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Alumina sol–gel/sonogel-carbon electrode based on acetylcholinesterase for detection of organophosphorus pesticides

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ABSTRACT

Two new amperometric biosensors based on immobilization of acetylcholinesterase on a sonogel-carbon electrode for detection of organophosphorous compounds are proposed. The electrodes were prepared applying high-energy ultrasounds directly to the precursors. The first biosensor was obtained by simple entrapping acetylcholinesterase in Al_2O_3 sol–gel matrix on the sonogel-carbon. The second biosensor was produced in a sandwich configuration. Its preparation involved adsorption of the enzyme and modification via a polymeric membrane such as polyethylene glycol and the ion-exchanger Nafion. The optimal enzyme loading was found to be 0.7 mIU. Both biosensors showed optimal activity in 0.2 M phosphate buffer, pH 7.0, at an operating potential of 210 mV. The detection limit achieved for chlorpyriphos-ethyl-oxon was 2.5×10^{-10} M at a 10-min incubation time.

1. Introduction

Organophosphorous pesticides (OPs) have been widely used for decades in agriculture, medicine, industry and even as chemical warfare agents in military practice. Meanwhile, their presence in the environment is very harmful for human health. The methodology optimization study for the detection of OPs is thus quite crucial for analytical researchers especially when the versatility and the cost of the analytical system are taken into account [1–6].

Electrochemistry affords high sensitivity, simple sample treatment, inexpensive instruments, easily operation procedure and it is an ideal analytical technique for in situ analysis. Amperometric biosensors based on the inhibition of acetylcholinesterase enzyme (AChE) using screen printed electrodes (SPE) have shown satisfactory results for real sample analysis [7], in which the acetylcholinesterase activity is employed as an indicator of quantitative measurement of OPs. The relevant reactions are described as follows:

$$\text{acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} \quad (1)$$

$$\text{thiocholine} \xrightarrow{\text{anodic oxidation}} \text{dithio-bischoline} + 2H^+ + 2e^- \quad (2)$$

The amperometric response of AChE biosensors, i.e. the anodic oxidation current resulting from the thiocholine (TCH) formed by enzymatic hydrolysis of acetylthiocholine (ATCH), is inversely proportional to the concentration of OPs in the sample.

The employment of sol–gel technology to produce ceramics based bio-sensitive materials [8–13]. This interest is due to inherent advantages such as its relative chemical inertness, simplicity of preparation, negligible swelling in solution, low-temperature encapsulation and high sensitivity; these properties contribute to the effective preservation of the chemical and biological activities of the entrapped molecules. Among these electrodes, the sonogel-carbon electrodes, developed by some of us [14,15], show very favourable electroanalytical properties that facilitate the easy incorporation of numerous receptor molecules, which can notably improve the selectivity and sensitivity of the electrode compared as compared with classical electrodes [16–22].

Methods used for the immobilization of acetylcholinesterase are integral to the success of the sensor. Studies exploring different techniques are of great interest. Several ways of immobilization are being developed at the present. Among these are the immobilization on 3-mercaptopropionic acid SAM gold electrode [23], or on gold nanoparticles embedded in sol–gel film [24]. Immobilization on screen printed electrodes was previously reported [25].

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Nevertheless, as far as we know, the immobilization on alumina sol–gel has not been explored. In the present paper, we propose alumina sol–gel for immobilization of AChE; furthermore we have employed a basis not used either with this aim and developed by some of us, the sonogel-carbon electrode. This constitutes the novelty of the paper, and it must be added to the good detection limits attained in the detection of organophosphorous pesticides. The objective proposed for the present studies was to seek new electrochemical biosensors for pesticide determination. The experimental parameters affecting the response of all resulting biosensors are discussed.

2. Materials and methods

2.1. Apparatus and reagents

Methyltrimethoxysilane was purchased from Merck, HCl from Panreac (Barcelona, Spain) and graphite powder (spectroscopic grade RBW) was from SGL Carbon (Ringsdorff, Germany). Acetylcholinesterase was purchased from Sigma (St. Louis, MO, USA); KH_2PO_4 and K_2HPO_4 for phosphate buffer were from Fluka (Buchs, Switzerland). High purity water was obtained by passing twice distilled water through a Mill-Q system (18 mΩ cm^{-1}, Millipore, Bedford, MA). All pesticide compounds tested in this work of analytical grade, and purchased from Sigma. Glass capillary tubes, i.d. 1.15 mm, were used as the bodies for the composite electrodes. Al_2O_3 sol–gel was prepared as described in the literature [26]. Chronoamperometric measurements were performed with an Autolab PGSTAT20 Potentiostat/Galvanostat (Ecochemie, Utrecht, The Netherlands) interfaced with a personal computer using the Autolab software GPES for wave form generation and data acquisition and elaboration. A 600-W model, 20 KHz ultrasonic processor (Misonix Inc., Farmingdale, NY) equipped with a 13-mm titanium tip was used. The ultrasonic processor was inside a closed sound proof chamber during operation.

Electrochemical impedance spectroscopy (EIS) measurements were performed with a Voltalab 10 type PGZ 100 from Radiometer. Scannig electron microscopy (SEM) studies were performed with a JEOL microscope, JSM 5400 type.

All electrochemical experiments were carried out in a cell containing 10 ml of an aerated 0.2 M phosphate buffer solution at pH 7.0 and 22($\pm$1)°C; the three electrodes system consisted of an AChE/Al_2O_3/modified sonogel-carbon, an Ag/AgCl (3 M KCl) and a platinum wire electrodes as working, reference and auxiliary electrodes, respectively. A magnetic stirrer and stirring bar were used to provide continuous convective transport during the measurements.

2.2. Biosensor fabrication

The unmodified sonogel-carbon electrode was prepared as described previously [14,15]. Before modification, the electrodes were polished with emery paper No. 1200 to remove extra composite material, wiped gently with weighing paper, thoroughly washed with deionized water and allowed to dry at room temperature.

The first type of biosensor was obtained by depositing 50 μl of AChE (0.7 mIU in Al_2O_3 sol), onto the sonogel-carbon electrode. The enzymatic membrane was subsequently gelatinized completely in the refrigerator (4 °C) for 24 h. All the prepared AChE/Al_2O_3/Sonogel-scarbon biosensors were stored in buffer solution (pH 7.0, 0.2 M) in the refrigerator (4 °C) before use.

The second, sandwich configured biosensor was prepared as follows: tyrosinase powder was dissolved in phosphate buffer of pH 7.0; in the first step, 50 μl of this solution (0.7 mIU) were placed onto the surface of an unmodified sonogel-carbon electrode and allowed to adsorb and dry at room temperature. In the second step, 1.5 μl of Nafion solution (0.5% in a mixture of lower-aliphatic alcohols and water) or PEG solution (5% in a mixture of water) were spread on the enzyme film. The resulting biosensors were stored for a minimum of 8 h to dry in a refrigerator at 4 °C. All biosensors were washed carefully with buffer solution before and after each manipulation, and were stored by immersing in a phosphate buffer solution of pH 7.0 at 4 °C when not in use.

3. Results and discussion

3.1. Cyclic voltammetric studies

The electrochemical behaviour of the proposed AChE/Al_2O_3/sonogel-carbon biosensor versus acetylthiocholine was examined. Fig. 1 shows the cyclic voltammogram of the sensor in phosphate buffer (pH 7.0) at a scan rate of 100 mV s^{-1}. No response was observed in absence of acetylthiocholine, whereas in presence of the substrate an anodic wave was clearly observed; this wave corresponds to the oxidation of the thiocholine released from acetylthiocholine via enzymatic hydrolysis.

3.2. Enzyme loading and pH studies

The influence of enzyme loading in the Al_2O_3 sol–gel matrix was studied and the responses of the biosensors in presence of a 1-mM substrate concentration are shown in Fig. 2. As can be seen, the response of the biosensor increases with the increasing amount of the enzyme, first rapidly and then slowly; in all cases the stability of the response was quite good. For a loading of 0.7 mIU of the enzyme, the current intensity was almost as good as for 1.5 mIU. Thus, AChE loading was set at 0.7 mIU for subsequent studies to conserve the enzyme while retaining comparable sensitivity.

The pH dependence of the enzyme electrode over the range 6.0–9.6 in 0.2 M phosphate buffer in the presence of 100 mM substrate is illustrated in Fig. 3. The resulting profile indicated maximum sensitivity of the enzyme electrode at pH 7.0, corresponding to the optimum pH for the reaction between the enzyme and the substrate. For AChE sensors, the matrix pH must be kept below 9 to allow for the requisite chemical hydrolysis to occur [27]. Thus, all experiments, including inhibition studies, were performed at pH 7.0.

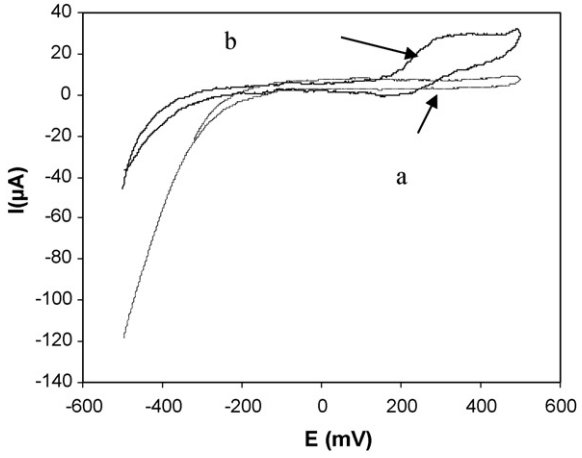

Fig. 1. Cyclic voltammograms of AChE/Al_2O_3/sonogel-carbon electrode in 0.2 M phosphate buffer, pH 7.0, (a) without acetylthiocholine and (b) with 1 mM acetylthiocholine.

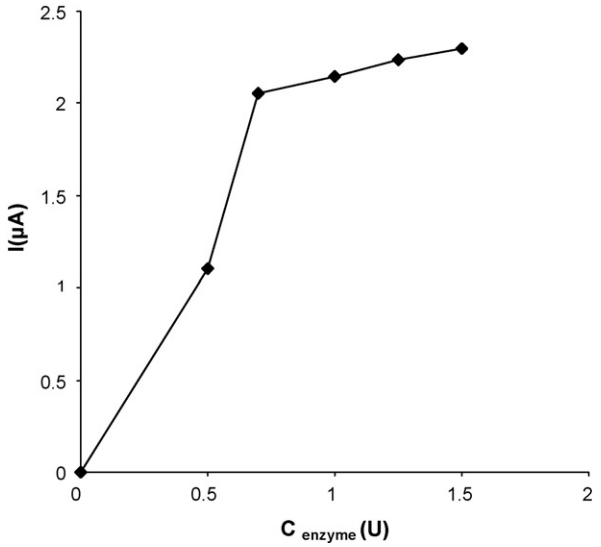

Fig. 2. Effect of enzyme loading into sol–gel matrix on the response of biosensor system, 0.2 M phosphate buffer, pH 7.0, applied potential 210 mV.

3.3. Reproducibility and stability of the enzyme electrodes

At a concentration of 1 mM acetylthiocholine, the Al_2O_3/sol–gel biosensors showed a signal of 2.02 μA and a quite good reproducibility. Electrodes prepared in the same batch demonstrated a standard deviation of 1.4% ($n = 11$). When the enzyme electrode was stored in the refrigerator at 4 °C between measurements, it retained 98% of initial current response after 20 assays. If the current response was measured once a day, after the intermittent use over a 50-day period, it retained 50% of its initial current response. In the same 50-day period we can use these sensors for more than 100 assays.

These results demonstrate that sol–gel film is very efficient for retaining the activity of acetylcholinesterase with a good long-term stability. This is attributed to the fact that the method provides a mild immobilization process. Specifically, the method does not involve any additive that results in chemical modification and fouling of the enzyme molecules. This allows the enzyme to maintain its activity to a large extent. Also, the sol–gel contains numerous hydroxyl groups which can form strong hydrogen bonds with acetylcholinesterase and sol–gel cages for enzyme loading.

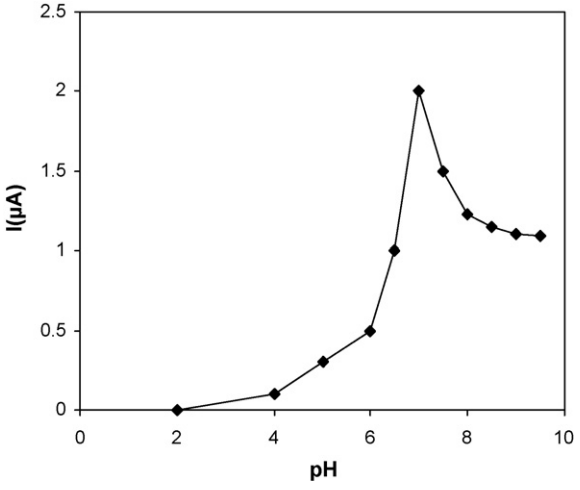

Fig. 3. Effect of pH on the enzyme response for a substrate concentration of 1 mM, 0.2 M phosphate buffer, pH 7.0, applied potential 210 mV.

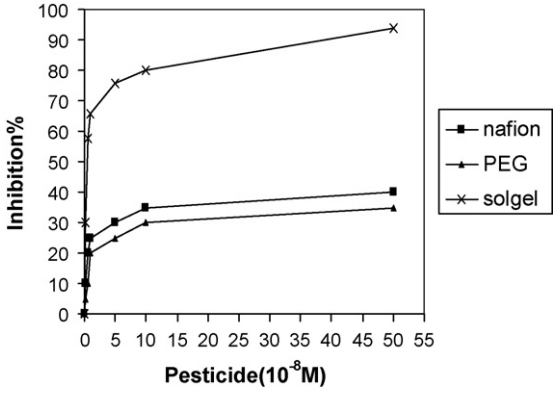

Fig. 4. Inhibition curves of AChE by Chlorpyrifos-ethyl-oxon after 10 min incubation. Measurement conditions: 0.2 M phosphate buffer, pH 7.0, applied potential: 210 mV, 1 mM acetythiocholine substrate concentration.

3.4. Detection of pesticides

It is well known that numerous pesticides can be determined using the signal inhibition that they cause in several (bio)sensors. AChE sensors have been used to carry out inhibition studies with three pesticides: Paraoxon, dichlorvos, and chlorpyriphos-ethyl-oxon. In order to obtain a lower-detection limit, an incubation time of 10 min was selected for inhibition measurements. The detection limit was calculated as the concentration of pesticide resulting in 20% inhibition [27]. Inhibition curves for chlorpyriphos-ethyl-oxon with immobilized AChE by two methods are presented in Fig. 4. The greatest inhibition was obtained with alumina sol–gel and may be attributed to the microenvironment surrounding the enzyme, because in the sol–gel. This may be due to the microenvironment encaging the enzyme. The sol–gel pores or cages are easily accessed by the substrate pesticide in the buffered electrolyte solution, whereas the PEG and Nafion membranes operate as diffusion barriers which impose kinetic limitation and restrict access to the active sites of the enzyme, thus affecting the detection limit and the sensitivity of these sensors.

Our results, presented in Table 1, are comparable or better than AChE screen-printed electrodes described in the literature [28–32]. In all these previous cases, the enzyme was immobilized by glutaraldehyde, PVA, silica sol–gel or metal-chelate affinity and a considerable enzyme amount was loaded on each screen. Fig. 5 shows the inhibition of the three pesticides with AChE immobilized in the same matrix (alumina sol–gel) employing an incubation time of 10 min. The higher inhibition is obtained for chlorpyriphos-ethyl-oxon.

3.5. EIS measurements

In order to confirm the presence of enzyme onto the surface of sonogel, EIS measurements were performed. The impedance spectra were collected at 210 mV (vs. SCE) in a frequency range

Table 1
Comparison between the three pesticides, 0.2 M phosphate buffer, pH 7.0, applied potential: 210 mV, 1 mM acetylthiocholine

Pesticide	Limit of detection
Paraoxon (M)	7.5×10^{-9}
Dichlorvos (M)	5.0×10^{-10}
Chlorpyrifos-ethyl-oxon (M)	2.5×10^{-10}

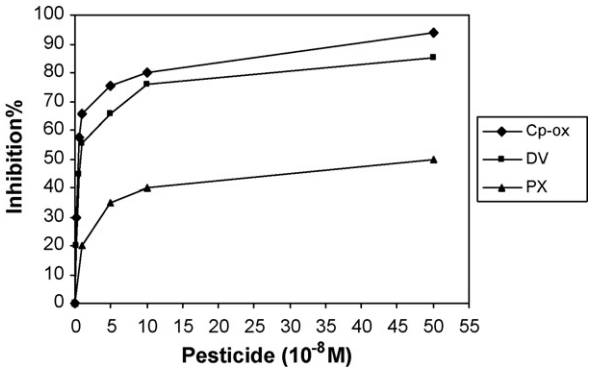

Fig. 5. Inhibition curve of AChE immobilized in Al_2O_3 sol–gel matrix by paraoxon (Px), dichlorvos (DV) and chlorpyriphos-ethyl-oxon (Cp-ox), 10 min incubation. Measurement conditions: 0.2 M phosphate buffer, pH 7.0, applied potential: 210 mV, 1 mM acetylthiocholine substrate concentration.

from 100 kHz to 10 MHz with AC amplitude of 5 mV. Fig. 6A shows the Nyquist plot for the AChE-free modified sonogel-carbon electrode. If we use the Randles equivalent circuit model, the calculated (Voltamaster 4.0 software) charge transfer resistance R_{ct} and the double layer capacitance C_d are successively: 50 kΩ cm^2 and 6 μF cm^{-2}, respectively.

On the other hand, Fig. 6B also exhibits an arc-like Nyquist plot for the AChE/Al_2O_3/sonogel-carbon electrode system. The electrical parameters calculated were: R_{ct} = 38 kΩ cm^2 and C_d = 41.8 μF cm^{-2}. It appears clearly from these data that the capacitance at the interface increases when the AChE onto the sonogel-carbon electrode. The observed decrease of the charge transfer resistance means also that AChE/Al_2O_3/sonogel-carbon system favours the electron transfer reaction at the electrode surface.

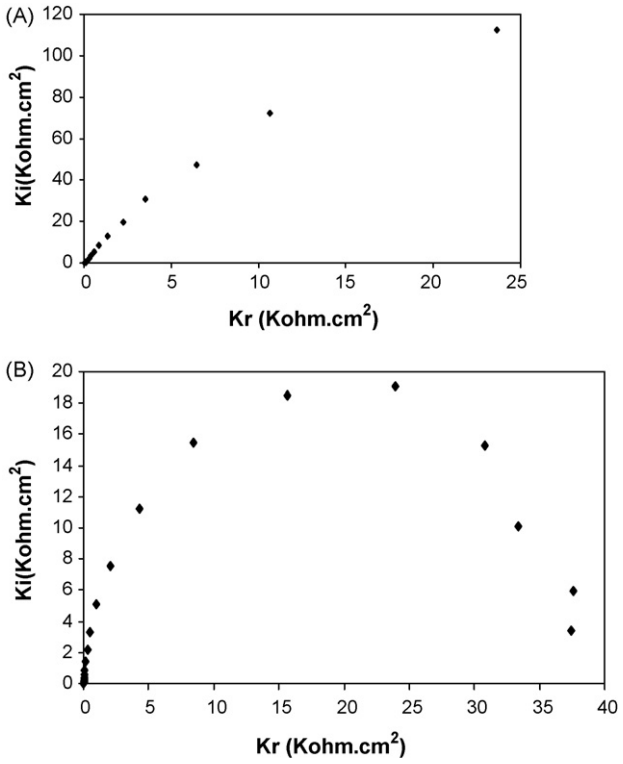

Fig. 6. Nyquist plot for (A) Al_2O_3/sonogel-carbon electrode and (B) AChE/Al_2O_3/sonogel-carbon electrode.

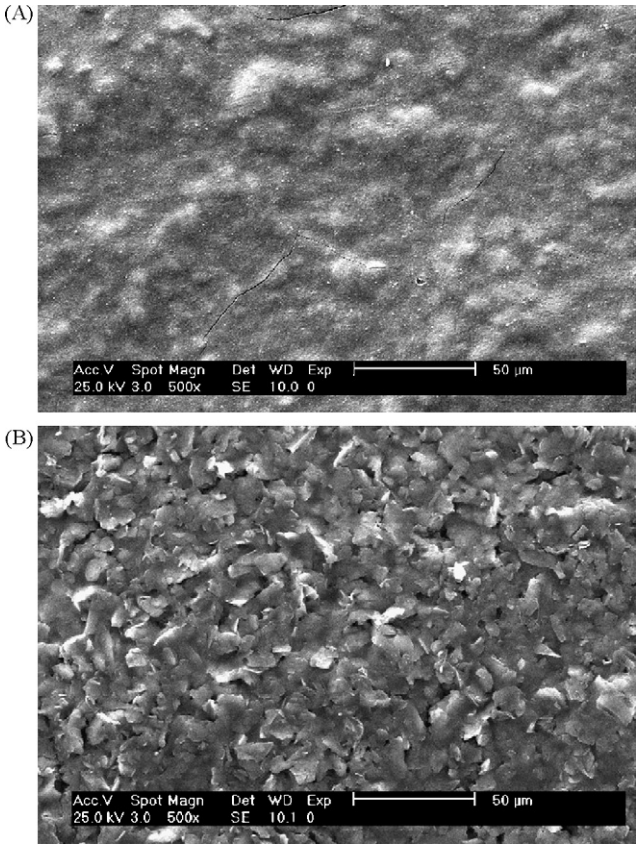

Fig. 7. Scanning electron micrograph of (A) Al_2O_3 sol–gel/sonogel-carbon electrode and (B) AChE/Al_2O_3 sol–gel/sonogel-carbon electrode, obtained at 25.0 kV with a 500× zoom.

3.6. SEM measurements

The results of morphological studies conducted on Al_2O_3/sonogel-carbon and AChE/Al_2O_3/sonogel-carbon are shown in Fig. 7A and B. The SEM of Al_2O_3/sonogel-carbon indicates that the film is uniform. The SEM of AChE/Al_2O_3/sonogel-carbon shows granular (porous) structure which confirms the presence of enzyme.

4. Conclusion

A new procedure to successfully immobilize acetylcholinesterase on a sonogel-carbon electrode is proposed. The enzyme is easily immobilized on the electrode surface employing Al_2O_3 and a simple sol–gel technology which requires minimal preparation time. The enzyme is well immobilized in sol–gel matrix, retains satisfactory enzymatic catalytic activities and the carbon provides the electric conductivity. The present study demonstrates that this simple encapsulation procedure for the development of electrochemical biosensors is highly sensitive to the pesticide studied. The developed biosensor showed quite stable and good responses towards substrate and inhibitors and could be applied to monitoring of other organophosphorous compounds.

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