A nanotechnological approach to biosensors sensitivity improvement: application to organophosphorus pesticides determination

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ABSTRACT
An electrochemical organophosphorus hydrolase-based sensor for direct organophosphorus pesticides determination was developed, analytically characterized and applied for paraoxon quantification. The biosensing platform was constructed by one-step electrodeposition onto the surface of a glassy carbon electrode of a bionanocomposite of chitosan with carbon nanotube (CNTs), hydroxyapatite (HA) and organophosphorus hydrolase. The sensor, optimized with respect to the CNTs and HA load, was characterized by the application of cyclic voltammetry and chronoamperometry. The limit of detection (LOD) was found to be as low as 0.1 \( \mu \text{mol L}^{-1} \), the linear concentration range was extended up to 80 \( \mu \text{mol L}^{-1} \), and the sensitivity was as high as 5.10 nA L \( \mu \text{mol}^{-1} \). The sensor possesses enhanced sensitivity towards organophosphorus pesticides (OPs), because of the CNTs and the HA nanoparticles synergistic action.

Introduction
The organophosphorus pesticides (OPs) are synthetic biocides: esters, amides and thiol derivatives of the phosphoric and phosphonic acids \([1–4]\). They are extensively used in the modern agricultural practice, because of their great effectiveness, large target spectrum and low persistence \([5]\). The associated risks are due to their high acute toxicity and quick breakdown in the environment \([4–7]\). These risks call for the development of sensitive and field-deployable screening methods of low cost for their rapid determination, as an alternative to the time-consuming and expensive chromatographic laboratory techniques, currently in use \([8–10]\). As reported in the literature, the electrochemical methods are well suited for such analytical purposes \([8,11–13]\).

The electrochemical methods of choice for organophosphorus pesticides determination are the biosensors based ones \([8,12,14,15]\). They take advantage of the cholinesterases activity inhibition by OPs or of the ability of the organophosphorus hydrolase (OPH) to catalyse their hydrolysis. Typically, the analytical signal is the current of oxidation of the electroactive products generated by the enzyme reaction. The cholinesterases-based sensors are very sensitive and allow reaching low limits of detection, but the determination is indirect and the sensor response is irreversible. In contrast, the OPH-based electrochemical sensors permit the direct OPs quantification, as the OPs act as enzyme substrates, but the sensitivity of the analysis is lower \([16,17]\). Therefore, current studies focus the increase of the sensitivity of the OPH-based electrochemical sensors for OPs determination. Promising results were obtained by using carbon nanotubes (CNTs) and/or nanoparticles modified electrodes, because of the large surface area and electrocatalytic properties of the nanomaterials \([13,14]\). Carbon nanotubes are suitable for nitrophenyl-substituted OPs determination, thanks to the \( \pi \)-conjugated interactions upon the benzene ring \([18,19]\). Furthermore, CNTs electrode modification favours the enzyme load increase, due to their hollow structure \([20]\). The carboxylic acid functionalized multi-walled carbon nanotubes (cMWCNT) in particular, in addition to their mechanical stability and good electric conductivity, display a large number of binding sites available for enzyme immobilization \([21]\). On the other hand, hydroxyapatite (HA, \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \)) has been reported as an excellent matrix for enzyme immobilization, because of its bio-compatibility and sorption capacity, based on electrostatic and functional groups interactions, and hydrogen bonds formation between the protein molecules and...
the Ca(II) and the phosphates on the HA surface [22,23]. It has also been demonstrated, that HA enhances the electrochemical oxidation of p-nitrophenol (PNP), which is the product of the OPH catalysed hydrolysis of the nitrophenyl-substituted OPs [24].

An appropriate medium for the cMWCNT and HA homogeneous dispersion is chitosan (CS). The nanotubes solubilization is favoured by the ability of chitosan to form covalent bonds with their carboxylated ends by the intermediary of its free amino-groups [25,26], while HA and chitosan interact by forming hydrogen- and coordination bonds [27,28]. Moreover, chitosan nanocomposites could be easily electrodeposited onto the surface of the electrochemical transducers, forming well-adhering and uniform films with controllable and reproducible characteristics [29,30]. Chitosan is also suitable for enzyme immobilization, because of its biocompatibility, porosity, hydrophilicity, gel-forming properties and functional groups availability [31]. So, chitosan and chitosan bionanocomposites are widely used as electrochemical biosensing platforms [29].

The objective of this work is the development of an electrochemical OPH-based sensor with enhanced sensitivity for the direct OPs determination, taking advantage of the synergistic action of the CNTs and the HA nanoparticles. Chitosan was used for nanomaterials dispersion and OPH entrapment. The biosensing platform was constructed by one-step electrodeposition of the chitosan-based bionanocomposite (CS-cMWCNT-HA/OPH) onto the surface of a glassy carbon electrode (GCE). The bionanocomposite performance was optimized with respect to the CNTs and HA load. PNP was used for the electrochemical characterization of the modified electrode by cyclic voltammetry and chronoamperometric measurements. The biosensor was tested for paraoxon determination.

Materials and methods

Reagents and instrumentation

The reagents used in this work: paraoxon-ethyl, p-nitrophenol, atrazine, chlorofos, and chitosan (medium molecular weight) were of analytical reagent grade and were purchased from Sigma (Mexico D.F., Mexico). Stock solutions of paraoxon (10 mmol L\(^{-1}\)), PNP (50 mmol L\(^{-1}\)), and chlorofos (10 mmol L\(^{-1}\)) were prepared in deionized water, whereas the stock solution of atrazine (10 mmol L\(^{-1}\)) was prepared in methanol. Chitosan was dissolved in acetic acid 0.1 mol L\(^{-1}\) to obtain a 0.2% w/v solution. All the measurements were carried out in a phosphate buffer solution (PBS) 0.1 mol L\(^{-1}\), pH 8.5, prepared by mixing appropriate quantities of K\(_2\)HPO\(_4\) and KH\(_2\)PO\(_4\). The selected pH value corresponded to the pH optimum of the immobilized enzyme.

Organophosphorus hydrolase (EC 3.1.8.1) was extracted from the stabilized enzymatic preparation DEFENZ\textsuperscript{TM} 130BG (Genencor International, Inc., Palo Alto, USA), as previously reported [32]. Briefly, the granulated product was used for the preparation of a 10% w/v aqueous dispersion, which was centrifuged at 3000 r/min (Hettige Universal 320 centrifuge) for 10 min. The supernatant served for the further analysis. The enzyme activity was evaluated by spectrophotometric assays, using a PC controlled Evolution 60S UV-VIS spectrophotometer. The enzyme activity unit (U) was defined as the increase in absorbance of 0.001 per minute at 400 nm, pH 8.5 and 25 °C in 1 mL reaction mix containing paraoxon.

Intelligent Materials Pvt. Ltd. (Dera Bassi, Punjab, India) provided COOH-functionalized multi walled carbon nanotubes with a diameter of 20–30 nm and length of 10–30 µm. Hydroxyapatite nanopowder with <200 nm particle size was acquired from Sigma. The nanomaterials were dispersed in the chitosan 0.2% w/v solution by ultrasonic agitation for 10 min with an UP-800 ultrasonic processor (E-Chrom Tech Co., Ltd., Taipei, Taiwan) to obtain CS-cMWCNT, CS-HA and CS-cMWCNT-HA homogeneous dispersions.

A three-electrode electrolysis cell of a conventional type and a CH Instruments model 440A electrochemical analyzer (CH Instruments Inc., Bee Cave, TX, USA) were used to perform the electrochemical measurements. The three-electrode system included a working glassy carbon electrode (GCE, Tokay GC 20, 3 mm diameter, bare or modified), Pt wire as an auxiliary electrode and Ag, AgCl/KCl\(_{\text{sat}}\) as a reference.

The morphological analysis of the surface of the modified electrodes was done by means of a JEOL scanning electron microscope JSM-6010PLUS/LV.
OH⁻ production, according to the following reaction:

\[ 2\text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2 + 2\text{OH}^- \]

A pH increase above 6.5 causes chitosan gelation through sol–gel transition [33]. An insoluble hydrogel network is formed:

\[ \text{CS} - \text{NH}_3^+ + \text{OH}^- \rightarrow \text{CS} - \text{NH}_2 + \text{H}_2\text{O} \]

The one-step fabrication of the bionanocomposite was possible because of the fact that chitosan retains its pH-responsive properties when mixed with proteins and nanomaterials [30], which allows their co-electrodeposition. Figure 1 illustrates the bionanocomposite-modified GCE fabrication.

The bionanocomposite-modified electrode was washed with PBS. Then chitosan was cross-linked by exposure to glutaraldehyde vapour (25% aqueous solution) for 15 min. Finally, the bionanocomposite film was washed again with PBS for 30 min. The as obtained biosensor was stored at 4°C.

The same technique was applied for the preparation of electrodes, modified with a chitosan blend and bovine serum albumin (BSA, 1 mg mL⁻¹), instead of OPH. These sensors were used for the non-enzymatic measurements.

**Results and discussion**

**Bionanocomposite performance optimization**

The bionanocomposite electrodeposition was carried out, as mentioned above, by application of a constant potential (−3 V vs. Ag, AgCl/KCl_sat) for a fixed period of time (5 min). Nevertheless, the quantity of the cMWCNTs (0.1, 0.25, 0.5 and 1.0 mg mL⁻¹) and the HA nanopowder (1.0, 2.5, 5.0 and 10 mg mL⁻¹) included into the chitosan blend was varied. This variation affected the sensitivity of the paraoxon determination using the constructed biosensors, as shown in Figure 2.

The amperometric responses of the chitosan nanocomposites biosensors to paraoxon (concentration increment: 40 µmol L⁻¹) were recorded applying a potential of +1.0 V vs. Ag, AgCl/KCl_sat. In the following conditions: PBS 0.1 mol L⁻¹, pH 8.5; 25°C, and a various chitosan blend composition.

It was suggested that the augmentation of the nanomaterials load would contribute to the enhancement of the sensitivity of the paraoxon quantification, because of...

![Figure 1. Bionanocomposite-modified GCE fabrication.](image)

![Figure 2. (A) Amperometric responses of the chitosan nanocomposites biosensors to paraoxon with chitosan blend composition of: (a) 0.2% w/v chitosan, 0.5 mg mL⁻¹ cMWCNT and 1 mg mL⁻¹ HA; (b) 0.2% w/v chitosan, 0.5 mg mL⁻¹ cMWCNT and 5 mg mL⁻¹ HA. (B) Variation of the sensitivity of the paraoxon amperometric determination as a function of the chitosan blend composition.](image)
Electrochemical characterization of the nanocomposites modified electrodes

As PNP is generated upon the OPH-catalysed hydrolysis of OPs, it was used for the electrochemical characterization of the CS-cMWCNT-HA/BSA modified GCE in comparison with the CS/BSA, CS-HA/BSA and CS-cMWCNT/ BSA modified GCEs. The registered cyclic voltammograms (PNP 50 μmol L⁻¹, scan rate 0.1 V s⁻¹, PBS 0.1 mol L⁻¹, pH 8.5, 25 °C) are shown in Figure 3.

Figure 3(A) demonstrates that the electrochemical behaviour of PNP in the selected potential range (+0.3 V to +1.15 V vs. Ag, AgCl/KClsat) applying various modified electrodes was identical. Therefore, the recorded anodic peak was ascribed to the irreversible PNP oxidation.

Nevertheless, a slight peak potential shift, as compared to the peak potential of the PNP oxidation at the CS/BSA modified GCE (+1.04 V vs. Ag, AgCl/KClsat) was observed, when CS was blended with nanomaterials. Figure 3(A) clearly shows that the presence of HA nanoparticles favours the PNP oxidation at a lower potential (+1.03 V vs. Ag, AgCl/KClsat), whereas the CS-cMWCNTs nanocomposite GCE modification caused a small peak potential increase (+1.05 V vs. Ag, AgCl/KClsat).

Electrode modification using various chitosan blends resulted also in 1.09-, 1.26- and 1.32-fold increase of the peak current of PNP oxidation at CS-HA/BSA, CS-cMWCNT/BSA and CS-cMWCNT-HA/BSA modified GCEs, correspondingly, in comparison to the anodic peak current, recorded at CS/BSA GCE (Figure 3(B)). This increase was attributed to the augmentation of the electroactive surface area of the nanocomposites modified electrodes and the enhancement of their catalytic activity, as confirmed by data presented in Table 1. These data were obtained by analysing the chronoamperometric responses (potential step 400 mV) of the modified electrodes in the presence of PNP in 0.1 mol L⁻¹ PBS at pH 8.5 (see Figure 4 as an example). The determination of the electroactive surface area was achieved by using the Cottrell equation, which describes the Cottrell plots derived from the chronoamperograms [37]:

\[ I = nFAC(D/\pi t)^{1/2} \]

where \( I \) is the current (A), \( n \) is the electron transfer number, \( F \) is the Faraday constant (96 485 C mol⁻¹), \( A \) is the electrode surface area (cm²), \( C \) is the PNP concentration (mol cm⁻³), \( D \) is the PNP diffusion coefficient (9.16 × 10⁻⁶ cm² s⁻¹ [38]) and \( t \) is the time elapsed (s).

The catalytic rate constant values were calculated from the slope of the \( I_c/I_L \) vs. \( t^{1/2} \) plot, according to the Galus method [39]:

\[ I_c / I_L = (\pi k_C t)^{1/2} \]

where \( I_c \) is the catalytic current of PNP oxidation at the modified electrodes (A), \( I_L \) is the limiting current in the absence of PNP (A), \( k_C \) is the catalytic rate constant (L mol⁻¹ s⁻¹), \( C \) is the PNP concentration (mol L⁻¹) and \( t \) is the time elapsed (s).

Table 1. Average values of the electroactive surface areas of the modified GCEs and of the catalytic rate constants of the PNP oxidation at modified GCEs.

| Electrode                | Active area (mm²) | Catalytic rate constant (L mol⁻¹ s⁻¹) |
|--------------------------|-------------------|--------------------------------------|
| CS/BSA GCE               | 14.93             | 1.72 × 10⁻¹                           |
| CS-HA/BSA GCE           | 18.63             | 6.94 × 10⁻¹                           |
| CS-cMWCNT/BSA GCE       | 24.38             | 5.02 × 10⁻¹                           |
| CS-cMWCNT-HA/BSA GCE    | 22.66             | 7.75 × 10⁻¹                           |
The data presented in Table 1 demonstrate that the CS-cMWCNT-HA/BSA modified GCE exhibited higher catalytic activity and larger electroactive surface area in comparison to the CS-HA/BSA and CS-cMWCNT/BSA modified GCEs, which was ascribed to the synergistic action of the individual components.

Morphological characterization of the bionanocomposites modified electrodes

The effectiveness of the dispersion of the selected nanomaterials in chitosan was confirmed by scanning electron microscopy (SEM). Figure 5 illustrates that the distribution of the cMWCNTs and the HA nanopowder is homogeneous. The bionanocomposites films are uniform. The incorporation of nanomaterials results in surface smoothing and porosity decrease, as compared to the chitosan film. The excellent performance of the bionanocomposites was attributed to the properties of chitosan as a dispersing agent for cMWCNTs and HA, as well as to the appropriate deposition technique.

Analytical performances of the chitosan nanocomposite OPH-based sensor

The CS-cMWCNT-HA/OPH modified GCE was tested for paraoxon determination, taking advantage of the synergistic action of the cMWCNT and the HA nanopowder. The analysis was based on the following conjugated enzymatic and electrochemical reactions: (i) OPH-catalysed hydrolysis of paraoxon and (ii) amperometric detection of the enzymatically produced PNP. The amperometric measurements were performed at a constant potential of +1.1 V vs. Ag, AgCl/KCl sat to ensure a high oxidation rate. The amperometric response of the biosensor to increasing paraoxon concentrations and the constructed calibration plot are shown in Figure 6.

The sensitivity of the determination, i.e. the slope of the calibration plot within the linear concentration range, was found to be 5.10 nA L⁻¹mol⁻¹. The limit of detection (LOD), calculated on the basis of S/N = 3, was 0.1 μmol L⁻¹. It is lower than that of most of the developed OPH-based sensors for paraoxon determination, as Table 2 demonstrates. The comparison of the data presented in Table 2 confirms that the electrode modification with the hybrid bionanocomposite CS-cMWCNT-HA/OPH contributes to the improvement of its analytical performance.

It is also important to note the high reproducibility of the analysis (RSD < 4%, n = 5), which was attributed to the chosen technique for electrode modification. Coatings with identical characteristics were obtained, due to the chitosan bionanocomposite electrodeposition at strictly controlled constant potential and electrodeposition time.

The repeatability of the determination was very satisfactory (RSD < 3%, n = 3), as compared to the CS/OPH-modified GCE [32], subject to electrode fouling. Electrode fouling is due to the production, upon the PNP electrochemical oxidation, of phenoxy radicals, which couple to form insulating polymers onto the electrode surface. The resistance to fouling of the CNT-modified electrodes is commented in the literature, but it is not yet fully understood [47].
The developed biosensor was applied for the selective determination of paraoxon in the presence of atrazine (200 \(\mu\text{mol L}^{-1}\)) and in the presence of chlorofos (200 \(\mu\text{mol L}^{-1}\)). Interferences were not observed, since OPH specifically catalyses the hydrolysis of the nitrophenyl substituted OPs.

The storage stability of the biosensor, kept dry at 4 °C, was tested by recording and evaluating its amperometric response to paraoxon (40 \(\mu\text{mol L}^{-1}\), 25 °C, pH 8.5). After a drop to about 36% of its initial value in 4 days of storage, the sensor response remained stable for 5 days. On the 10th day, it quickly decreased to 10% of its initial value. The loss of stability was evidently due to the intrinsic properties of the enzyme preparation DEFENZ™ 130BG, used in this work. The spot life (time for which the enzyme is active in aqueous solution) of the DEFENZ line enzymes is of 8 h [48]. Enzyme immobilization obviously resulted in enzyme activity preservation for a longer period.

The developed biosensor was successfully applied for paraoxon determination in spiked tap water. The recovery, in accordance to the IUPAC recommendations [49], was estimated by calculating the ratio of the concentration of paraoxon found to that of the paraoxon added at three different levels within the linear concentration range (20, 40 and 60 \(\mu\text{mol L}^{-1}\)). It varied in the range

![Figure 5. SEM images of the electrodeposited films of CS/OPH, CS-HA/OPH, CS-cMWCNT/OPH and CS-cMWCNT-HA/OPH.](image)

Table 2. Analytical performances of the OPH-based sensors for paraoxon determination.

| Electrode     | LOD (\(\mu\text{mol L}^{-1}\)) | Sensitivity (nA L \(\mu\text{mol}^{-1}\)) | Linear range (\(\mu\text{mol L}^{-1}\)) | Reference |
|---------------|--------------------------------|------------------------------------------|---------------------------------------|-----------|
| SWCNT/GCE     | 0.01                           | 2.40                                     | 0.5-8.5                               | [40]      |
| Carbon paste  | 0.02                           | 12.00                                    | 0.02-0.18                             | [41]      |
| MC/CB/GCE     | 0.12                           | 198.00                                   | 0.2-8.0                               | [42]      |
| SWCNT/GCE     | 0.15                           | 25.00                                    | 0.25-4.0                              | [43]      |
| MWCNT/GCE     | 0.31                           | 25.95                                    | 0.5-2.0                               | [44]      |
| fMS/GCE       | 0.40                           | -                                        | -                                     | [45]      |
| Carbon paste  | 0.90                           | 1.45                                     | 4.6-46.0                              | [46]      |
| CS/GCE        | 2.0                            | 22.2                                     | 5-82                                  | [32]      |
| CS-c-MWCNT-HA/GCE | 0.10         | 5.10                                     | 5.0-80.0                               | This work |

Note: CB, carbon black; fMS, functionalized mesoporous silica; MC, mesoporous carbon; MWCNT, multiwalled carbon nanotubes; SWCNT, single-walled carbon nanotubes.
98.4% to 101.2%, which demonstrates the high accuracy of the determinations.

Conclusions

The CNTs and the HA nanoparticles synergistic action was employed for the development of an electrochemical OPH-based sensor for direct OPs determination, possessing enhanced sensitivity. A simple approach was applied for the biosensing platform construction: one-step electrodeposition of a chitosan-based bionanocomposite (CS-cMWCNT-HA/OPH) onto glassy carbon electrode surface. The CNTs and the HA load was optimized and the sensor was characterized by cyclic voltammetry and chronoamperometry. It was applied for paraoxon determination within the linear concentration range of 5–80 μmol L⁻¹, with a LOD of 0.1 μmol L⁻¹ and a sensitivity of 5.10 nA L μmol⁻¹. The suggested biosensor is very suitable for the rapid \textit{in situ} monitoring of detoxification processes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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