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ORIGINAL PAPER

Surface properties of sensors based on aminophenol-polymerized film

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Abstract In this work, carbon electrodes modified with aminophenols were developed for the production of pesticides biosensors based on acetylcholinesterase. The polymers were potentiodynamically deposited on a graphite electrode surface by the oxidation of monomers, 2-aminophenol, 3-aminophenol and 4-aminophenol. The electrochemical behaviour and surface analysis of the electrodes modified by polyaminophenols non-immobilized and immobilized on acetylcholinesterase were studied by cyclic voltammetry, electrochemical impedance spectroscopy and atomic force microscopy. Roughness values obtained for graphite electrodes modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase were 174 and 86 nm, respectively. The acetylcholinesterase enzyme was immobilized on a graphite and a graphite modified with poly(4-aminophenol), and these electrodes were coupled in the flow system. Potentiometric response due to hydrogen ions generated by an enzymatic system in the presence of acetylcholine chloride substrate was evaluated. The results showed that the graphite/poly(4-aminhophenol) sensor presents high sensitivity to hydrogen ions when compared with other graphite/polyaminophenols sensors. The biosensor coupled in a continuous flow system was employed for the detection of dichlorvos. The detection and quantification limits were 0.8 and 2.4 μmol L^{-1} dichlorvos, respectively. This sensor reveals an efficient and promising material for biomolecules immobilization.

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Introduction

Organophosphate pesticides residue in fruits and vegetables is a serious problem in food safety. Cleary, the development of a simple and rapid analytical method to detect pesticide residue is essential for on-site detection. The mode of action of pesticides is based on inhibition of acetylcholinesterase, and the same principle is utilized for pesticides analysis. Therefore, fabricating acetylcholinesterase-based biosensor is of considerable analytical interest. Due to the demand for higher selectivity and sensitivity, the use of chemically modified electrodes has become very attractive [1]. Chemically modified electrodes are devices that contain a supporting electrode and a layer of a chemical modifier. The main objective of this modification is to control the chemical and physical properties of the electrode/solution interface, thus improving the reactivity and selectivity of the surface.

Modified electrodes with polymeric films formed by electropolymerization of organic monomers have caused great interest in electrochemical research [2, 3]. The preparation of modified electrodes using polymeric films is simple. These electrodes present reproducibility been applied to the detection of drugs, pesticides and heavy metals [4–7].

A chemical sensor is a device that transforms chemical information into an analytically useful signal. Chemical sensors contain, usually, two basic components: a chemical recognition system (receptor) and a physico–chemical transducer. Amperometric and potentiometric sensors have

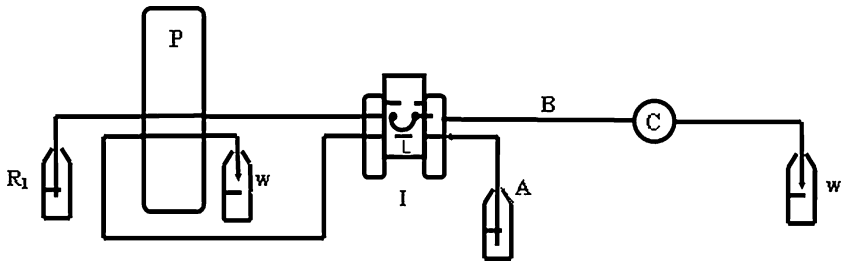

Fig. 1 Schematic diagram of the flow injection system. R_1 carrier solution (1.0 mmol L^{-1} phosphate buffer in 0.1 mol L^{-1} KCl) and flow rate of 1.5 mL min^{-1}, *P* peristaltic pump, *W* waste, *L* loops of 200 μL, *I* injection valve, *A* standard solutions of acetic acid or acetylcholine chloride, *B* reaction coil of 35 cm, *C* potentiometric detector

been the most widely used [8, 9]. The measurements of potentiometric sensors are based on the difference of the potential values between the working electrode (sensor) and a reference electrode. Researchers have used these transducers in the development of biosensors based on acetylcholinesterase [10–12]. Accordingly, immobilization, storage and operational stability of cholinesterase are very important for biosensor exploitation. Immobilization of enzymes onto a carbon surface is difficult since graphite or amorphous carbon surfaces usually possess insufficient active groups for direct immobilization of enzymes. There are many methods suitable for the immobilization of enzymes onto amino-containing matrices [13]. Many of these methods result in decreased enzyme stability in water–organic mixtures and storage instability in dry conditions.

In the present paper, carbon electrodes were modified with aminophenol-polymerized film for the immobilization of acetylcholinesterase with the aim of improving the efficiency of organophosphate biosensors. The proposed biosensor was employed for the detection of dichlorvos.

Experimental

Reagents and chemicals

Acetylcholinesterase (VI-S, 403 units mg^{-1} protein) was obtained from *Electrophorus electricus*. Acetylcholine chloride and dichlorvos (2,2-dichlorovinyldimethyl phosphate) were purchased from Sigma–Aldrich (Steinheim, Germany). Aminophenols (2-aminophenol, 3-aminophenol and 4-aminophenol) were purchased from Acros Organics (NJ, USA). All other chemicals were of analytical grade, and ultrapure water was obtained from the Gehaka Master System (São Paulo, Brazil) and was employed in all experiments.

Apparatus

A graphite disk (28.26 mm^2) (99.9%, Alfa Aesar, Karlsruhe, Germany) was used as a working electrode. As counter and reference electrodes, platinum plate and Ag/AgCl (KCl 3.0 mol L^{-1}) were used, respectively. A potentiostat (model 760C) from CH Instruments (Austin, TX, USA) was used

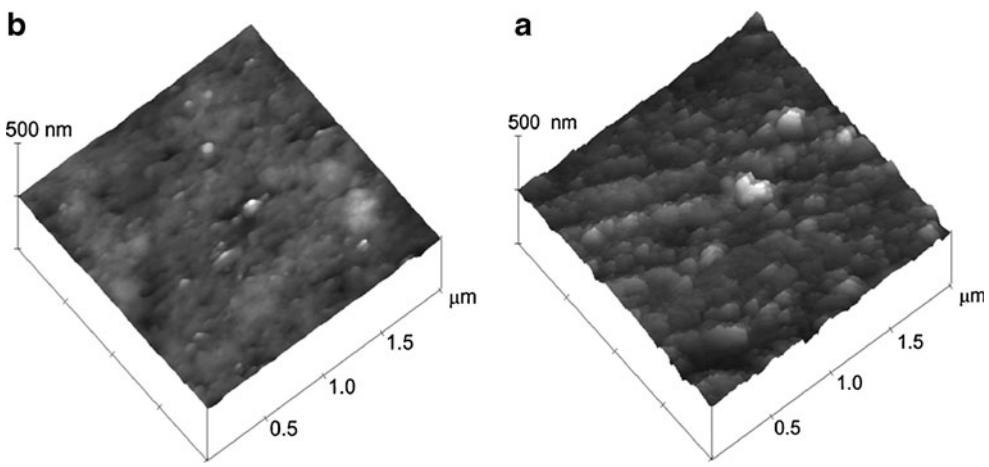

Fig. 2 Atomic force microscopy images of graphite electrodes modified with **a** poly(4-aminophenol) and **b** poly(4-aminophenol)/acetylcholinesterase

for electrochemical measurements in a three-compartment electrochemical cell. Electrochemical impedance spectroscopy (10^6–10^{-2} Hz frequency interval using a signal amplitude of 10 mV) and cyclic voltammetry (potential range −0.10 V at +0.50 V and 100 mV s^{-1}) were performed using a solution containing 5 mmol L^{-1} $K_3Fe(CN)_6$, 5 mmol L^{-1} $K_4Fe(CN)_6$ and 50 mmol L^{-1} KNO_3. The morphology of the graphite electrodes modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase were analyzed on a Nanoscope III Atomic Force Microscopy from Digital Instruments (Ottawa, ON, Canada).

Figure 1 shows the flow injection system used in this work. Polyethylene tubing (i.d., 0.8 mm) were used for the loops, transmission line and coils. All solutions were pumped using a Minipuls 3 peristaltic pump from Gilson (Villiers le Bel, France) and Tygon® pumping tubes.

The graphite electrodes were prepared by utilizing a mechanically polished graphite disk with alumina (0.3 μm) slurry, ultrasonicated, washed with deionized water and dried with ultrapure N_2. The procedure of electropolymerization was carried through in accordance with that described in the literature [14, 15]. The analytical response of the sensors was evaluated using acetic acid solutions (0.050–100 mmol L^{-1}) containing 1.0 mmol L^{-1} phosphate buffer/0.1 mol L^{-1} KCl.

The enzyme was immobilized on a graphite and a graphite modified with poly(4-aminophenol) electrodes according to the procedure described in the literature [10]. The response of the sensor containing immobilized enzyme in the presence of 200 μL substrate (0.4–100 mmol L^{-1} acetylcholine chloride solutions in 1.0 mmol L^{-1} phosphate buffer/0.1 mol L^{-1} KCl) was evaluated by monitoring the open circuit potential.

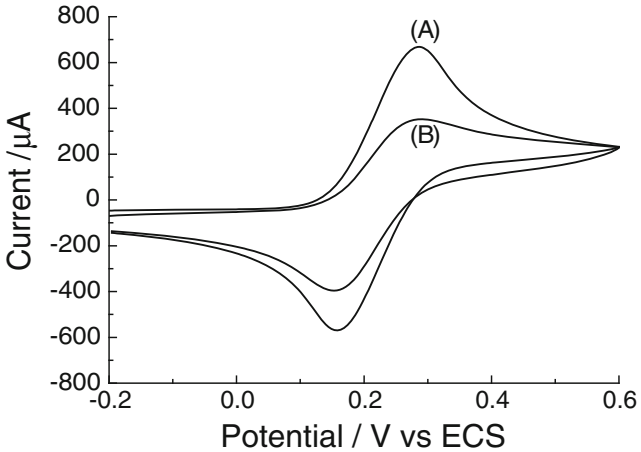

Fig. 3 Cyclic voltammetry, poly(4-aminophenol) (*A*) and poly(4-aminophenol)/acetylcholinesterase (*B*). Solution, 5.0 mmol L^{-1} $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ containing 1.0 mol L^{-1} KCl

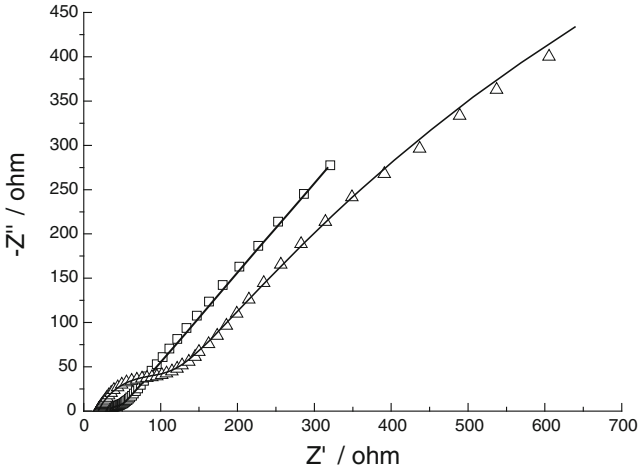

Fig. 4 Nyquist diagrams (−*Z*″ vs. *Z*′) for graphite electrodes modified with poly(4-aminophenol) (*unfilled square*) and poly(4-aminophenol)/(acetylcholinesterase) (*unfilled triangle*). Solution, 5.0 mmol L^{-1} $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ containing 1.0 mol L^{-1} KCl

Application of the proposed biosensor

The method was applied for dichlorvos determination in potato samples. The extract was obtained according to the following procedure: 88 μL dichlorvos (0.113 mol L^{-1}) stock solution and 12 g of sample were triturated with 60 mL acetone/water (2:1) mixture and afterwards the material was filtrated. Twelve millilitres from this extract was homogenized in a separation flask containing 15 mL of dichloromethane and 15 mL of petroleum ether. The aqueous phase was transferred to another flask and, again, it was extracted with 15 mL of petroleum ether. The organic

Table 1 Results obtained from the simulation of experimental data of electrochemical impedance spectroscopy for graphite electrode modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase

Parameter	Surface	
	Poly(4-aminophenol)	Poly(4-aminophenol)/ acetylcholinesterase
R_s/Ω	32.8	18.66
R_{tc}/Ω	15.52	36.30
R_{cm}/KΩ	–	2.7
Q_{dl}/μF	0.27	0.27
Q_{cm}/μF	–	3.7
W	0.006	0.068
χ^2	0.01	0.01

R_s solution resistance, Q_{dl} electric double-layer capacitance, R_{tc} charge transfer resistance, W Warburg impedance, R_{cm} charge transfer resistance to modified layer, Q_{cm} electric double-layer capacitance to modified layer, χ^2 error in simulation of the experimental data

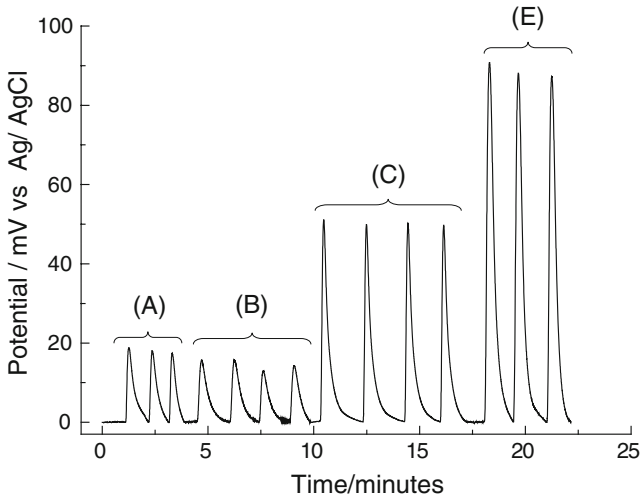

Fig. 5 Potenciometric response of graphite electrodes modified with poly (2-aminophenol) (*A*), poly(3-aminophenol) (*B*), poly(4-aminophenol) (*C*) and graphite electrode (*E*). Solution, 5 mmol L^{-1} acetic acid

phases were preconcentrated by rota-evaporation. The residue was washed with a carrier solution and the volume filled until 10 mL.

Results and discussion

Atomic force microscopy, electrochemical impedance spectroscopy and cyclic voltammetry characterization of poly(aminophenol) modified graphite electrode

Aminophenols are interesting to modify the surface of electrodes with its respective polymers. In this work, the electropolymerization of poly(aminophenol) film on the graphite electrode was made as described in the literature [14, 15]. During the electropolymerization, an increase in current values is observed after the first cycle in the potential range of the growing polymer. Also, the increase in current values is observed with an increase in the number of potential scans, showing the covering of the electrode surface by a polymeric film. A similar electrochemical behaviour was observed for the electropolymerization of polymeric films from 2-aminophenol, 3-aminophenol and 4-aminophenol [15–17].

The morphology of the electrodes modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase was investigated by atomic force microscopy (AFM) images. Figure 2 shows the AFM photographs of graphite electrode modified with poly(4-aminophenol) before (Fig. 2a) and after modification with acetylcholinesterase (Fig. 2b). The surface of the graphite electrodes modified with poly(4-aminophenol) exhibit high heterogeneity, and it is made up of many cavities, grooves and stacked flakes of differing sizes. Figure 2b shows the AFM picture of the surface morphology of the poly(4-aminophenol) graphite electrode modified with acetylcholinesterase. It is possible to see that once adsorption of acetylcholinesterase on to the surface takes place, the surface of poly(4-aminophenol) graphite electrode is covered by an almost homogeneous polymer film and becomes smooth. This may be due to the cavities that as seen on the modified graphite electrode are filled by acetylcholinesterase molecules. This indicates the presence of a polymer membrane on the electrode surface. Roughness values obtained for graphite electrodes modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase were 174 and 86 nm, respectively. Higher roughness value for poly(4-aminophenol) without enzyme is in agreement with the high current values of the modified electrode [15, 16]. Lower roughness value for electrode modified with poly(4-aminophenol)/acetylcholinesterase shows that the enzyme covers the poly(4-aminophenol) electrode surface.

Studies by electrochemical impedance spectroscopy and cyclic voltammetry in the presence of 5.0 mmol L^{-1} $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ containing 1.0 mol L^{-1} KCl were made to evaluate the rate of electron transfer of the electrodes modified with poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase by the active species in the solution (Figs. 3 and 4). Cyclic voltammetry studies

Table 2 Figures of merit for graphite and graphite modified with polyaminophenols sensors

Merit figures	Graphite	Graphite (electrode-modified)		
		Poly(2-aminophenol)	Poly(3-aminophenol)	Poly(4-aminophenol)
Linear range/mmol L^{-1}	5.0–100	5.0–100	3.0–100	1.0–100
Detection limit/μmol L^{-1}	68	1,600	840	140
Relative standard deviation[a]/%	2.2	3.9	8.6	1.1
Slope/mV $Lmol^{-1}$	57.2±3.3	44.1±1.2	55.2±1.5	58.8±0.7
Velocity[a] of response/mV s^{-1}	6.6±1.2	2.0±0.8	1.2±0.2	5.6±0.4

[a] 5.0 mmol L^{-1} acetic acid, N=5

Table 3 Figures of merit for graphite/acetylcholinesterase and graphite/poly(4-aminophenol)/acetylcholinesterase sensors

Merit figures	Sensor	
	Graphite/acetylcholinesterase	Poly(4-aminophenol)/acetylcholinesterase
Linear range/mmol L^{-1}	0.5–10	0.5–10
Detection limit/μmol L^{-1}	160	140
Quantification limit/mmol L^{-1}	1.2	0.4
ΔE^{a}/mV	17.4±1.0	12.4±1.6
Linearity	ΔE=5.42+1.73 [CAC]	ΔE=1.74+2.01 [CAC]
	r^2=0.99056	r^2=0.99533
K_M^{app}/mmol L^{-1}	5.53	7.29
Velocity[a] of response/mV s^{-1}	1.0±0.1	1.0±0.2

CAC acetylcholine chloride, *ΔE* difference of potential

[a] 5.0 mmol L^{-1}

showed the current values of 666.8 and 352.3 μA for graphite/poly(4-aminophenol) and graphite/poly(4-aminophenol)/acetylcholinesterase, respectively.

Figure 3 shows the cyclic voltammograms of 5.0 mmol L^{-1} $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 1.0 mol L^{-1} KCl at an electrode modified with poly(4-aminophenol) (curve a) and poly(4-aminophenol) electrode modified with acetylcholinesterase (curve b). The cyclic voltammogram shows a pair of redox peaks at 150 and 300 mV at electrode modified with poly(4-aminophenol). When this electrode is modified with acetylcholinesterase, the redox peaks of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple were obtained almost the same potentials. However, it is observed that the current response of the graphite/poly(4-aminophenol)/acetylcholinesterase electrode was less than the graphite/poly(4-aminophenol) electrode. The difference was about 307 μA due to the presence of the enzyme that produced a lower transfer charge of electroactive species in solution. Figure 4 shows Nyquist diagrams ($-Z''$ vs. Z') for poly(4-aminophenol) and poly(4-aminophenol)/acetylcholinesterase electrodes. The linear region describes information about the transport of ions while the semicircle describes faradaic processes of the transfer of electrons between the electrode and the surface. It was observed that the semicircle for the poly(4-aminophenol) film is less pronounced, indicating that Z' and $-Z''$ did not change significantly or that the processes of electron transfer between the electrode and the surface are not significant, i.e. the formation of isolated polyphenol film does not cause signal attenuation. Electrochemical impedance spectroscopy also showed the greatest resistance to the transfer charge in the graphite/poly(4-aminophenol)/acetylcholinesterase (Fig. 4) that can be confirmed by a simulation data (Table 1). A simulation of the experimental data was performed using the following circuit: $R_s(Q_{dl}[R_{tc}W])(R_{cm}Q_{cm})$, where R_s is the solution resistance, Q_{dl} is the electric double-layer capacitance, R_{tc} is the charge transfer resistance, W is the Warburg impedance, R_{cm} and Q_{cm} are the charge transfer resistance and electric double-layer capacitance to modified layer, respectively. The charge transfer resistance (R_{tc}) for the graphite/poly(4-aminophenol)/acetylcholinesterase sensor (36.30 Ω) is higher than the graphite/poly(4-aminophenol) sensor (15.52 Ω). These results are consistent with the values of current observed in cyclic voltammetry for graphite/poly(4-aminophenol) and graphite/poly(4-aminophenol)/acetylcholinesterase in the presence of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple.

Electrochemical behaviour of electrodes

The performance of graphite electrodes modified with polyaminophenols as potentiometric sensors was studied in flow system (Fig. 1). In the first case, the standard solutions of acetic acid were injected in carrier solution (1.0 mmol L^{-1} phosphate buffer in 0.1 mol L^{-1} KCl). Graphite and modified graphite electrodes with these polymeric films presented sensitivity to hydrogen ions (Fig. 5). Hence, these modified electrodes can be used as

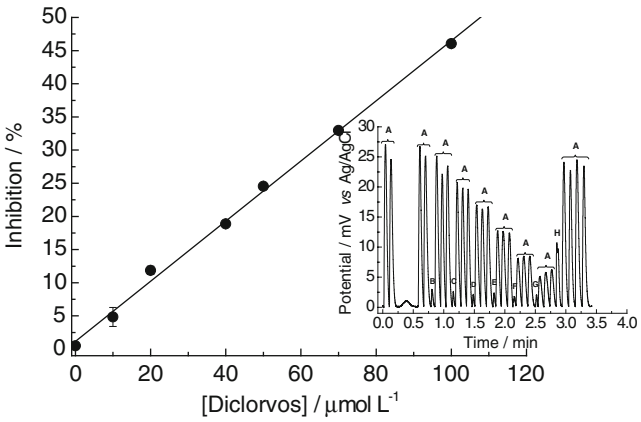

Fig. 6 Analytical signals for proposed biosensor. *A*, biosensor signal in the presence of 5.0 mmol L^{-1} substrate solution; *B*, *C*, *D*, *E*, *F*, *G* incubation time at 0 min, in the presence of 10, 20, 40, 50, 70 and 100 μmol L^{-1} pesticide dichlorvos solutions; *H*, regeneration of enzyme using 0.5 mol L^{-1} 1,1'-Trimethylene bis(4-formylpyridinium bromide) respectively. Conditions, 200 μL of 5.0 mmol L^{-1} acetylcholine chloride prepared in 1.0 mmol L^{-1} phosphate buffer solution containing 0.10 mol L^{-1} KCl at pH 7.40. Flow rate, 1.5 mL min^{-1}

a potentiometric detection system. The results show that the graphite/poly(4-aminophenol) sensor presents higher sensitivity (58.8 mV $L mol^{-1}$) and better reproducibility (RSD 1.1%; N=5) when compared with other graphite/polyaminophenols sensors studied in this work (Table 2). The response characteristics of the electrodes were evaluated according to the method recommended by IUPAC [18]. The electrodes have a linear response to acetic acid in the concentration range of 1.0–100 mmol L^{-1} (R^2=0.999). The limit of detection based on the three r/s, where r is the standard deviation of 10 measurements of the blank and s is the slope of the calibration graphs, was 68 μmol L^{-1} for graphite electrode and 140 μmol L^{-1} for graphite/poly(4-aminophenol). The modified electrodes are easily constructed at a relatively low cost and have a fast response time (5.6 mV s^{-1}) with no significant changes in its performance characteristics.

The acetylcholinesterase enzyme was immobilized on a graphite and a graphite modified with poly(4-aminophenol), and these electrodes were coupled in the flow system (Fig. 1). Potentiometric response due to hydrogen ions generated by enzymatic system in the presence of acetylcholine chloride substrate was evaluated (Table 3). The electrodes have a linear response to acetylcholine chloride in the concentration range of 0.5–10 mmol L^{-1}. The limits of detection were 160 and 140 μmol L^{-1} for graphite/acetylcholinesterase and graphite/poly(4-aminophenol)/acetylcholinesterase, respectively. Table 3 shows the analytical figures of merit for the proposed electrodes. Kinetic parameters (K_M^{app}, Michaelis–Menten apparent constant) were obtained from the plot of the double-reciprocal Lineweaver–Burk (data not shown) were 5.53 and 7.29 mmol L^{-1}, respectively. The greatest value of K_M^{app} indicates less interaction between the enzyme and substrate probably due to links between the enzyme and poly(4-aminophenol). Despite this, the surface modified with poly (4-aminophenol) shows suitable to respond hydrogen ions generated in the enzymatic system.

Determination of dichlorvos

The proposed method was applied for the determination of dichlorvos pesticide in potato samples. Inhibition effects were investigated measuring the biosensor response using 5.0 mmol L^{-1} acetylcholine chloride in the presence of dichlorvos ranging from 0.1 to 100 μmol L^{-1}. Dichlorvos standard solutions (200 μL) were injected into the flow system (Fig. 1) without stopped flow. After the establishment of the baseline, acetylcholine chloride solution was injected again and the potential difference, due to hydrogen ions produced in the enzymatic system, was obtained. In the presence of the pesticide, acetylcholinesterase decreases the enzyme activity. The difference between the two signals (before and after inhibition) was related to the pesticide concentration. Figure 6 shows the calibration curve (inhibition curve) for dichlorvos pesticide in the studied range. For these pesticide concentrations, an inhibition rate from 8.30% to 47.3% was observed. Many researchers developed biosensors using the acetylcholinesterase enzyme. We also have used this enzyme; however, the flow system was used without stopped flow. It was found to be associated with a high sensitivity and good repeatability (Table 3). The results of the inhibition rate obtained from potato samples and potato samples spiked with 100 μmol L^{-1} of the dichlorvos were 3.0±0.1% and 41.7±2.5%, respectively. A recovery rate of 65.7±2.5% of dichlorvos (N=5) was obtained using the proposed method. The graphite/poly(4-aminophenol)/acetylcholinesterase biosensor was employed more than 100 times during 12 days. A decrease of 45% was observed in the response of the biosensor.

Conclusions

The electrodes modified with poly(4-aminophenol) showed higher sensitivity to hydrogen ions than electrodes modified with poly(2-aminophenol) and poly(3-aminophenol). Graphite electrode modified with poly(4-aminophenol) is an efficient and promising material for immobilization of acetylcholinesterase enzyme, and the biosensor producer can be applied for the quantification of the inhibitors substances of this enzyme.

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