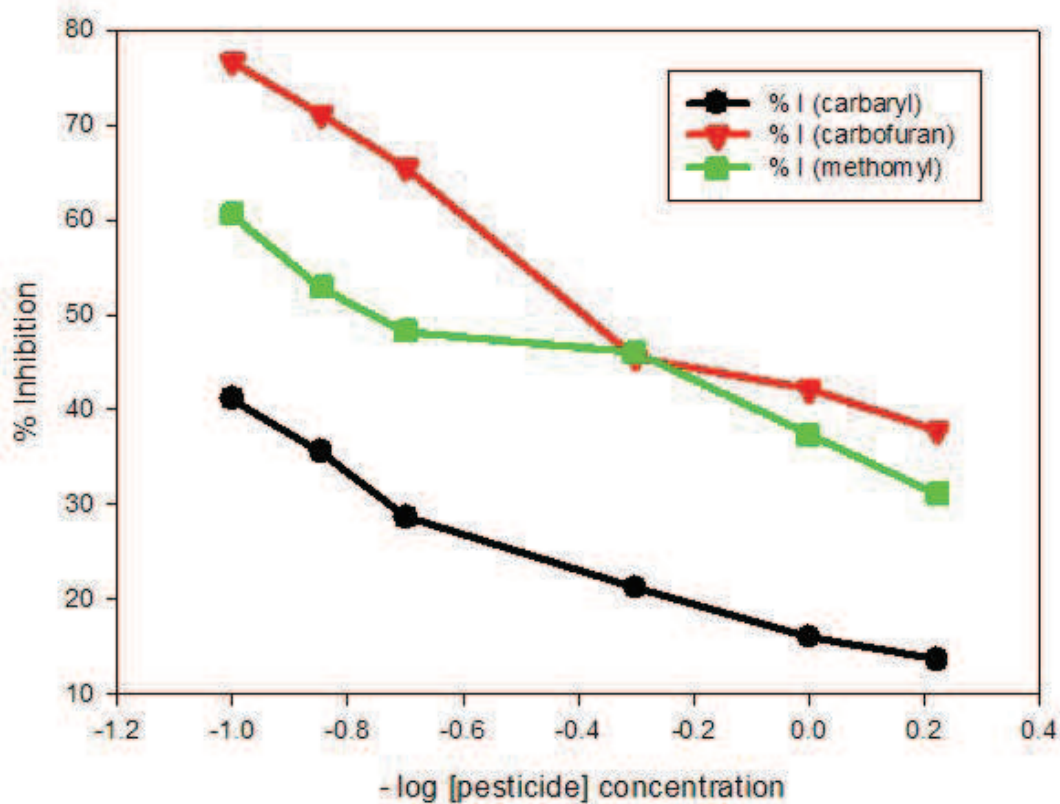


Fig. 9. Graph of percentage inhibition vs. $-\log$ [pesticide] concentration for three different carbamate pesticides investigated with the Au/MBT/PANI/AChE/PVAc biosensor.

Carbamate pesticides			
Pesticide	Sensitivity (%I/decade)	Detection limit (nM)	Regression coefficient
carbaryl	-21.92	0.880	0.9581
carbofuran	-33.20	0.249	0.9590
methomyl	-21.04	0.111	0.94552

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

4. Conclusion

The results described in this paper have successfully demonstrated the construction and use of an Au/MBT/PANI/AChE/PVAc thick-film biosensor for the detection of organophosphorous and carbamate pesticides in polar organic solvents. This study has also 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. Furthermore, very good detection limits for the standard OP and CM pesticide standard samples were obtained with the Au/MBT/PANI/AChE/PVAc biosensor. The results for the detection limit values for the individual organophosphate pesticides were 1.332 nM (parathion-methyl), 0.189 nM (malathion), 0.018 nM (chlorpyrifos). The detection limit values for the individual carbamate pesticides were 0.880 nM (carbaryl), 0.249 nM (carbofuran) and 0.111 nM (methomyl).

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