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Mono-Enzyme Biosensor for the Detection of Organophosphorous Compounds

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Organophosphorous compounds are important neuroactive molecules whose presents significant analytical challenges. New monoenzyme biosensor introduced here, is used for the measurement of the level of two organophosphorous compounds, paraoxon (POX) and ethyl parathion (EPA), in phosphate buffer. Prussian blue electrodepositionized on a graphite electrode served as a template for immobilization of monoenzyme layer. A monoenzyme layer containing choline oxidase was immobilized along with nafion and bovine serum albumin, by cross-linking with glutaraldehyde. The results revealed that the response of the proposed biosensor was linear between 5 μM and 80 μM in the presence of POX and EPA. Detection limits were 0.4 μM in both cases. Also, for the optimization of the biosensor performance, temperature and pH dependency, concentration of choline chloride as substrate of choline oxidase and incubation time were determined. The results showed that the optimum values for temperature, pH, substrate concentration and incubation time were room temperature, pH 7, 4 mM and 30 min, respectively.

Keywords: Paraoxon, Ethylparathion, Choline oxidase, Organophosphorous compounds, Prussian blue, Monoenzyme biosensor, Electrochemistry, Biosensor parameters

INTRODUCTION

In recent years, the use of organophosphorous pesticides has become widespread in agriculture [1,2]. Because of possible health risks and the consequent contamination of water and food sources, research is continuing towards the development of fast and accurate methods for the detection and measurement of organophosphorous pesticides [3]. In this respect, several research groups have introduced some enzyme-modified electrodes [4]. Since the organophosphorous agents are cholinesterase inhibitors, most of them have used acetylcholinesterase (AChE) or buterylcholinesterase (BChE) as the enzymatic component of their sensors [5]. In these sensors, the inhibition of AChE or BChE, can be measured by electrochemical techniques such as pH-shift potentiometry [3]. The main disadvantage of this technique is its strict requirement for low buffer capacity of analyte solution. This may lead to significant complexities and uncertainness in the measurement of the organophosphourous compounds.

Since the sensitivity of pH-based analytical techniques, in general, is less than that of amperometric methods, the current measurement technique has been developed for the detection of organophosphates [6]. Amperometric biosensors typically rely on an enzyme system that catalytically converts electrochemically non-active analytes into products that can be oxidized or reduced at a working electrode. Generally, the produced current is linearly proportional to the electroactive product concentration which, in turn, is proportional to the

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non-electroactive enzyme substrate concentration. Enzymes typically used in ampermetric biosensors are oxidases such as choline oxidase (ChOx) [7].

For the detection of organophosphates, amperometric biosensors are based on coupling two consecutive cholinesterase (AChE or BChE) and (ChOx) enzymatic reactions at the appropriate electrode. The sequence of enzymatic reaction is as follows: Butryl or acetylcholine is catalyzed by AChE or BChE, accordingly, choline as a product is catalyzed by ChOx and the product of this enzymatic reaction (hydrogen peroxide; H_2O_2) is detected *via* horse-radish peroxidase (HRP) catalyzed electron transfer [8].

The most problematic areas in bienzyme amperometric biosensors are related to the interface between the enzymes layers and the solid phase of an electrode. For this reason, different methods have been developed to overcome the problem. For example, the problem may be overcome by the use of chemical mediators such as Ferric ferrocyanide ($Fe_4^{III}[Fe_2^{II}(CN)_6]_3$) or Prussian Blue (PB) [9]. In this method, Prussian Blue (PB) films immobilized on the electrode surface, are extensively used as redox mediator. The reasons for employing PB films include their simple preparation and potential for electrocatalytic applications in aqueous and non-aqueous media. In addition, this redox system has the advantages of well-defined electron stoichiometry, insensitivity of mediation to changes in acidic or neutral pH, ionic strength and a high value of the rate constant for electron transfer between the enzyme and electrode. Moreover, the modification of graphite electrodes with PB has been particularly effective in enhancing the selectivity of oxidase enzymes [10].

Although sensitive, multienzyme biosensors based on AChE inhibition have some limitations. Firstly, since AChE is inhibited by neurotoxins which include not only organophosphate agents such as POX and EPA, but also carbomate pesticides and many other compounds, are not thus selective. Secondly, immobilization protocols involve multi steps requiring optimization of physical condition (such as potential, temperature and so on) and chemical condition (such as pH and kinetic parameters) of three important enzymes including BChE or AChE, ChOx and HRP which are almost impossible. Finally, reactivation/regeneration in many cases is not possible due to irreversible inhibition of BChE or AChE (reversibility is necessary if the electrode has to be reused) [11].

This paper describes the construction of a simple amperometric monoenzyme biosensor for the direct, sensitive and selective determination of POX and EPA, using ChOx immobilized *via* cross-linking on the surface of graphite/PB modified electrode. Moreover, this paper assesses some important analytical parameters of the newly constructed biosensor. We hope to take advantage of the improvements made by the use of ChOx and the substitution substrate (choline chloride) to make a new, simple, inexpensive and more effective organophosphorous biosensor.

EXPERIMENTAL

Choline oxidase (ChOx; E.C. 1.1.3.17) from *Alcaligenes Species*, with an activity of 17 units/mg solid, choline chloride as substrate, bovine serum albumin (BSA), glutaraldehyde 25% (v/v), nafion 5% (v/v), paraoxon (POX) and ethyl parathion (EPA) were all purchased from Sigma chemical company. $FeCl_3$ and ferric ferrocyanide ($Fe_4^{III}[Fe_2^{II}(CN)_6]_3$) or Prussian blue (PB) were bought from Merck. Unless otherwise stated, all solutions were prepared with 0.1 M phosphate buffer + 0.1 M KCl, pH 7.

Graphite/PB-modified electrode construction was prepared based on protocol as follows: graphite rods with 5 cm length were cleaned in methanol, rinsed with double-distilled water and air-dried. For insulation purposes, the graphite rods were covered with thermal insulator except 0.1 cm at each end. A fresh solution of 2.5 mM $K_3Fe(CN)_6$ (PB) in distilled water was prepared and for the deposition of PB, one end of the graphite rods was dipped in the solution. Also, a fresh solution of 2.5 mM $FeCl_3$ in 0.1 M HCl, KCl and 2.5 mM of $K_3Fe(CN)_6$ (PB) in distilled water was prepared, separately. Then, the graphite/PB-modified electrodes were dipped in the 4 ml solution, 3 ml of $FeCl_3$ and 1 ml of PB, and were cathodically polarized using ZAG Chmie Co Digital Microamperometer instrument. For polarization, the current density of 40 μA cm^{-2} was used for 1 min. For optimization of electrodes, pre-treatment procedure was performed by using cyclic voltammograms in a phosphate buffer solution. Also, the stability of PB layer at electrode surface was studied by cyclic voltametery in the range of potential between -50 mV

and +400 mV with a scan rate of 100 mV s^{-1} for 40 cycles in the buffer solution.

ChOx deposition at the surface of graphite/PB-modified electrode was based on protocol as follows: A solution was prepared by mixing 5 µl nafion, 5 µl glutaraldehyde (2.5%); as cross-linker, 25 µl ChOx (3.6 mg ml^{-1} ChOx dissolved in 0.1 M phosphate buffer pH7) and 1 mg BSA in a vial. The BSA as inert protein in the solution of ChOx was used to get the most sensitive response. For homogeneity, the solution was mixed on vortex shaker. Then, 5 µl of this solution was drawn by micropipette and carefully inserted on the surface of the graphite/PB-modified electrode. Finally, electrodes were air-dried, rinsed and connected *via* wire to the instrument and used for the measurement of the output current of the proposed biosensor.

Electrochemical measurements were performed using a computer-interfaced EG&G (Prinsceton, NJ, USA) 263 A model Potentiostat-Galvonostat. The instrument was interfaced to a computer with PAR M270 software. A three-electrode setup was used in all the electrochemical measurements consisting of a graphite/PB-ChOx-BSA-modified working electrode (1 mm diameter) called "proposed biosensor" throughout this article, a platinum wire counter electrode and an Ag/AgCl reference electrode. All electrodes were purchased from Metreohm.

Temperature and pH were controlled in all of the experiments. For this purpose, all measurements were made in 2 ml of phosphate buffer, thermostated to desired temperature, in a 2 ml working volume jacketed glass cell. The water was circulated in the jacket of the glass cell; thus, the temperature of the water in the cell was controlled by a circulating water-bath instrument (model Pharmaci Biotech Multi Temp III). The changes of pH were recorded by a pH analyzer (Model 691-pH meter, Ω-Metreohm, Swiss made).

The proposed biosensor was calibrated by immersing the tip into instrument cell containing 2 ml 0.1 M phosphate buffer pH 7, in deionized water. Then, the amperometric current as a biosensor response was measured by adding aliquots of a stock solution of the appropriate concentration of choline chloride as substrate. Seperately, the organophosphates (POX/EPA) were dissolved in pure ethanol, and then added to the cell with various concentrations. The changes of the currents due to changes of the organophosphates concentrations or changes of the incubation time were recorded by instrument. To be on the safe side, in an independent experiment, the ineffectiveness of ethanol on the chronoamperometric base line was tested.

RESULTS AND DISCUSSION

Biosensors with the potential for organophosphates monitoring have been the subject of a number of recent investigations. These biosensors include bienezymatic (containing AChE and ChOx) or trienzymatic (containing AChE, ChOx and HRP) systems [12,13]. In this report we introduce a monoenzymatic biosensor. The components of the proposed biosensor include Graphite/PB-ChOx-BSA as a working electrode, a platinum wire as a counter electrode and an Ag/AgCl as a reference electrode. In the rest of this article, the analytical characteristics of the proposed biosensor are reported.

So far, a great deal of effort has been made to improve the selectivity and sensitivity of graphite-based electrochemical sensors, mainly through their modification with redox mediators such as PB [14]. In graphite/PB-modified electrodes, the potential stability is a critical point, especially at neutral pH. Therefore, cyclic voltametry was performed to evaluate electrochemical properties and stability of the PB layer on the surface of graphite electrode in the proposed biosensor. Cyclic voltametry was carried out from -50 mV to 400 mV with scan rate 100 mV s^{-1} in phosphate buffer solution at pH 7. In Fig. 1 the cyclic voltammogram of the PB-modified graphite electrode is illustrated. The sharpness of peaks in cyclic voltammogram indicates that the PB layer is well bound onto the graphite electrode surface.

Before immobilization of ChOx on the surface of the graphite/PB-modified electrode, the control experiment was carried out. For this propose, the steady state current of the graphite/PB-modified electrode in the presence of choline chloride was measured by chronoamperometric experiment. Steady state currents were measured by exerting -50 mV potential pulses during 30 s. Figure 2A shows the chronoamperometric plots of the bare (without ChOx deposition) graphite/PB-modified electrode. As illustrated in this figure, in the absence of ChOx in the PB-modified layer, chronoamperometry profiles showed no changes in current at

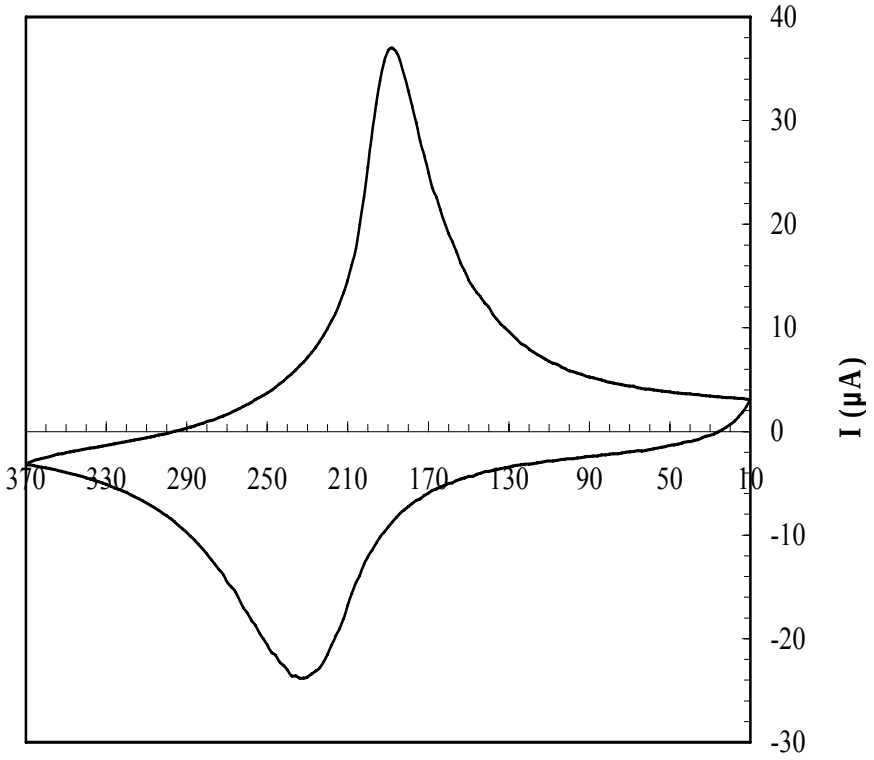


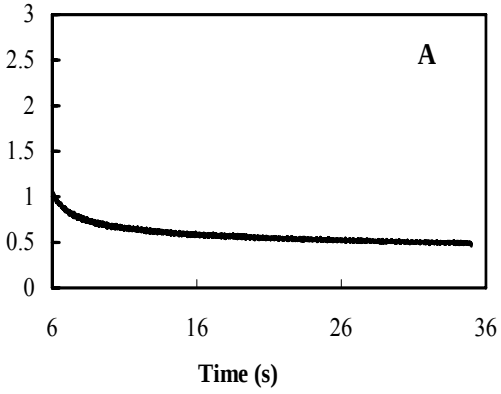
Fig. 1. Cyclic voltammogram of the PB modified graphite electrode. Cyclic voltammogram of PB is obtained in 0.1 M phosphate buffer +0.1 M KCl, pH 7 and room temperature versus Ag/AgCl. Potential range = -50 mV to 400 mV and scan rate = 100 mV s^{-1}.

different choline chloride concentrations. The selected potential (-50 mV) and time (30 s) for chronoamperometric studies were the optimum potential and time because of the high signal for H_2O_2 and the low interference with other samples. In addition, at the selected potential, the time needed to reach a stable current was as low as 30-60 s which is a suitable time for chronoamperometric studies [10].

After the immobilization of ChOx, in the presence of different concentrations of choline chloride, the enzyme activity on the surface of graphite/PB-ChOx-BSA modified electrode was monitored using chronoamperometry experiment. For this purpose, the current decay was measured in the same condition as the control experiment during 30s potential pulse (-50 mV). The current response of the proposed biosensor with time (Fig. 2B) showed that an increase in substrate concentration (choline chloride) caused an increase in amperometric current (biosensor response). Likewise, concentrations above 4 mM caused saturation of the biosensor response. Since 4 mM choline chloride gave an acceptable current response, this substrate concentration was selected for the other experiments.

In order to evaluate the proficiency, calibration curves, chronoamperometric response and the sensitivity (which is defined as the change of currents per unit change concentrations of POX or EPA) all as the analytical parameters of the proposed biosensor, were investigated. The experiments showed that they all were functions of the incubation time, pH of phosphate buffer and temperature of the electrochemical cell. Therefore, the following experiments were performed in order to find out the optimum function conditions for the proposed biosensor.

The effects of pH on the modified electrode were studied over the range 6.0-8.0 in a 0.1 M phosphate buffer containing 4 mM choline chloride solution and the peak current responses



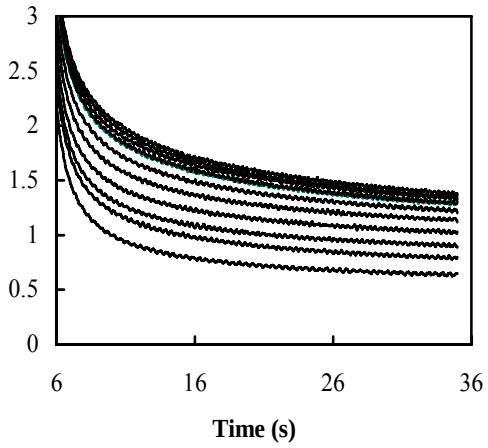


Fig. 2. Chronoamperometric responses of graphite/PB (A) and graphite/PB-BSA-ChOx modified electrodes (for procedure of modification see text). Chronoamperometric responses of modified electrodes were obtained in 0.1 M phosphate buffer +0.1 M KCl, pH 7 at room temperature *vs*. Ag/AgCl. The concentrations of choline chloride increased from 0 to 4 mM (in graph B, a, b, c, d, e, f, g, h, i, indicates 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 mM of choline chloride concentration, respectively). In graph A some chronoamperometric responses were obtained in presence of ethanol. Applied potential was -50 mV.

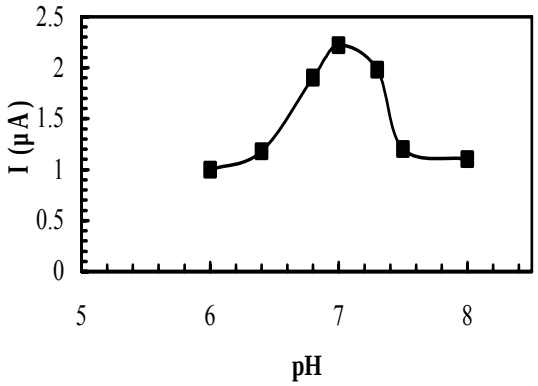


Fig. 3. pH dependence of the proposed biosensor (graphite/PB-BSA-ChOx modified electrode). The peak current was obtained in presence of 4 mM choline chloride in 0.1 M phosphate buffer +0.1 M KCl at room temperature *vs*. Ag/AgCl. Applied potential was -50 mV and current was measured after 30 s.

were monitored. Based on Fig. 3, pH 7 can be chosen as the optimum pH for the proposed biosensor. The 1.5 µA decrease in current at pH 6 can be attributed to the reduction in enzyme catalytic activity, because this is not the optimum pH for ChOx. Also, at higher pH (more than optimum pH), the current was drastically decreased again and this can be explained as follows. Although the enzymatic activity of ChOx increases with the pH up to a maximum plateau of pH 7-8 [15], the stability of PB is a crucial point which must be considered because hydroxyl ions (OH^-) are able to solubilize the PB at high pH [14]. Furthermore, the hydrolysis of POX and EPA may occur at pH > 7 [16] which makes one choose pH 7 as the optimum pH. Again, the current decrease in pH > 8, besides reduction of ChOx activity, might be due to the enzyme and PB instability at surface of the proposed biosensor. Therefore, pH 7 which gave the maximum current, without any undesired side-effects on the components of the proposed biosensor, was used as optimum pH in subsequent experiments**.**

The effects of temperature on the proposed biosensor were independently studied. Figure 4 shows the temperature dependence of the biosensor's response in 4 mM choline chloride solution at pH 7. As can be seen, the peak current responses increased with temperatures up to 60 ºC and then decreased with further temperature increase. Similar thermal behavior for the biosensor has been reported in the literature [17]. The initial increase might be attributed to the increase of both the enzymatic reaction and mass transport rates both of which increased with augmentation of temperature [18]. The

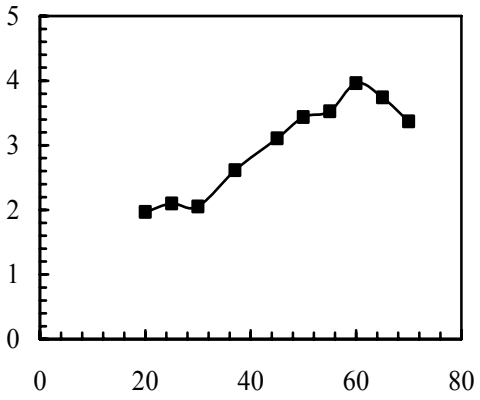


Fig. 4. Temperature dependence of proposed biosensor (graphite/PB-BSA-ChOx modified electrode). The peak current was obtained in presence of 4 mM choline chloride as substrate in 0.1 M phosphate buffer +0.1 M KCl at pH 7 *vs*. Ag/AgCl. Applied potential was -50 mV and current was measured after 30 s.

decrease in current at higher temperatures might be due to enzyme conformational changes or even denaturation. Although 60 ºC was determined to be the optimum temperature for the proposed biosensor operation at short-term experiments, for long-term stability of the proposed biosensor components, especially PB, subsequent experiments were further performed at room temperature (20 ºC). In addition, room temperature was selected in order to overcome the problem of excessive evaporative losses during the course of the experiments and ease of operations. Figure 4 shows that at temperatures higher than 30 ºC the responses of the biosensor were unstable and this was an additional reason for selecting room temperature for further experiments.

In Fig. 5 (A and B) calibration curves for POX and EPA (0 to 800 µM), using the proposed biosensor, are reported. Measurements were carried out by incubating the electrode for 30 min, with an increasing concentration of POX or EPA in 0.1 M phosphate buffer pH 7. The optimum incubation time was obtained from an independent experiment (will be explained and discussed). At the end of the incubation time, the peak currents were measured by adding 4 mM choline

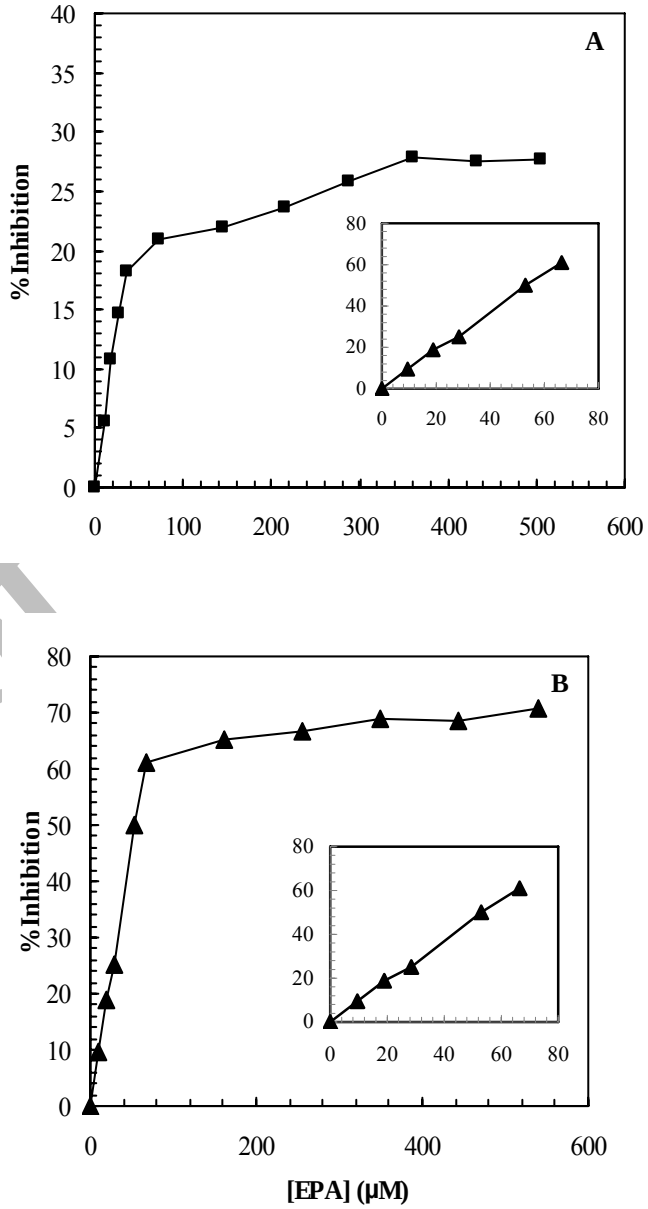


Fig. 5. Normalized calibration curves of graphite/PB-BSA-ChOx modified electrode for choline oxidase inhibitors. Data are from chronoamperometric analysis and expressed as percent inhibition. The linear range data is shown in an independent plot (Inset). The peak current was obtained in presence of POX (■), EPA (▲) and in presence of 4 mM choline chloride as substrate in 0.1 M phosphate buffer +0.1 M KCl at pH 7 *vs*. Ag/AgCl. Applied potential was -50 mV and current was measured after 30 s.

chloride in buffer solution. The percentage of uncompetitive inhibition [19] was quantified by the equation:

$$\text{Percentage of inhibition} = i_o - i/i_o \times 100$$

where i_o and i are the steady-state currents obtained in the absence or presence of POX/EPA, in the phosphate buffer, respectively [20]. A steady-state response was obtained after 30 seconds and the percentage of inhibition was used to draw calibration plots for the POX and EPA.

The analytical characteristics of the proposed biosensor for POX and EPA detection are summarized in Table 1. This table presents the slopes of the linear portion of the calibration curves at two ranges of EPA/POX concentration, 0-100 µM and 100-800 µM. The slopes indicate the sensitivity of the proposed biosensor defined as the changes of currents per unit changes concentrations of POX/EPA. It can be seen that inhibition percentage of ChOx increased with an increase in the concentration of POX (Fig. 5A) or EPA (Fig. 5B) until reaching a plateau. The calibration plots were linear over the range 5-80 µM and also were linear over the range 100-800 µM with a lower slope (Table 1) with a detection limit of 0.4 µM for both organophosphorous. Detection limits were calculated based on the number of data points acquired in the sample (n = 3) and the measured standard deviation for each value (RSD = 5%) with a confidence interval of 99% (statistical method) [21,22].

In order to obtain the sensitivity of the biosensor, the slope of the calibration curves at two ranges were calculated. The criterion for proposed biosensor sensitivity was the slopes of the calibration curves. The proposed biosensor gave a significantly lower sensitivity to EPA relative to POX (Table 1). The sensitivity of biosensor also significantly decreased at higher concentrations of both POX and EPA (up to 100 µM) and this could be interpreted as follows. When the buffer contained POX/EPA at low concentrations, namely, 5-80 µM, the enzyme ChOx, which catalysed the enzymatic reaction, was partially inhibited by these organophosphorous compounds, while, at higher concentrations, POX/EPA had severe inhibitory effect on ChOx.

The values of R^2 in Table 1 indicate the square of regression correlation coefficient and its root, R, is attributed to the linearity of calibration curve [23]. The values of R are calculated 0.9787 for POX and 0.9788 for EPA between 5-80 µM. Although the values of R^2 are comparable with literature [24], they may improve by more optimization of experimental conditions which is current in our laboratory.

The reactions of the ChOx and PB in the proposed biosensor are shown on the basis of the following scheme in series [10]:

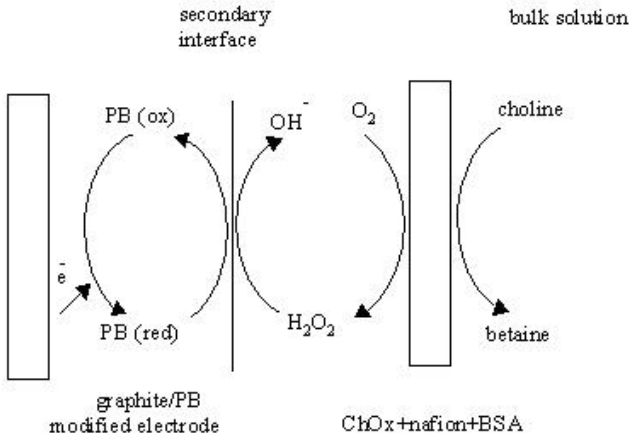


Table 1. Analytical Characteristics of Graphite\PB-ChOx-BSA Modified Amperometric Enzyme Electrode

Organophosphorous	Dynamic range (µM)	Linear regression	Sensitivity (nA μM^{-1})	Detection limit ((µM)	R^2
EPA	0-80	y = 7.4079x+50.011	7.4079	0.4	0.9579
EPA	100-800	y = 0.3865x+403.77	0.3865	0.4	0.9648
POX	0-80	y = 10.5620+68.316	10.562	0.4	0.9581
POX	100-800	y = 0.941x+669.34	0.941	0.4	0.9633

This scheme shows that, in the presence of POX/EPA the rate of enzymatic recycling of PB decreases due to inhibitory effect of these organophosphates on ChOx, and consequently the response current of the proposed biosensor will decrease. Besides, as is shown in Fig. 5 (A and B), a correlation was found between the increase in inhibition of ChOx and the reduction of H_2O_2 concentration in the enzymatic reaction catalyzed by ChOx. Consequently, the peak current in the measurement apparatus, decreased. Furthermore, the response of biosensor in the presence of high concentrations range of POX/EPA may be limited by the mass-transfer rate of substrate, hence, decrease in slope and consequently decrease in sensitivity can be due to such a phenomenon.

Preliminary experiments showed that, besides pH and temperature, the pre-incubation time played an important role in the sensitivity and better performance of the proposed biosensor. Therefore, the degree of inhibition was investigated by changing the pre-incubation time at fixed concentration of POX (800 μM). Then, the sensitivity of the proposed biosensor to the choline chloride, as substrate, was determined before and after incubation with POX. The data presented in Fig. 6 shows that with an increase in pre-incubation time, the peak current and initial rate decreases. So the sensitivity to the substrate diminished during the initial 30 min and 120 min exposure of the biosensor to POX. In addition, as shown in Fig. 6, pre-incubation of ChOx with 4 mM POX for 60 min, completely abolished the biosensor activity, which might be due to ChOx conformational changes. It should be mentioned that the whole unfolding (denaturation) occurred at higher concentrations of POX/EPA, namely over 4 mM, according to the results of the independent spectrophotmetrical studies which were performed in our laboratory, [19]. Thereupon, electrochemical data confirmed the spectrophotometric results. These evidences indicate the time and concentration dependency of inhibitory effect of POX on ChOx. According to the obtained data, 30 min incubation and concentrations less than 4 mM of POX, were selected to carry out further experiments.

In biosensors, recovery process is one of the important analytical characteristics. So, by using the selected potential, temperature, pH, incubation time and inhibitors (POX) and substrate (choline chloride) concentrations at the optimum values, the recovery process of the proposed biosensor was

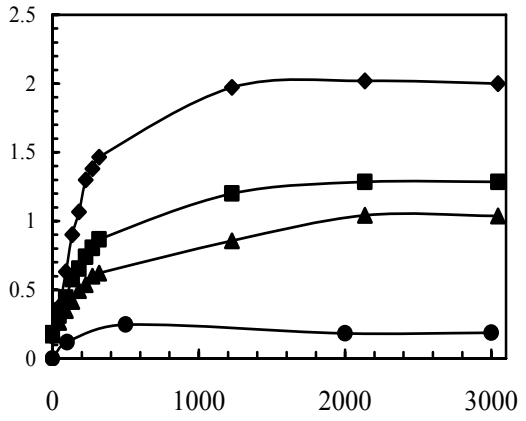


Fig. 6. The effect of incubation time in presence of various POX concentrations on the response of the proposed biosensor (graphite/PB-BSA-ChOx modified electrode) to increasing choline chloride as substrate. The peak current measured after 0 (♦), 30 (■) and 120 (▲) min incubation of modified electrode in 0.1 M phosphate buffer at room temperature containing 800 μM POX. Also, for complete depression, proposed biosensor was incubated by 4000 μM POX for 60 min (●).

investigated. To this end, the recovery experiment of the proposed biosensor was carried out in two stages. In the first stage, three peak currents in the presence of 4 mM choline chloride and absence of POX were measured between 0-30 min, and as shown in Fig. 7A, the current did not decrease. Instead, when the POX was added after 30 min, a decay in current began due to inhibition of the enzyme and reduction in products (betaine and H_2O_2). The maximum reduction of current occurred after 30 min incubation (60 min after starting of recovery experiment) which corresponded to optimum incubation time. In the second stage, through immersing the proposed biosensor in fresh phosphate buffer at pH 7, the peak current in the presence of 4 mM choline chloride was measured. Variations in the peak current over a period of 200 min are plotted in Fig. 7B. As shown in this figure, in the presence of added substrate and using the proposed biosensor, a steady state amperometric response was improved. Thus, the proposed biosensor response for the short-term stability test

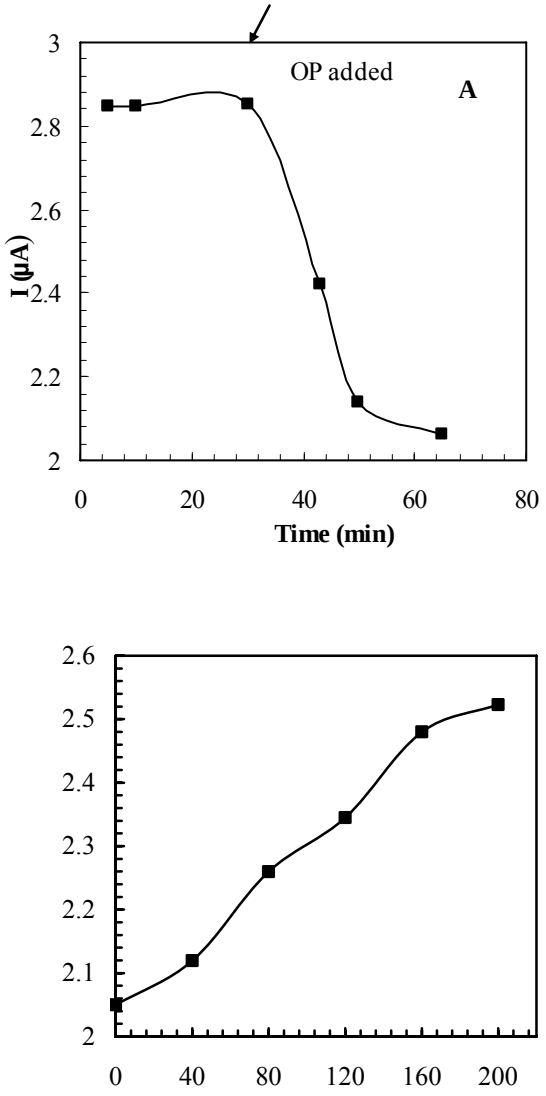


Fig. 7. Recovery of proposed biosensor. After 30 min, 800 μM POX injected into 0.1 M phosphate buffer containing 4 mM choline chloride as substrate 7A. In starting of recovery process, the proposed biosensor immersed into the fresh phosphate buffer and in different time, in the presence of 4 mM choline chloride, peak current was measured chronoampermerically 7B. Applied potential = -50 mV.

was almost reversible after 200 min of incubation in fresh phosphate buffer, whereas for longer time only a partial recovery of the biosensor occurred. The results of the short-term stability test for the evaluation of recovery process of the proposed biosensor indicated that, unlike AChE in bienzyme biosensor, the ChOx immobilization method successfully retained the enzyme activity and provided an environment for suitable and stable operation.

The analytical parameters including detection limit, sensitivity, stability, response time of the proposed biosensor are compared with similar organophosphorous biosensors based on choline oxidase enzyme. Although these parameters are deeply affected by various factors such as biosensors fabrication method, experimental conditions and type of organophosphorous material, nonetheless, the analytical parameters of the proposed mono-enzyme biosensor favorably compare with most of the data reported in the literature. For instance, in the case of incubation time, La Rosa [25] used an incubation time of 3-5 min for paraoxon and carbaryl detection while Bernabei and Campanella used 30 and 20 min, respectively [26,27] for aldicarb and paraoxon detection. Nunes used 10 min incubation time for aldicarb, and an incubation time as high as 1 h was reported by Skladal and Cho [28,29]. All of these researchers used bienzyme biosensors by co-immobilizing acetylcholinestearase (AChE) and ChOx [24]. Generally, for screen printed electrode compared with other biosensors, a low incubation time has been reported in literature [25].

Detection limit is another important analytical parameter in organophosphorous biosensors based on ChOx. The immobilization method of enzymes (AChE/ChOx) and type of electron transferring mediators causes diversity in detection limit value as reported in literature. For example, when AChE and ChOx were co-immobilized at the surface of working electrode, 3.6 μM was reported for detection limit [30,31]. In this report, concentration of POX was measured by amperometric method similar to the present investigation. This value of detection limit was approximately ten times higher than that determined for the proposed biosensor (0.4 μM). It was demonstrated here that the detection limit of monoenzyme biosensor is one of its strengths.

In addition, the long-term stability of the mono-enzyme biosensor was investigated. In dry or wet storage (in buffer phosphate pH 7, 4 ºC), no significant change in the activity of immobilized ChOx could be detected for two months. In the literature, there are data about stabilities of

organophosphorous biosensors based on co-immobilization of ChOX and AChE. Navera found that in bienzyme biosensor, the activity of biosensor decreased to 20% of its original value in 6 weeks [32]. Also, Rouillon reported that while their immobilized enzymes (AChE/ChOx) were stable for 3 months in dry storage, a significant decrease was observed in wet storage (50% in a month) [33]. Cho used polyurethane for immobilization of AChE/ChOx and reported a 33% loss in enzyme activity after 1 month [34,35]. It seems that the proposed biosensor during dry or wet storage is more stable than those reported about bienzyme biosensors.

Response time was another analytical parameter which was surveyed in this research. Response time is the sum of the incubation time and amperometric current measurements durations. In fact, POX/EPA detection was carried out in two steps. First, the proposed biosensor was incubated in the presence of each organophosphorous compound (30 min), in the second step, the response current in saturated substrate was measured, (30 s). The total response time was therefore 30 min for the proposed biosensor. A wide range of response times has been reported in the literature. For example, Cagnini and Skladal reported 12 min and 20 min for their fabricated organophosphorous bienzyme biosensors, respectively. They used disposable screen-printed electrodes and did not carry out reactivation process. Several researchers the reported longer response times mainly because of the long incubation times required. For example, Bernabei, Botre [36] and Cho reported response times of 35, 40 and 60 min, respectively [33,34,35]. Consequently, then, the time of the proposed biosensor is reasonable for POX/EPA detection.

CONCLUSIONS

This report described choline-oxidase based monoenzyme biosensor using Prussian blue as a mediator for the electrochemical detection of paraoxon (POX) and ethyl parathion (EPA). The choline oxidase was immobilized by chemical cross-linking with glutaraldehyde and using bovine serum albumin at the surface of working electrode. The optimum conditions such as substrate concentration (4 mM choline chloride), pH (pH = 7), temperature (room temperature) and incubation time (30 min) were determined for the proposed biosensor. Then, some analytical parameters of this new mono-enzyme biosensor were investigated. The results showed that at low concentration of POX and EPA (less than 80 μM) the sensitivity of the biosensor for detection of POX (10.5620) was more than that for detection of EPA (7.3079), while the detection limits were the same for both organophosphates (0.4 μM). In addition, the investigation of the recovery ability for the new biosensor showed that after 200 min treatment in 0.1 M phosphate buffer pH 7, the biosensor could be reusable. So, the simplicity of electrode modification, enzyme immobilization and the relative sensitivity due to the nature of Prussian blue may offer some advantages in practice. Also, the reusability and low-cost, renders the present biosensor quite economical. In our laboratory, further work will deal with the optimization of the procedure in terms of analytical parameters and with an extension of this mono-enzyme biosensor to other pesticides.

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