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AChE biosensor based on zinc oxide sol–gel for the detection of pesticides

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ABSTRACT

Zinc oxide has been used as a matrix for immobilization of acetylcholinesterase (AChE) and detection of the pesticide paraoxon. The immobilized enzyme retained its enzymatic activity up to three months when stored in phosphate buffered saline (pH 7.4) at 4 °C. An amperometric biosensor for the detection of paraoxon was designed. The biosensor detected paraoxon in the range 0.035–1.38 ppm and can be used to detect other AChE inhibiting organophosphate pesticides.

1. Introduction

Over the last decade, biosensors based on acetylcholinesterase (AChE) enzyme have emerged as a promising technique for toxicity analysis, environmental monitoring, food quality control and military investigations [1–3]. The main application of AChE biosensors is for the detection of organophosphate and carbamate pesticides based on enzyme inhibition. These devices are designed to complement or replace the existing reference analytical methods (chromatographic and coupled chromatographic–spectrometric procedures) by simplifying or eliminating sample preparation, thus decreasing the analysis time and cost. Most AChE biosensors reported to date are utilizing an electrochemical detection system. Due to the nature of the inhibition process and the difficulties of regenerating the active sensor surface disposable screen-printed electrodes (SPE) are often preferred.

Fabrication of AChE biosensors involves immobilization of the AChE enzyme onto an electrode surface. Various immobilization strategies and materials have been used for this purpose including physical and covalent binding and affinity interactions. One attractive method that ensures stability of the enzymatic layer is to entrap the enzyme in a silica material prepared by low temperature sol–gel processing. Silica sol–gel technology is a method of synthesizing oxide glasses and ceramics starting from alkoxysilanes or aqueous silicate precursors such as sodium silicate or colloidal silica in a two-step process of hydrolysis and polycondensation. A variety of enzymes, including AChE, have been immobilized in silica sol–gel glasses with retention of enzymatic activity [4–13]. Several reports demonstrated the possibility of combining sol–gel technology with screen-printing protocols for biosensor fabrication [14,15]. Other reports have indicated the potential of the sol–gel method as a versatile and efficient technique for conserving enzyme activity in organic solvents [15–19]. Studies have demonstrated that immobilization in a sol–gel material requires lower enzyme loading as compared to covalent binding via glutaraldehyde to obtain comparable current values [20]. This is due to the absence of chemical bound formation, thus preserving the enzymatic activity during the immobilization process. Andreescu et al. used this method to immobilize genetically modified AChE onto SPEs and reported an enhanced enzymatic stability [21]. However, there are several drawbacks of using silica gels for enzyme immobilization, such as brittleness and an aggressive chemical environment that can lead to enzyme denaturation and negatively impact the sensor performance. Therefore, alternative non-silica materials with increased biocompatibility have more recently been investigated, such as vanadium pentoxide [22], zirconium dioxide [23], alumina [24], or titania [25]. This study reports the use of an alternative sol–gel material, zinc oxide (ZnO), as a matrix for AChE immobilization onto the surface of SPE electrodes and its application for the detection of pesticides.

Zinc oxide is a non-silica biocompatible sol–gel material with attractive properties that have been exploited for applications such as gas sensors photocatalysts or solar energy conversion [26]. Nanoporous ZnO films have also been used for the immobilization

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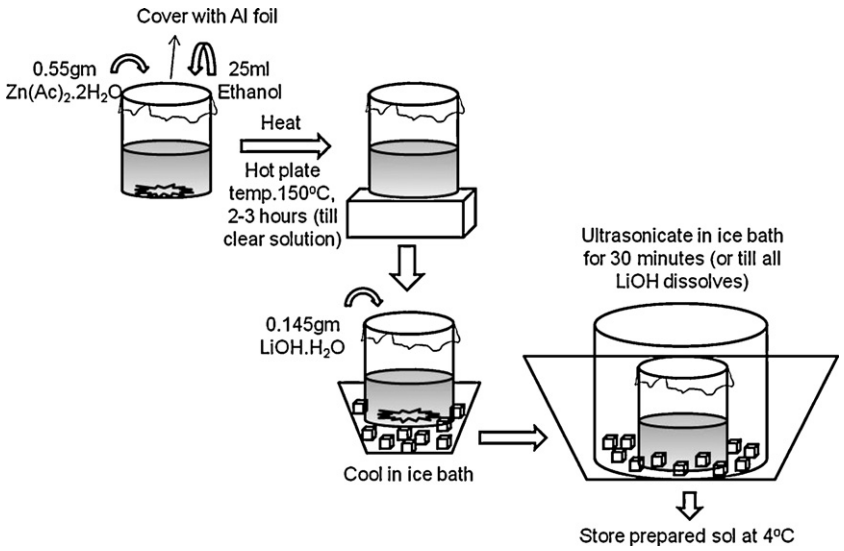

Fig. 1. Schematic of sol–gel preparation process of the ZnO matrix.

of proteins [27]. ZnO nanoarrays have been recently used for the detection of cytokines [28] and ZnO microspheres have been used for the detection of H_2O_2 and $NaNO_2$ [29]. Liu et al. reported on the fabrication of a tyrosinase biosensor based on a ZnO sol–gel matrix [30]. However, despite their attractive properties including bioactivity and biocompatibility [30], ZnO sol–gel materials have been very little investigated for the design of enzymatic biosensors. To our knowledge, there is no study directed at the design of AChE biosensors based on ZnO for pesticide detection. Among the few studies that used ZnO for enzyme immobilization, Yang et al. [31] used immobilization of AChE into a ZnO membrane deposited on a Pt electrode to design an acetylcholine and choline sensor, but did not design a pesticide biosensor. In this work, we report on the immobilization of AChE into a sol–gel ZnO matrix deposited on SPE, and on the design of an amperometric biosensor for the detection of organophosphorus pesticides using paraoxon as example.

2. Experimental

2.1. Reagents and equipment

Acetylcholinesterase (AChE), acetylthiocholine chloride (ATChCl), phosphate buffered saline containing 0.1 M KCl (PBS, 0.01 M, pH 7.4), hydrochloric acid (37%), dithiobis(2-nitrobenzoic acid) (DTNB), zinc acetate dihydrate, lithium hydroxide monohydrate, ethanol, sodium chloride, poly ethylene glycol (PEG) 6000, and paraoxon were purchased form Sigma Aldrich. Enzyme solution was prepared in PBS and its activity determined by Ellman's assay [32]. All electrochemical measurements were carried out with a BASi Epsilon electrochemistry system coupled with a C3 cell stand (Bioanalytical Systems Inc.). Spectroscopic measurements were carried out with a SpectraMax Plus Microplate Reader from Molecular Devices. Screen printed electrodes (SPE) from Pine Instrument Company in a three electrodes configuration were used to fabricate the AChE biosensors. The SPE electrodes were treated electrochemically to induce hydrophilicity to the electrode surface, and improve adhesion of the sol–gel layer. For this, the electrodes were immersed in a cell containing 3 ml PBS and subjected to 1.75 V voltage for 300 s. Next, the electrode was cycled starting from 0.3 mV and going between 1.25 V and −1.3 V until a stable response was registered.

2.2. Preparation of zinc oxide matrix

The zinc oxide solution was prepared using a modified sol–gel method reported in literature [30]. The preparation process is schematically presented in Fig. 1.

Briefly, 0.55 g of $Zn(Ac)_2{\cdot}2H_2O$ was added to 25 ml of absolute ethanol, the solution vessel was covered with aluminum foil, and heated on a hot plate set at 150 °C for three hours, until a clear solution was obtained. The solution was cooled down to 0 °C in an ice bath. Next, 0.145 g of $Li(OH){\cdot}H_2O$ was added to the solution and ultrasonicated for 30 min, while kept at 0 °C. The resulting solution (ZnO sol) was stored at 4 °C and subsequently used for enzyme immobilization. The amount of ZnO sol in the enzymatic solution was optimized as described in the results section. To improve the sensor stability and reproducibility, a solution of 20% (w/v) PEG 6000 was added to the ZnO sol in a 1:5 ratio. This configuration was used in all the experiments. To immobilize the enzyme, a solution containing 5–10 U ml^{-1} AChE enzyme was mixed with the ZnO–PEG sol in a 1:1 ratio, and 3 μl of this mixture was spread onto the surface of the pretreated SPE. These electrodes are denoted as ZnO–AChE biosensors. The electrodes were dried in air at room temperature for about 1–2 hours and then stored in PBS at 4 °C.

2.3. Electrochemical measurements and determination procedures

All electrochemical measurements were carried out with the AChE-modified SPE immersed in a cell containing 3 ml PBS solution at pH 7.4. Cyclic voltammetric and amperometric measurements were used to characterize the ZnO–AChE biosensor. For amperometric measurements, the electrochemical cell was stirred with a small magnetic bar at 150 rpm. Measurements were carried out at fixed applied potential of 235 mV vs. Ag/AgCl screen-printed pseudo-reference electrode. After stabilization of the capacitive current, the enzymatic reaction was initiated by addition of 1 mM ATCh substrate and the response of the sensor was measured. The operational stability of the biosensor was evaluated by measuring repetitively the electrode response to the same quantity of ATCh substrate (final concentration 1 mM) and rinsing the cell between measurements. The storage stability was evaluated by measuring the response of electrodes, stored under the same conditions,

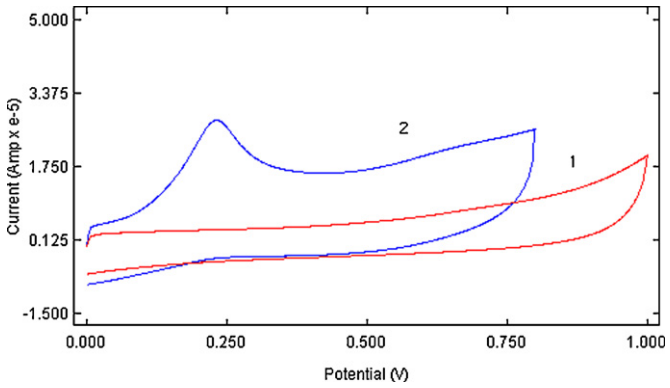

Fig. 2. Cyclic voltammetry of AChE loaded electrodes with varying the pH of ZnO sol. Curve 1 represents the CV obtained at a pH of 9.5. Curve 2 represents the CV obtained at a pH of 6.4.

at various time intervals. The reproducibility was evaluated from the response of identically prepared electrodes. Inhibition measurements were carried out in a two steps batch procedure [21] by measuring the response of the sensor to additions of a constant amount of ATCh substrate (1 mM) before and after inhibition with pesticides. All inhibition measurements were carried out with paraoxon as a model organophosphate pesticide. The electrodes were incubated for 10 min in DI water in the absence and presence of concentrations of paraoxon ranging from 10^{-8}–10^{-5} M.

3. Results and discussion

3.1. Electrochemical characterization of the ZnO–AChE biosensors

ZnO–AChE biosensors (SPE electrodes with the AChE immobilized in the ZnO sol–gel) were first characterized by cyclic voltammetry (CV) in the presence of 1 mM ATChCl. Fig. 2 shows the CVs of the ZnO–AChE electrode in the presence and absence of ATCh. SPEs were selected for this work due to their low cost and high surface area. Since the biosensor is designed to be disposable, this is another reason SPE electrodes were selected. A well-defined oxidation peak between 220 and 260 mV with a maximum at about 235 mV and a wide shoulder at ~ 700 mV vs Ag/AgCl were observed. A similar behavior, with a well-defined peak at 200 mV and a shoulder at ~700 mV, was reported with the use of multiwall carbon nanotubes on a glassy carbon electrode [33]. This peak corresponds to the oxidation of the thiocholine (R-SH), the product of the AChE catalyzed hydrolysis of ATCh to dithio-bischoline (R-S-S-R) as shown in the following reactions:

$$\text{AChE; R-S-CO-CH}_3 + \text{H}_2\text{O} \rightarrow \text{R-SH} + \text{CH}_3\text{COOH}$$

$$2\text{R-SH} \rightarrow \text{R-S-S-R} + 2e^- + 2\text{H}^+$$

The oxidation peak was obtained at relatively low potential values (between 220 and 250 mV) as compared to those reported in literature with other electrode materials such as carbon (750 mV) [33]. Low oxidation potentials have been reported previously with the use of electronic mediators like tetracyanoquinodimethane (TCNQ) (100 mV) [21] and cobalt hexacyanoferrate (CoHCF) (350 mV [34]) and with multiwall carbon nanotubes (150 mV [35]). The observed low oxidation potential of thiocholine can be due to a catalytic effect of ZnO. Decreasing the ZnO sol in the sol–gel immobilization solution in ratios ranging from 1:1 to 1:8 ZnO:DI water generated a reduction in current response. The signal also decreased as the pH increased from 6.4 to 9.5. No redox peak was observed at a pH of 9.5 (results not shown), which can indicate that a low or no enzyme is present onto the electrode surface at this pH value. The isoelectric point of ZnO is 9.5 and that of AChE is 5.5. Between pH 5.5 and 9.5 the enzyme and ZnO particles are oppositely charged and hence greater electrostatic is expected. Outside this pH range, electrostatic interactions are not favored, resulting in low enzyme attachment and poor sensor stability. The low applied potential obtained with this system could be effective in decreasing the effect of interfering substances that could be involved in redox reactions at higher potentials. Other reports in literature indicated that ZnO nanoparticles and a poly N-acetylaniline (pnAn) polymer deposited on Pt electrodes were used for AChE immobilization [31]. This system was used for detection of Ch and ACh/Ch and the detection limit for Ch was of 5.0×10^{-7} M [31], which supports our findings that ZnO could be a suitable alternative matrix for AChE immobilization.

3.2. Optimization of the ZnO–AChE biosensor. Amperometric measurements

To optimize the ZnO–AChE biosensor, the effect of water content, pH and substrate were studied by constant potential amperometry at 235 mV vs the Ag/AgCl reference electrode. To characterize the biosensor, the calibration curve, stability, and reproducibility were determined for each of the tested variables. Because the amount of water present in a sol–gel system dramatically affects its properties, this parameter was first to be investigated in the composition of the ZnO–PEG sol. Generally, the larger the amount of water is added to a sol stock solution, the larger the size of the pores of the sol–gel matrix. The pore dimensions affect significantly the sensitivity, stability and response time of the biosensor as a result of a decrease in the electrode film thickness and a better access of the analyte to the immobilized enzyme. However, larger pore sizes could also be responsible for enzyme leaching and thus pore size needs to be strictly controlled and enzyme stability needs to be evaluated.

Several AChE biosensors were prepared with ZnO–PEG sols containing various amounts of water, in rations ranging from 1:1 to 1:8 ZnO:water (v/v). The biosensor response to successive additions of ATCh substrate and the operational stability were tested. The cali-

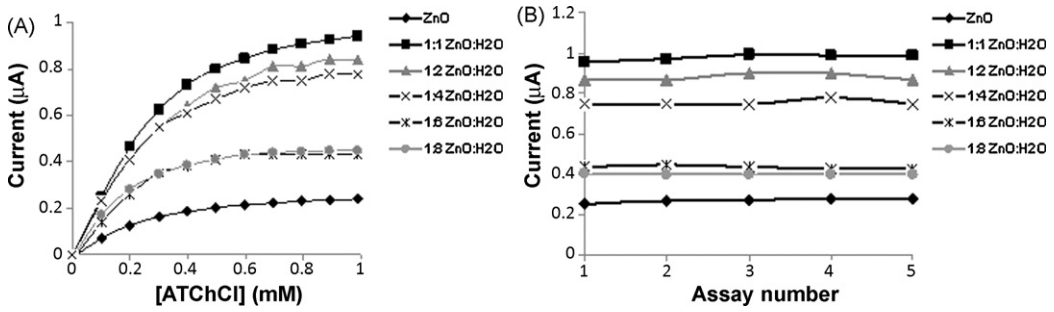

Fig. 3. Calibration curves (A) and sensor stability (B) of ZnO–AChE biosensors prepared with various ratios of ZnO:DI water.

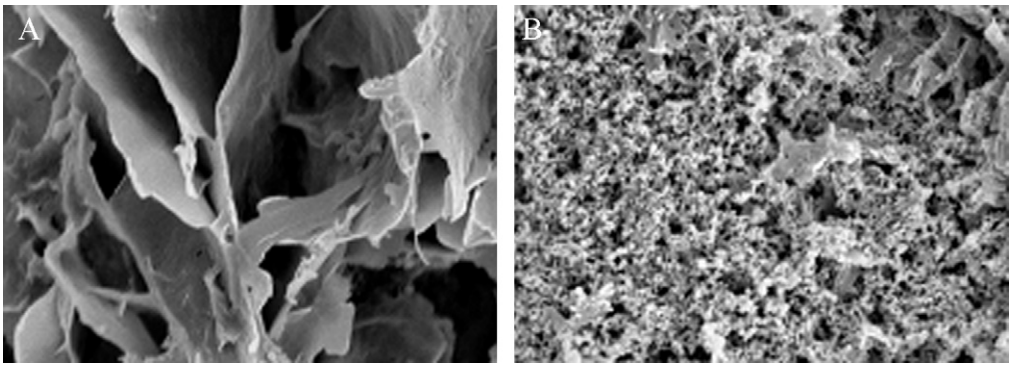

Fig. 4. SEM micrographs of the surface of SPE electrodes with deposited (A) undiluted ZnO–PEG/AChE (B) ZnO–PEG/AChE with the ZnO sol diluted in a 1:8 ratio with DI water before enzyme immobilization.

bration curve and the operational stability are presented in Fig. 3. The electrode prepared with ZnO without water added displayed the lowest current response. In turn, the electrode prepared with a 1:1 ratio of ZnO:water showed the highest amperometric response and the highest sensitivity for ATCh substrate. The response continued to decrease with increasing the amount of water (Fig. 3A). This can be attributed to an increase in the porosity of the matrix, which may favor low enzyme loading, or by less ZnO material available for the coverage of the electrode to provide appropriate catalytic activity.

Fig. 4 shows scanning electron microscope (SEM) images of electrodes with 1:0 and 1:8 ZnO–PEG:water ratios. This continuity in the form of flakes, even though at a small level, seems to help holding the enzyme and prevent leaching. In the 8 times diluted sample some small flakes were seen. We also noticed concentration of the ZnO in several areas of the electrode, while other remained uncovered. This uneven coverage of the electrode is probably the main cause of the lower response obtained with a higher water amount.

To evaluate the operational stability of the electrode, the response of the biosensor to additions of 1 mM substrate, with rinsing the cell between measurements, was repetitively measured. All electrodes showed good operational stability (Fig. 3B), with no obvious enzyme leaching. This can be due to the strong electrostatic binding between the enzyme and the ZnO matrix at the test pH value (7.4). All biosensors were stable for at least up to 5 assays, with a response variation of less than 5%. The response time of the biosensors was around 2 min, which is close to that reported with the AChE immobilized in a conventional silica material [21]. When the amount of enzyme was decreased without diluting the ZnO sol, the response time was increased by 1 min. Storage stability experiments were performed with 1:1 ZnO:water electrodes. The electrodes showed retention of nearly entire enzymatic activity after storage of up to 3 months. For the storage experiments, the electrodes were dipped in PBS and stored at 4 °C in a covered container to prevent PBS evaporation and contamination. These storage conditions are mild and easy to achieve compared to what had previously been required to retain enzyme activity i.e. storage under vacuum [21] and −20°C temperature [7,21]. The extended lifetime of the biosensors could be explained by the protective effect of the ZnO matrix on the enzyme activity as well as the mild immobilization process and the biocompatibility of the ZnO layer.

To determine the optimum operational pH for AChE immobilization, the pH was varied between 6.4 and 9.5 and the response of the biosensor was measured electrochemically. The highest current response was obtained at a pH of 7, which is in good agreement with the optimum pH value for the free AChE, while the lowest current response was obtained at a pH of 9.5 (Fig. 5). At this pH value, there is very little or no enzyme immobilized in the ZnO matrix since the matrix becomes either neutral or slightly negatively charged and therefore interacts unfavorably with the negatively charged residues of the AChE.

To test the reproducibility of the ZnO–AChE biosensors, electrodes prepared in identical conditions, containing the same amount of enzyme (5 mU AChE/electrode) and stored in PBS at 4 °C were tested. The average standard deviation of $n = 6$ electrodes to injections of 0.1 mM substrate was 1.43 μA (±0.16). It is worth mentioning that these electrodes require low enzyme loading; the amount of enzyme deposited within the ZnO matrix per electrode (5 mU AChE/electrode) is among the lowest reported in literature. The lowest amount of AChE immobilized in a sol–gel matrix was 1 mU per electrode with the enzyme immobilized in a silica sol–gel SPE electrode [29].

3.3. Paraoxon detection

Inhibition measurements and paraoxon detection experiments were performed at a substrate saturation concentration of 1 mM ATChCl. The irreversible phopshorylation of the AChE active site when biosensors are incubated with pesticides leads to a decrease in the electrode response. The detection of the paraoxon is quantified by measuring the AChE inhibition response, as a percent decrease of the current before and after inhibition, which is directly proportional to the concentration of paraoxon in the tested solution. To determine paraoxon, the initial electrode response in the absence of the inhibitor was determined. The electrodes were then immersed in an aqueous solution containing paraoxon at a given concentration and incubated for a fixed amount of time (between 10 and 30 min), and the response was measured again. The average

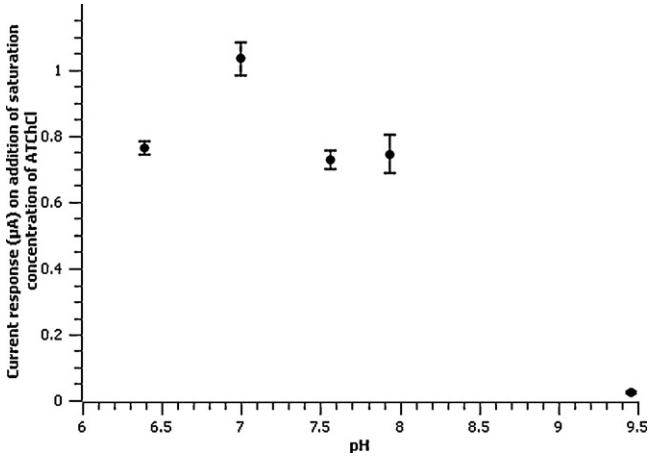

Fig. 5. Variation of electrode response with pH of ZnO sol.

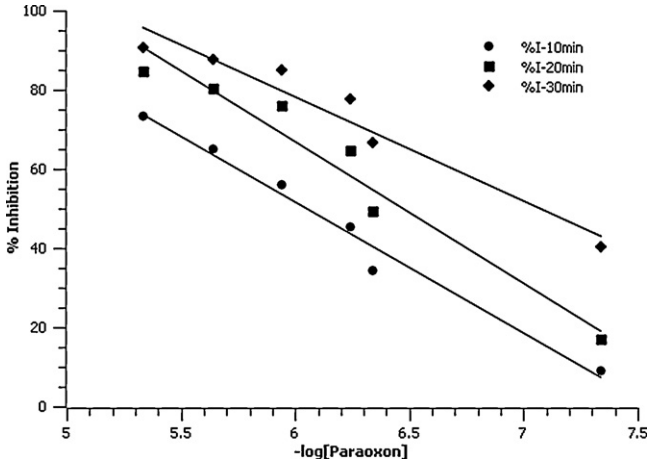

Fig. 6. Inhibition curves of ZnO–AChE biosensor by paraoxon after 10, 20 and 30 min incubation (logarithmic scale). Measurement conditions: 0.01 M PBS, applied potential: 235 mV vs Ag/AgCl reference electrode, 1 mM ATChCl substrate.

percentage of AChE inhibition was calculated and plotted against paraoxon concentration, at different incubation times (Fig. 6). As expected, the longer the incubation time, the higher the enzyme inhibition. The inhibition for 10 min incubation shows a good linear correlation with the log of concentration of paraoxon, as can be seen from the R^2 value of the linear fit for this data. Fig. 6 is linear for high concentrations of paraoxon and low incubation times. At lower concentrations, the detection resolution becomes poor. To overcome this problem, higher incubation times were tested. At higher incubation times, the sensitivity improves for low concentrations but the graph's shape changes from linear behavior to saturation plateau at higher concentrations. We therefore selected 10 min as an optimum incubation time to get linear behavior over a wide concentration range. This fitting line can be used to predict the paraoxon concentration for intermediate inhibition levels. A detection limit of 0.035 ppm of paraoxon was determined, corresponding to 20% inhibition (also known as I_{20}). The biosensor demonstrated to detect paraoxon up to 1.38 ppm. Following the procedure detailed here, the biosensor could be used to detect other organophosphates, for example methyl parathion. Since paraoxon is a stronger cholinesterase inhibitor than parathion, its detection limit is lower. A lower detection limit is possible by reducing the amount of enzyme on the electrodes, but at the cost of a higher background, which reduces the sensitivity and the linear range of the biosensor.

4. Conclusions

This paper reports the use of a zinc oxide sol–gel as a non-silica matrix for immobilization of AChE onto the surface of disposable SPE electrodes. The immobilization process performed at a mild pH value close to neutral and the biocompatibility of the ZnO matrix led to good electrode performance in terms of stability, reproducibility, enzyme amount and current response. The ZnO matrix offered the possibility to operate the biosensor at a lower overpotential (235 mV) than that reported with other electrode materials, in the absence of electrochemical mediators, and provided an effective method for stable attachment of the AChE enzyme via strong electrostatic interactions. The amount of enzyme loading onto the electrode is low, in the order of 5–10 mU per electrode. The electrodes showed excellent operational stability, good reproducibility and retained enzymatic activity for up to three months. The detection limit for paraoxon was found to be in the sub-ppm range. In summary, we demonstrated that zinc oxide has the potential to serve as an alternative enzyme immobilization material to silica for biosensor design. An added advantage of this immobilization method was the simple biosensor preparation method. Once prepared, the ZnO sol could be stored for up to three months without noticeable variation and using this sol, biosensor preparation is reduced to mixing with enzyme, depositing on electrode and drying.

Acknowledgements

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