

A Biosensor Using Poly(4-Aminophenol)/Acetylcholinesterase Modified Graphite Electrode for the Detection of Dichlorvos

Edmar Isaías Melo, Diego Leoni Franco, André Santiago Afonso, Hélen Cristine Rezende, Ana Graci Brito-Madurro, João Marcos Madurro and Nívia Maria Melo Coelho*

Instituto de Química, Universidade de Uberlândia, Av. João Naves de Ávila, 2121; 38400-902; Uberlândia - MG - Brasil

ABSTRACT

The properties of poly(4-aminophenol) modified graphite electrode as material for the immobilization of acetylcholinesterase were investigated by the Cyclic Voltammetry, Electrochemical Impedance Spectroscopy and Atomic Force Microscopy. The polymer was deposited on graphite electrode surface by the oxidation of 4-aminophenol and then acetylcholinesterase was immobilized on the surface of the electrode. The biosensor coupled in the continuous flow system was employed for the detection of dichlorvos. The detection and quantification limits were 0.8 and 2.4 $\mu\text{mol L}^{-1}$ dichlorvos, respectively. Graphite electrodes modified with the poly(4-aminophenol) showed to be an efficient and promising material for immobilization of acetylcholinesterase enzyme. The proposed method requires simple parts which are easy to build, involves only one biosensor and the potentiometric detection is simple.

Key words: graphite, poly(4-aminophenol), biosensor, dichlorvos

INTRODUCTION

Organophosphate pesticides residue is a serious problem in the food safety and its detection on site is essential. Usually the detection methods are based on the inhibition of acetylcholinesterase by the pesticides. Chemically modified electrodes have become very attractive due to high selectivity and sensitivity (Pilar et al., 2002). Chemically modified electrodes are devices containing a supporting electrode and a layer modified chemically. The main purpose for 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 the electropolymerization of organic monomers have encouraged research in the field of electrochemistry (Stozhko et al., 2004; Zhao and Zhen-Nan, 2004). Methods to prepare the modified electrodes by the polymeric films are very simple involving only the immersion of the electrode into the polymer solution. These electrodes have been used for the detection of drugs, pesticides and heavy metals (Yuqing et al., 2004; Durrieu and Tran-Minh, 2002; Dzyadevych et al., 2002; Tsai et al., 2003). Amperometric and potentiometric sensors have been the most widely used detection systems (Kochana et al., 2008; Lupu et al., 2007). In this case, the measurements are based on the values of potential difference

*Author for correspondence: nmmcoelho@ufu.br

between the working electrode (sensor) and reference electrode. These transducers have been used for the construction of biosensors based on acetylcholinesterase (Ivanov et al., 2000; Snejdarkov et al., 2004; Tran-Minh et al., 1990). Accordingly, immobilization, storage and operational stability of cholinesterase are very important for the biosensor exploitation. However, the immobilization process of enzymes on the surface of carbon is difficult, since graphite or the amorphous carbon surfaces usually possess insufficient active groups for direct immobilization of the enzymes. There are many methods suitable for the immobilization of the enzymes on amino-containing matrices (Hermanson et al., 1992). Many of these methods result in the decreased enzymatic stability in the water-organic mixtures and storage instability in the dry conditions. The aim of this work was to study the properties of poly(4-aminophenol) modified graphite electrode for the immobilization of acetylcholinesterase to use this electrode for the detection of dichlorvos in the continuous flow system.

MATERIALS AND METHODS

Reagents and chemicals

Acetylcholinesterase (VI-S, 403 units/mg protein) and acetylcholine chloride were purchased from the Sigma-Aldrich (Steinheim, Germany). Poly (4-

aminophenol) was purchased from the Acros Organics (New Jersey, USA). Other reagents were analytical grade. Solutions were prepared using deionized water obtained from the Gehaka Master System (São Paulo, Brazil).

Apparatus

A graphite disk (28.26 mm²) (99.9%, Alfa Aesar - Karlsruhe, Germany) was used as working electrode. The counter and reference electrodes such as platinum plate and Ag/AgCl (KCl 3.0 mol L⁻¹) were used, respectively. A potentiostat from CH Instruments model 760C (Austin, USA) was used for the electrochemical measurements in a three-compartment electrochemical cell. Electrochemical Impedance Spectroscopy (10⁶ to 10⁻² Hz frequency interval using signal amplitude of 10 mV) and Cyclic Voltammetry (potential range -0.10V to +0.50 V, and 100 mV.s⁻¹) were performed using a solution containing 5 mmol L⁻¹ K₃Fe(CN)₆, 5 mmol L⁻¹ K₄Fe(CN)₆ and 50 mmol L⁻¹ KNO₃. The morphology of the modified graphite electrode was analyzed by the Nanoscope III Atomic Force Microscopy from the Digital Instruments (Ottawa, Canada). Figure 1 shows the flow injection system used in this work. The polyethylene tubing (i.d.: 0.8 mm) were used for the loops, transmission line and coils. The solutions were pumped using a Minipuls 3 peristaltic pump from Gilson (Villiers Le Bel, France) and Tygon® pumping tubes.

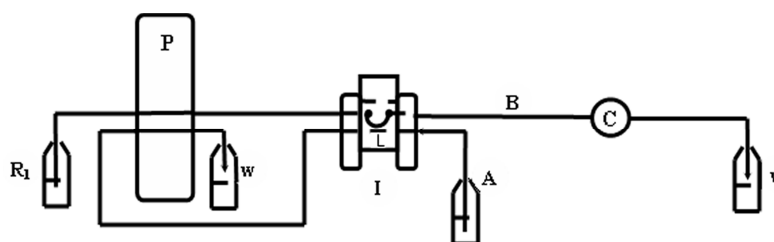


Figure 1 - Schematic diagram of the flow injection system. R1, Carrier solution (1.0 mmol L⁻¹ phosphate buffer in 0.1 mol L⁻¹ KCl) and flow rate of 1.5 mL min⁻¹; P, peristaltic pump; W, Waste; L, loops of 200μL; I, injection valve; A, standard solutions of acetic acid or acetylcholine chloride; B, reaction coil of 35 cm; C, potentiometric detector.

Preparation of poly (4-aminophenol)/acetylcholinesterase modified graphite electrode

The graphite electrode was prepared utilizing a mechanically polished graphite disk with alumina (0.3 μm) slurry, ultrasonicated, washed with ultra-pure water and dried with the ultra-pure N₂. The

procedure of electropolymerization was performed as described in the literature (Brito-Madurro et al., 2007; Franco et al., 2008a). The analytical response of the sensor was evaluated using acetic acid solutions (0.05 to 100 mmol L⁻¹), containing 1.0 mmol L⁻¹ phosphate buffer in 0.1 mol L⁻¹ KCl. The enzyme was immobilized on the poly(4-

aminophenol) modified graphite electrode (Ivanov et al., 2000). The response of the sensor containing the immobilized enzyme in the presence of 200 μL substrate (0.5 to 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 as a time function.

RESULTS AND DISCUSSION

Properties of electrodes

In this article, electropolymerization of poly (4-aminophenol) film on the graphite electrode was performed as described in the literature (Brito-Madurro et al., 2007; Franco et al., 2008a). During the electropolymerization, higher value of current was observed after the first cycle in the stage deposition of the polymer on surface. Also, higher value of current was observed increasing the number of potential scans, showing the covering of the electrode surface by the polymeric film. Similar electrochemical behavior was observed for the electropolymerization of polymeric films from 2-aminophenol and 3-aminophenol (Franco et al., 2008a; Franco et al., 2008b; Ferreira et al., 2008).

The performance of poly(4-aminophenol) modified graphite electrode as potentiometric sensor was studied in the continuous flow system (Fig. 1) using acetic acid solutions (0.05 to 100 mmol L^{-1}). The results indicated that the poly(4-aminophenol) graphite sensor presented high sensitivity (58.8 mV L mol^{-1}) to hydrogen ions and good reproducibility (RSD= 1.1%; N= 5). Hence, this electrode could be used as potentiometric detection system.

The morphology of the poly (4-aminophenol)/acetylcholinesterase modified graphite electrode was investigated by the Atomic Force Microscopy (Fig. 2). Roughness value of 86 nm was obtained. Low value of roughness for poly(4-aminophenol)/acetylcholinesterase modified graphite electrode showed that the enzyme covered the poly(4-aminophenol) electrode surface.

Studies by the Electrochemical Impedance Spectroscopy and Cyclic Voltammetry in the presence of 5.0 mmol L^{-1} $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ containing 1.0 mol L^{-1} KCl were made to evaluate the rate of electron transfer of the poly(4-aminophenol) acetylcholinesterase modified graphite electrode.

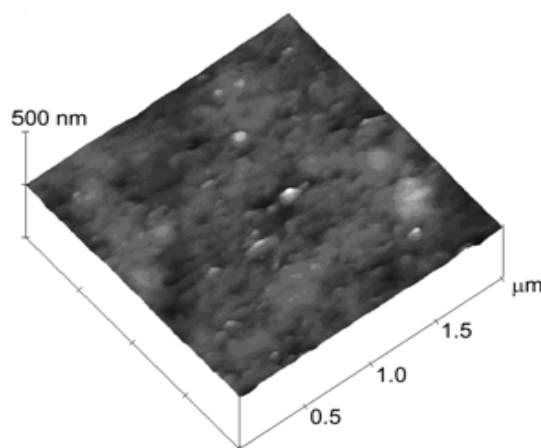


Figure 2 - Atomic Force Microscopy image of graphite electrode modified with poly (4-aminophenol)/acetylcholinesterase.

Cyclic Voltammetry studies showed the current values of 666.8 μA for graphite/poly(4-aminophenol) and 352.3 μA for graphite/poly(4-aminophenol)/acetylcholinesterase. 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 was the solution resistance, Q_{dl} was the electric double layer capacitance, R_{tc} was the charge transfer resistance, W was the Warburg impedance, R_{cm} and Q_{cm} were the charge transfer resistance and electric double layer capacitance to modified layer, respectively.

Results are presented in Table 1. The charge transfer resistance (R_{tc}) for the poly(4-aminophenol) acetylcholinesterase modified graphite electrode (36.30Ω) was higher than the graphite/poly(4-aminophenol) sensor (15.52Ω). These results showed that the enzyme immobilized

on the electrode produced a lower transfer charge of electroactive species in solution.

This result was consistent with the values of current observed in the Cyclic Voltammetry in the presence of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ couple, i.e., $352.3 \mu\text{A}$.

Table 1 - Results obtained from 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
$R_{cm}/K\Omega$	---	2.7
$Q_{dl}/\mu\text{F}$	0.27	0.27
$Q_{cm}/\mu\text{F}$	---	3.7
W	0.006	0.068
χ^2 *	0.01	0.01

* Error in simulation of the experimental data.

Evaluation of the flow parameters

After the immobilization of acetylcholinesterase enzyme on the poly(4-aminophenol) modified graphite electrode, the biosensor was coupled in the flow system (Fig. 1). Potentiometric response due to hydrogen ions generated by the enzymatic system in the presence of acetylcholine chloride substrate was evaluated. In order to find a compromise between the sensitivity and sampling frequency, the effects of the phosphate buffer and substrate concentrations and pH were investigated. The response of the biosensor was evaluated using $200 \mu\text{L}$ of acetylcholine solution (0.5 to 100 mmol L^{-1}) prepared in carrier solution (1.0 mmol L^{-1} phosphate buffer in 0.1 mol L^{-1} KCl). The pH of carrier solution was studied in the range from 6.00 to 8.00 and phosphate buffer concentration was evaluated in the range from 0.001 to 0.10 mol L^{-1} . The analytical signal for consecutive injections of $200 \mu\text{L}$ of acetylcholine chloride increased

proportionally with the substrate concentration until 10 mmol L^{-1} . Higher concentrations were not used because there was a large time for the analytical signal to return the baseline and to maintain the compromise with the analytical frequency, 5.0 mmol L^{-1} acetylcholine chloride was used for further experiments. Analogously, increasing pH values, varying from 6.00 to 8.00 , increased the signal. Appropriate results were obtained with a pH of 7.40 , favoring the stability of the biosensor. In order to obtain sufficient sensitivity, the phosphate concentration was investigated. Therefore, phosphate solutions were prepared at pH 7.40 and concentration was varied from 0.001 to 0.10 mol L^{-1} . The analytical signal decreased with the high concentration values.

The best compromise for the phosphate concentration was obtained for 0.001 mol L^{-1} .

Table 2 shows analytical figures of merit for the proposed biosensor.

Table 2 - Figures of merit for poly (4-aminophenol)/acetylcholinesterase modified graphite electrode in function of the acetylcholine chloride concentration.

Figures	Response
Linear range (mmol L^{-1})	$0.5 - 10$
Detection limit (mmol L^{-1})	0.1
Potential (mV) ^(a)	12.4 ± 1.6
Equation ^(b)	$E = 1.74 + 2.01/[CAC]$
Correlation coefficient	$r^2 = 0.9953$
Velocity of response (mV s^{-1}) ^(a)	1.0 ± 0.2

^(a) 5.0 mmol L^{-1} ^(b) E is potential (mV) and $[CAC]$ is acetylcholine chloride concentration.

Response of biosensor for dichlorvos

The detection of dichlorvos was made using the proposed biosensor coupled in the continuous flow system (Fig. 1). The substrate concentration was 5.0 mmol L⁻¹ acetylcholine chloride prepared in 1.0 mmol L⁻¹ phosphate buffer solution containing 0.1 mol L⁻¹ KCl at pH 7.40. The response of biosensor was obtained before and after the inhibition step. Dichlorvos standard solutions (200 μL) were injected in the flow system without stopping the flow. The percentage of acetylcholinesterase inhibition was obtained by the equation:

$$I (\%) = \frac{\Delta E_1 - \Delta E_2}{\Delta E_1} \times 100\%$$

where I is the percent of inhibition, ΔE₁ is the potential difference produced in the enzymatic system before inhibition and ΔE₂ is the potential difference produced in enzymatic system after inhibition. The regeneration step of the biosensor

was carried out in the flow system using 200 μL of 5.0 μmol L⁻¹ TMB-4 (1,1-trimethylene-bis (4-formylpyridinium bromide) dioxime). Figures 3 and 4 show the calibration curve for dichlorvos and analytical signals for proposed biosensor in the studied range, respectively. For these concentrations, an inhibition rate from 2.3 to 47.3% was observed. Under the optimized conditions, the poly(4-aminophenol) acetylcholinesterase modified graphite electrode was employed more than 100 times during 12 days. In this case, a decrease of 45% analytical signal was observed. The detection and quantification limits based on the 3*r*/*s* and 10*r*/*s* where *r* was the standard deviation of 10 measurements of a blank and *s* was the slope of the calibration graphs, were 0.8 and 2.4 μmol L⁻¹ dichlorvos, respectively. The selected conditions were judged from a good slope and linearity of the calibration graph obtained with a reasonable analysis time.

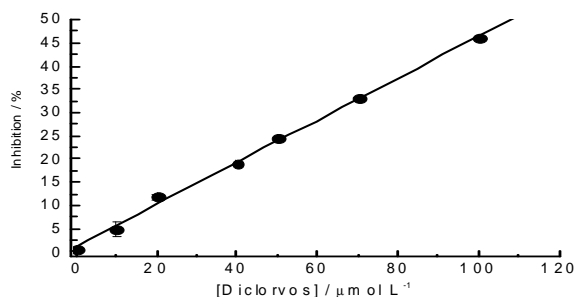


Figure 3 - Calibration curve obtained for dichlorvos standard solutions.

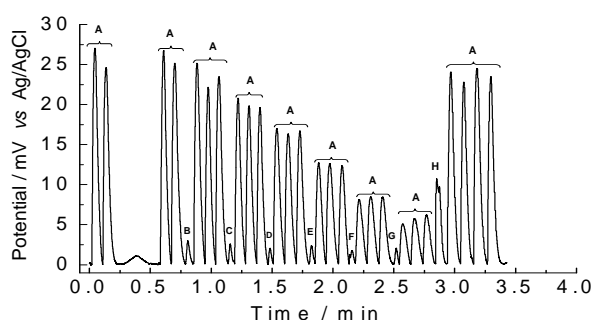


Figure 4 - Analytical signals for proposed biosensor (A) 5.0 mmol L⁻¹ acetylcholine chloride; (B, C, D, E, F and G) 10, 20, 40, 50, 70 and 100 μmol L⁻¹ dichlorvos, respectively, (H) regeneration of enzyme.

CONCLUSIONS

The graphite electrodes modified with poly(4-aminophenol) showed to be an efficient and

promising material for the immobilization of acetylcholinesterase enzyme. The results showed that the poly(4-aminophenol) acetylcholinesterase modified graphite electrode presented high

sensitivity for dichlorvos. Hence, this electrode could be used as detection system for pesticides residue. The FI procedure proposed here could to be employed as an inexpensive alternative to those procedures using separation methods of analysis. The proposed method could be very favorable for rapid and precise detection of dichlorvos. It would require simple parts easy to build and would involve only one biosensor and the potentiometric detection was simple.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

- Brito-Madurro, A.G.; Ferreira, L.F.; Vieira, S.N.; Ariza, R.G.; Filho, L.R.G.; Madurro, J.M. (2007), Immobilization of purine bases in poly-4-aminophenol matrix, *J. Mat. Sci.*, **42**, 3238-3243.
- Durrieu, C.; Tran-Minh, C. (2002), Toxicity tests employing microalgae to determine the ecotoxic concentration of heavy metals provide important information for predicting the environmental impact of their pollution, *Ecotoxic. Environ. Saf.*, **51**, 206-209.
- Dzyadevych, S.V.; Soldatkin, A.P.; Chovelon, J.M. (2002), Assessment of the toxicity of methyl parathion and its photodegradation products in water samples using conductometric enzyme biosensors, *Anal. Chim. Acta*, **459**, 33-41.
- Hermanson, G.T.; Malia, A.K.; Smith, P.K. (1992), Immobilized affinity ligand techniques, Academic Press, San Diego, USA.
- Ferreira, L.F.; Boodts, J.F.C.; Brito-Madurro, A.G.; Madurro, J. M. (2008), Gold electrodes modified with poly(4-aminophenol): incorporation of nitrogenated bases and na oligonucleotide, *Polym. Intern.*, **57**, 644-650
- Franco, D.L.; Afonso, A.S.; Ferreira, L.F.; Gonçalves, R.A.; Boodts, J.F.C.; Brito-Madurro, A.G.; Madurro, J.M. (2008a), Electrodes modified with polyaminophenols: Immobilization of purines and pyrimidines, *Polym. Engin. Sci.*, **48**, 2043-2050.
- Franco, D.L.; Afonso, A.S.; Vieira, S.N.; Ferreira, L.F.; Gonçalves, R.A.; Brito-Madurro, A.G.; Madurro, J.M. (2008b), Electropolymerization of 3-aminophenol on carbon graphite surface: electric and morphologic properties, *Mat. Chem. Phys.*, **107**, 404-409.
- Ivanov, A.N.; Evtugyn, G.A.; Gyurcsányi, R.E.; Tóth, K.; Budnikov, H.C. (2000), Comparative investigation of electrochemical cholinesterase biosensors for pesticide determination, *Anal. Chim. Acta*, **404**, 55-65.
- Kochana, J.; Nowak, P.; Jarosz-Wilkolazka, A.; Bieroń, M. (2008), Tyrosinase/laccase bienzyme biosensor for amperometric determination of phenolic compounds, *Microchem. J.*, **89**, 171-174.
- Lupu, A.; Valsesia, A.; Bretagnol, F.; Colpo, P.; Rossi, F. (2007), Development of a potentiometric biosensor based on nanostructured surface for lactate determination, *Sensors and Actuators B*, **127**, 606-612.
- Pilar, M.D.; Taboada, S.; Tanaka, A.A.; Kubota, L. T. (2002), Development of an amperometric enzymeless biosensor for phenol compounds using a nafion membrane doped with copper dipyrindyl complex, *J. Electroanal. Chem.*, **536**, 71-81.
- Snejdarkov, A.M.; Svobodova, L.; Evtugyn, G.; Budnikov, H.; Karyakin, A.; Nikolelis, D.P.; Hianik, T. (2004), Acetylcholinesterase sensors based on gold electrodes modified with dendrimer and polyaniline: A comparative research, *Anal. Chim. Acta*, **514**, 79-88.
- Stozhko, N.Y.; Lipunova, G.N.; Maslakova, T.I.; Aleshina, L.V.; Brainina, K.Z. (2004), A thick-Film carbon-containing electrode modified with formazan for determining copper, lead, cadmium and zinc, *J. Anal. Chem.*, **59**, 179-184.
- Tran-Minh, C.; Pandey, P.C.; Kumaran, S. (1990), Studies on acetylcholine sensor and its analytical application based on the inhibition of cholinesterase, *Biosensors and Bioelectronics*, **5**, 461-471.
- Tsai, H.C.; Doong, R.A.; Chiang, H.C.; Chen, K.T. (2003), Sol-gel derived urease-based optical biosensor for the rapid determination of heavy metals, *Anal. Chim. Acta*, **481**, 75-84.
- Zhao, Q.; Zhen-Nan, G.; Zhuang, Q.K. (2004), Electrochemical study of tetra-phenyl-porphyrin on the SWNTs film modified glassy carbon electrode, *Electrochem. Commun.*, **6**, 83-86.
- Yuqing, M.; Jianrong, C.; Xiaohua, W. (2004), Using electropolymerized non-conducting polymers to develop enzyme amperometric biosensors, *Trends Biotechnol.*, **22**, 227-231.

Received: August 10, 2010;
Revised: March 24, 2011;
Accepted: August 09, 2011.