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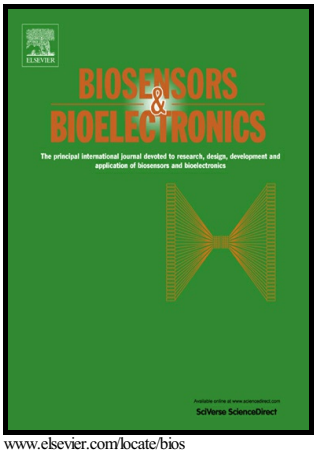

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Functionalized polyacrylamide as an acetylcholinesterase-inspired biomimetic device for electrochemical sensing of organophosphorus pesticides

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Abstract

A plethora of publications has continuously reported electrochemical biosensors for detection of pesticide. However, those devices rarely accomplish commercial application due to technical issues associated with the lack of stability and high cost of the biological recognition element (enzyme). Alternatively, the biomimetic catalysts have arisen as a candidate for application in electrochemical biosensors to overcome the enzymatic drawbacks, combining low cost scalable materials with superior stability. Herein, for the first time, we propose a biomimetic biosensor for organophosphorus pesticide detection employing a functionalized polyacrylamide, polyhydroxamicalkanoate (PHA), which mimics the performance of the acetylcholinesterase (AChE) enzyme. The PHA bears functional groups inserted along its backbone chain working as active sites. Thereby, PHA was immobilized on screen printed electrodes (SPE) through a blend formation with poly(ethylene glycol) methyl ether (mPEG) to prevent its leaching out from the surface. Under optimum conditions, the biomimetic sensor was employed for the amperometric detection of paraoxon-ethyl, fenitrothion and chlorpyrifos ranging from 1.0 and 10.0 μmol L^{-1} with a limit of detection of 0.36 μmol L^{-1}, 0.61 μmol L^{-1}, and 0.83 μmol L^{-1}, respectively. Typical AChE-based interfering species did not affect the PHA performance, which endorsed its superior behavior. The proposed biomimetic biosensor, denoted as SPE/PHA/mPEG, represents a significant advance in the field, offering a new path for low cost devices by means of an artificial enzyme, simple configuration and superior stability. Moreover, the biosensor performance can be further improved by modifying the electrode surface to enhance electronic transfer rate.

Keywords: biomimetic biosensor, acetylcholinesterase, organophosphorus pesticide, screen printed electrode, polyacrylamide.

1. Introduction

The human population has been constantly exposed to higher level of pesticides over the years since these hazardous species play a relevant role in agricultural productivity. Consequently, their intentional release results in heavy environmental contamination, reaching non-target organisms in addition to human exposure to pesticides from food and drink sources (Van Dyk and Pletschke, 2011). Notably, organophosphorus (OP) compounds account for over 38% of the total pesticides used worldwide (Singh, 2009). Research findings endorse that continuous low-level exposure to OP pesticides may alter neurodevelopment and growth in developing organisms (Eskenazi et al., 1999). The aforementioned issues make the environmental monitoring of OP levels crucial.

The most employed analytical tools for OPs detection consist of gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry due to their accuracy, sensitivity, reliability and robustness (Tankiewicz et al., 2010). However, those techniques are laborious since involve time-consuming extraction and cleanup steps. In addition, they are not adapted for in situ and real time detection, and require expensive equipment and highly trained personnel for their operation.

Accordingly, electrochemical biosensors have arisen as a promising analytical tool for the detection of those pollutants based on their rapid response, low-cost and simplicity. Moreover, they are able to provide real-time and in situ information without or with a minimal sample pre-treatment (Andreescu and Marty, 2006; Arduini et al., 2010; Pundir and Chauhan, 2012). Biosensors based on the inhibition of acetylcholinesterase (AChE) enzyme have been widely developed for the detection of OP pesticides (Andreescu and Marty, 2006; Arduini et al., 2010; Periasamy et al., 2009; Zhang et al., 2014b). Despite the advantages shown by those devices, AChE-biosensors are rarely applied to real samples owing to low long-term stability, unsatisfactory sensitivity, and lack of robustness (Luque de Castro and Herrera, 2003; Scognamiglio et al., 2015). Mostly, the predominant concerns are related to the sensitivity and stability of biological recognition element immobilized on the sensor surface (Scognamiglio et al., 2010), in addition to the high cost of AChE. Furthermore, AChE can be inhibited by the presence of metal cations and organic molecules leading to unreliable results.

As an alternative, biomimetic materials which exhibit the desirable catalytic properties of natural enzymes may overcome such drawbacks, then boosting biosensor performance with regard to sensitivity and stability (Scognamiglio et al., 2015). Enzyme mimics enable a finely tailored performance by attaching suitable groups of functional moieties similarly as those in the active centre of enzymes (Kuah et al., 2016). Bio-inspired sensors allow the development of more reliable biosensing systems in view of the enhanced stability under experimental conditions (pH and temperature) and low-cost scalable production, while combine catalytic efficiency.

Particularly, a biomimicry system based on AChE enzyme aiming for electrochemical sensing application may imitate some factors involved in the enzymatic catalysis: i) to hydrolyze the electroactive substrate acetylthiocholine (ATCh), and ii) to interact with OP pesticides. Thereby, the biomimetic AChE-based model may be structurally designed with artificial active sites that contains proper functional groups to drive and react with the target molecules. We have previously demonstrated that a functionalized polyacrylamide, polyhydroxamicalkanoate (PHA), which contained hydroxamic acid and carboxylic acid moieties inserted along its backbone chain, acting as an artificial active site, exhibited a potential AChE-like behavior (Sgobbi et al., 2017). We scrutinized the kinetic performance of PHA towards the catalysis of ATCh hydrolysis in which we found an exceptional enhancement in the reaction rate over 10^8-fold in pH 7.0 and over 10^7-fold in pH 8.0 in relation to spontaneous hydrolysis. Additionally, we verified the interaction between PHA and an OP pesticide model (paraoxon-ethyl) through spectrophotometric assay, which revealed its potential in OP sensing. Theoretical insights shed light on the mechanism of interaction between ATCh and PHA, besides paraoxon-ethyl and PHA. Those outcomes bolster the application of the proposed biomimetic AChE-based polymer for the development of a new sensing platform for OP detection.

To the best of our knowledge, this is the first report of a biomimetic AChE-based system with catalytic behavior applied in electrochemical sensing field. Herein, we developed a simple bio-inspired platform employing PHA as the AChE mimic for the amperometric detection of OP pesticides. To further improve the immobilization efficiency, a polymeric blend was formed combining PHA and poly(ethylene glycol) methyl ether (mPEG) on the screen printed electrode (SPE) surface, and then the sensor was denoted SPE/PHA/mPEG. The SPE/PHA/mPEG sensor fabrication was characterized and optimized, and its performance in OP pesticide detection was

evaluated. Finally, the simple and highly stable biomimetic sensor was evaluated for the detection of OP pesticides in water supply samples.

2. Experimental

2.1. Chemicals

Polyacrylamide (M_w 1500 g mol^{-1}) was purchased from Scientific Polymer Products, Inc. (Ontario, USA) and hydroxylamine hydrochloride from Merck (Darmstadt, Germany). Acetylthiocholine chloride (ATCh), poly(ethylene glycol) methyl ether (mPEG, M_n 5000 g mol^{-1}), potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6$, paraoxon-ethyl, chlorpyrifos and fenitrothion were obtained from Sigma-Aldrich (St. Loius, USA). All the chemicals were of analytical grade and used without additional purification. All the chronoamperometric and differential pulse voltammetric (DPV) measurements were performed in Britton-Robinson (BR) buffer solution at pH 8.0 as the supporting electrolyte. The BR buffer solution was prepared with equivalent mixtures of 0.1 M of acetic acid, 0.1 M boric acid, and 0.1 M phosphoric acid. The pH value was adjusted with a 1.0 M NaOH solution. Borax buffer solution 0.1 M at pH 10.0 was prepared with anhydrous sodium tetraborate and the pH value was adjusted using a 1.0 M NaOH solution. All aqueous solutions were prepared with ultrapure water obtained from a Milli-Q Plus system (Millipore Corporation, Darmstadt, Germany). The stock solutions of all OP pesticides were prepared by dissolving the standards in ethanol.

2.2. Apparatus

Morphological characterization of PHA, mPEG and the polymeric blend was observed through field emission scanning electron microscopy (FESEM) (XL 30, Philips, Netherlands,) operating at a voltage of 5.0 or 10.0 kV.

The cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on a model PGSTAT302 Autolab® electrochemical system (Eco Chemie, Netherlands) controlled by the GPES software (version 4.9 - Eco Chemie, Netherlands). The electrochemical impedance spectroscopy (EIS) measurements were performed on a model PGSTAT302 Autolab® electrochemical system (Eco Chemie, Netherlands) controlled by the

FRA software (version 4.9 - Eco Chemie, Netherlands). The complex plane spectra were analyzed with the ZView electrochemistry software (version 3.1c – Scribner Associates Inc., North Carolina, USA). The EIS spectra were recorded under open circuit conditions, in a frequency range of 100 kHz to 0.1 Hz with AC voltage amplitude of 10 mV. The frequency interval was divided into 61 logarithmically equidistant measure points. The measurements were performed in 0.1 mol L^{-1} KCl containing 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. All electrochemical measurements were performed in a droplet of 50 μL deposited on the disposable electrode surface covering the work, reference and counter electrodes. None electrochemical cell was used.

2.3. Synthesis of polyhydroxamicalkanoate (PHA)

The biomimetic polymer PHA was synthesized towards the functionalization of the polyacrylamide polymer based on the methodology reported by (Mello et al., 2011) as follows. Briefly, a solution of hydroxylamine hydrochloride (2 g, 75.0 mmol, 25mL) was poured into a polyacrylamide solution (5.0 g, 3.0 mmol, 20mL) under magnetic stirring. After 2 h, 75 mmol of NaOH solution was added in the reactional mixture. After proceeding for 48 h at room temperature under stirring, the solution pH was adjusted to 1.0 using concentrated HCl, followed by methanol addition, obtaining a white precipitate. Afterwards, the mixture was cooled to 4.0 °C overnight and filtered, then washed several times with methanol and dried for 12 h, yielding a pale pinkish solid.

2.4. Screen-printed electrodes (SPEs)

The disposable SPE system composed by working, reference and counter electrodes was fabricated using screen-printed machine (DEK 248) and three different photolithograph masks as reported by Aragay et al. (2011). Three different inks were purchased from Gwent Group of Companies (UK): carbon ink (C2030519P4) for the working and counter electrodes, silver/silver chloride ink (C61003P7) for the reference electrode layer and the dielectric blue paste (D2071120P1) to isolate the working electrode area protecting the connections from solution contact. Hence, the production of SPEs consists of three principal steps: i) the carbon ink was

printed onto a polyethylene terephthalate (PET) sheet and the ink was cured at 60°C for 20 min, ii) Ag/AgCl ink was printed and cured at 60 ºC for 20 min, iii) the insulating layer was printed and cured overnight.

2.5. Fabrication of the biomimetic sensor SPE/PHA/mPEG

Prior to the surface modification, SPE was electrochemically conditioned by applying −2 V for 30 s in H_2SO_4 0.1 mol L^{-1}, and then washed it to remove the bubbles. Sequentially, CV measurements were performed in H_2SO_4 0.1 mol L^{-1} in a potential range from 0 to 1 V to achieve cycling stability. After the pre-treatment, the biomimetic PHA was immobilize on the SPE surface based on a polymeric blend formation as proposed by Swamy and Siddaramaiah (2007). To prevent the PHA leaching from the electrodic surface, we made a polymeric blend between PHA and mPEG polymers, which promoted the film stabilization on the SPE surface. The polymeric blend between PHA and mPEG polymers with 50/50 composition of each polymer was prepared using a stock solution of 4.5% w/v of each polymer. The blend formation was characterized by FESEM, and then it was compared with the films composed by PHA and mPEG, individually.

Subsequently, 5 μL of the blend was dropped on the working electrode surface, and dried overnight at room temperature. The modified electrode, denoted as SPE/PHA/mPEG was characterized by EIS and CV measurements in 0.1 mol L^{-1} KCl solution containing 5.0 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$. For CV measurements, the potential was scanned from -0.7 to +1.1 V at 50 mV s^{-1}.

2.6. Electrochemical measurements

After the SPE surface modification, CV measurements were performed in a single drop (50 μL) of BR buffer solution at pH 8.0 using the potential range from 0.0 to +1.0 V at 50 mV s^{-1} until the stability of voltammetric profiles. Afterwards, DPV was employed using the following parameters: 0.1 s of interval time, potential step of 1.0 mV, 50 mV of amplitude and potential range from 0.0 to +1.0 V.

The immobilized PHA activity was determined by the electrochemical monitoring of thiocholine (TCh) formed upon hydrolysis of ATCh catalyzed by the proposed biomimetic polymer. Freshly prepared ATCh solutions with concentration range from 1.0 to 10.0 mmol L^{-1} in 0.1 mol L^{-1} BR

buffer (pH 8.0) were used. The detection of the electroactive thiocholine product was carried out after 20 min of incubation in those ATCh solutions.

Chronoamperometry was used as the measurement technique for biosensor operation obtaining the calibration curve through the application of +0.5 V during 60 s, rinsing the electrode with deionized water between each measurement.

For inhibition tests, the original chronoamperometric signal was first recorded in 0.1 mol L^{-1} BR buffer (pH 8.0) containing 8 mmol L^{-1} ATCh. Then, the SPE/PHA/mPEG sensor was rinsed with deionized water and incubated in 50 μL of given concentrations of OP pesticides solutions during 20 min. Solutions containing the OP pesticides, paraoxon-ethyl, fenitrothion, chlorpyrifos, were freshly prepared at concentrations between 1.0 and 10.0 μmol L^{-1} in 0.1 mol L^{-1} Borax buffer pH 10.0. After inhibition step, the residual signal was measured at the identical condition as the previous chronoamperometric measurement in the absence of pesticide in 8 mmol L^{-1} ATCh solution. The degree of inhibition was calculated using the relation,

$$Inhibition(\%) = \frac{(I_0 - I_1)}{I_0} \times 100\% \qquad \text{Equation 1}$$

Where I_0 represents the chronoamperometric response of the biosensor to 8 mmol L^{-1} ATCh before incubation, and I_1 is the response after incubation in inhibitor solution.

3. Results and discussion

3.1. FESEM analysis

In order to immobilize the bio-inspired polymer PHA on the surface of the SPE in a way of preventing the film leaching off the electrodic surface, we obtained an immiscible polymeric blend between PHA and mPEG dissolved in water (Huraux et al., 2007; Swamy and Siddaramaiah, 2007). Fundamentally, those blends exhibit phase separation during drying of the solvent. Besides, mPEG and PHA interact through hydrogen bonding between ether groups and amide groups in which mPEG molecular chains aggregate around PHA clews (Lue et al., 2010). The changes in morphology of PHA/mPEG blend in comparison to the individual PHA and

mPEG films were investigated by FESEM (Fig.1). A smooth, glassy surface is exhibited by PHA film (Fig. 1A) whereas a uniform unwrinkled surface is presented by mPEG film (Fig. 1B). Meanwhile, Fig. 1C reveals the formation of two different phases with crystalline morphology when the blend composition is 50/50 w/v. The higher magnification of FESEM image (Fig. 1D) provides clearer information about the presence of two phases. The blend formation was optimized considering the ratio between mPEG and PHA in order to immobilize the PHA on the SPE surface. Proportions less than 50% led to the PHA leaching from SPE surface. On the other hand, proportions higher than 50% increased the system resistivity to charge transfer.

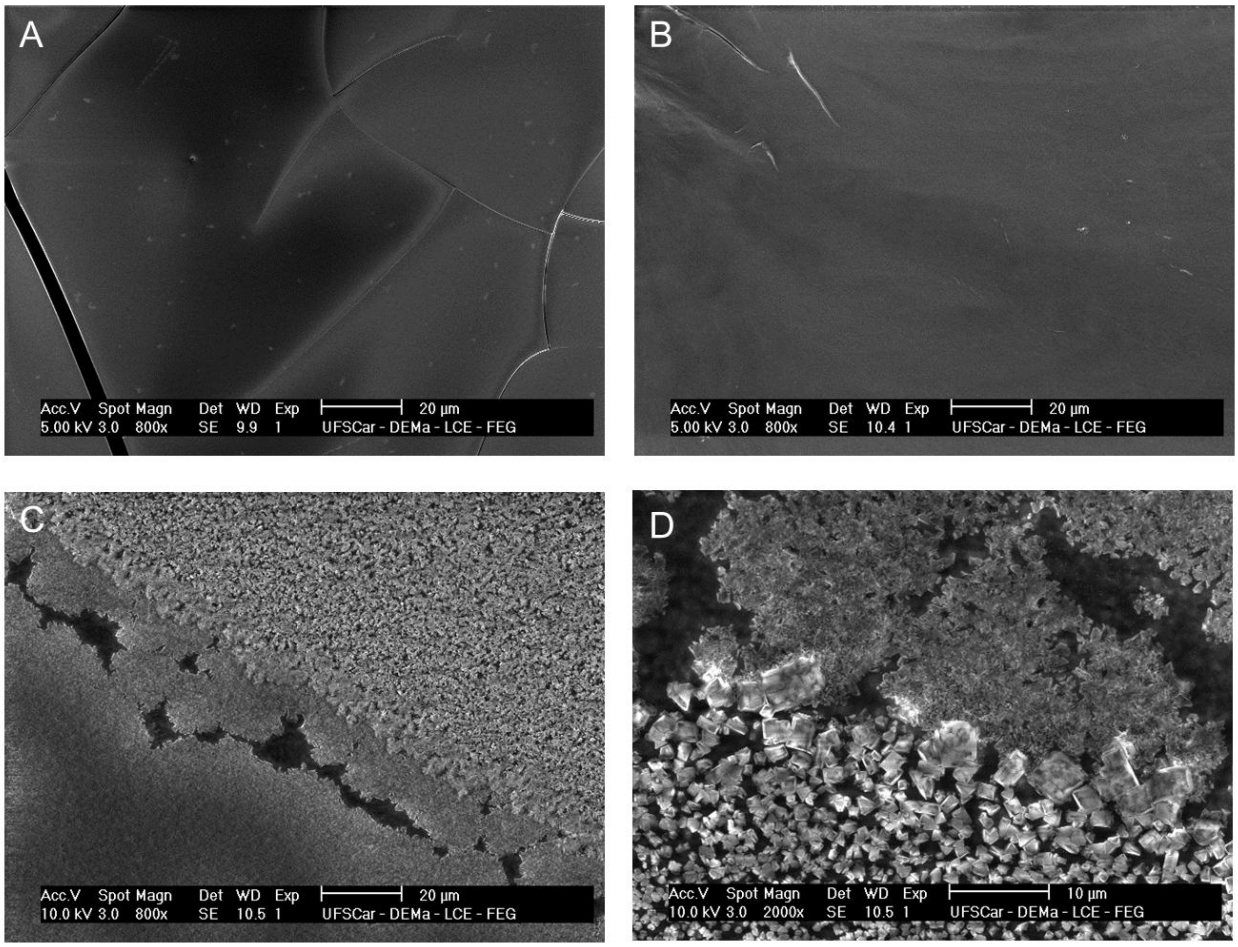

Figure 1. FESEM images of (A) PHA film, (B) mPEG film, (C) PHA/mPEG blend magnified by 800x, and (D) PHA/mPEG blend magnified by 2000x.

3.2. Electrochemical behavior of the SPE/PHA/mPEG sensor

The effect of the SPE surface modification with PHA/mPEG blend was studied by CV and EIS techniques. Fig. 2A shows the CVs of bare SPE, and SPE modified with PHA/mPEG blend in 0.1 mol L^{-1} KCl containing 5 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at 50 mV s^{-1}. On the bare SPE (black curve), a pair of well-defined redox peaks, characteristic of a diffusion-limited process, was observed. When the blend composed by PHA/mPEG was cast on SPE surface, we observed a peak separation increased due to the higher resistivity of the electrode surface, related to the non-conducive blend nature. Moreover, although the values of oxidation peak currents seem quite different for both surfaces, if a baseline correction is performed for both cyclic voltammograms, the peak current values become quite similar. In this way, the difference should be mainly attributed to the inclination of the modified electrode profile due to the increase in the resistivity and in the capacitive current.

EIS studies were performed to evaluate the kinetics of charge transport by bare SPE in addition to the SPE/PHA/mPEG sensor under open circuit conditions in Faradaic mode (Fig. 2B). Electrochemical impedance data are conventionally expressed by Nyquist plots, considering the imaginary impedance component (Z'', out-of-phase) against the real impedance component (Z', in-phase) at different excitation frequencies. The Randles circuit was used as the equivalent circuit model for impedance data fitting. In this circuit (inset Fig. 2B), the parameter R_{ct} denotes the charge transfer resistance, CPE is the capacitance, R_s comprises the solution resistance and Z_w is the Warburg impedance. In Fig. 2B, the semicircle diameter for the SPE/PHA/mPEG increased in comparison to the bare SPE one resulting in a higher R_{ct} value of 4238 Ω in comparison to 3325 Ω, respectively, attributed to the surface modification with non-conductive material. The comparison between the CV and EIS results for the bare SPE and SPE/PHA/mPEG systems shows that the increase in the R_{ct} corroborates the increase in the ΔE_p observed in the CV measurement associated with the immobilization of a non-conductive material.

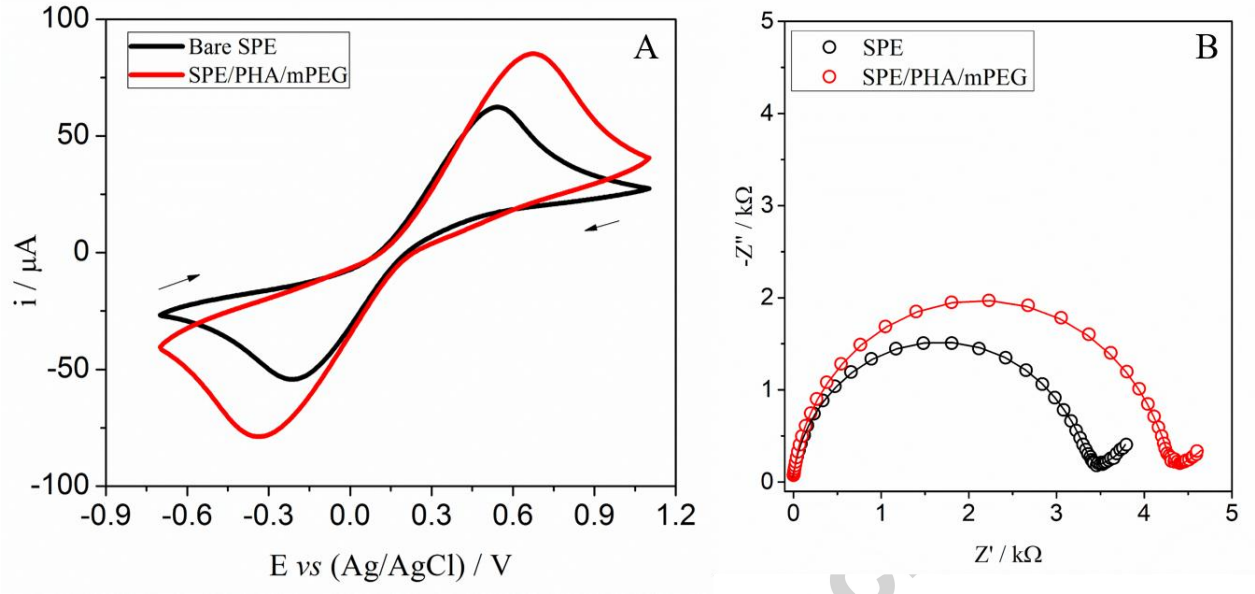

Figure 2. A) CV of bare SPE and SPE/PHA/mPEG at 50 mV s^{-1} and B) EIS of the bare SPE in relation to SPE/PHA/mPEG in 0.1 mol L^{-1} KCl solution containing 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-}$ /$[Fe(CN)_6]^{4-}$.

3.3. Optimization of experimental parameters for thiocholine detection

As the original definition, a biomimetic sensor should operate similarly to the electrochemical AChE-based devices in which ATCh substrate is hydrolyzed by the biological recognition element into thiocholine and acetate. The PHA polymer exhibits a similar AChE-based behavior, where it first electrostatically drives the positive-charged ATCh substrate towards the carboxylate group in the artificial active site enabling the nucleophilic attack from the hydroxamic acid group. Fig. 3 depicts the principle of operation of the SPE/PHA/mPEG sensor, considering a fragment of PHA immobilized on the SPE surface. At pH 8.0, the immobilized PHA is monoprotonated; and therefore, the carboxylate group attracts the positive quaternary amine moiety in the ATCh, facilitating the nucleophilic attack promoted by the hydroxamic acid moiety in the PHA. Afterwards, the produced thiocholine is oxidized under the applied voltage, forming the disulfide dimer. The incubation of the SPE/PHA/mPEG sensor in pesticide sample was performed in a different solution at pH 10.0 to fully deprotonate the acidic groups in the active site of the polymer chain (pK_a 9.2), thus increasing its reactivity in relation to OP interaction. As the ATCh

molecule undergoes spontaneously hydrolysis at such high pH values, the decay of the biomimetic sensor response was evaluated in the previous ATCh solution at pH 8.0.

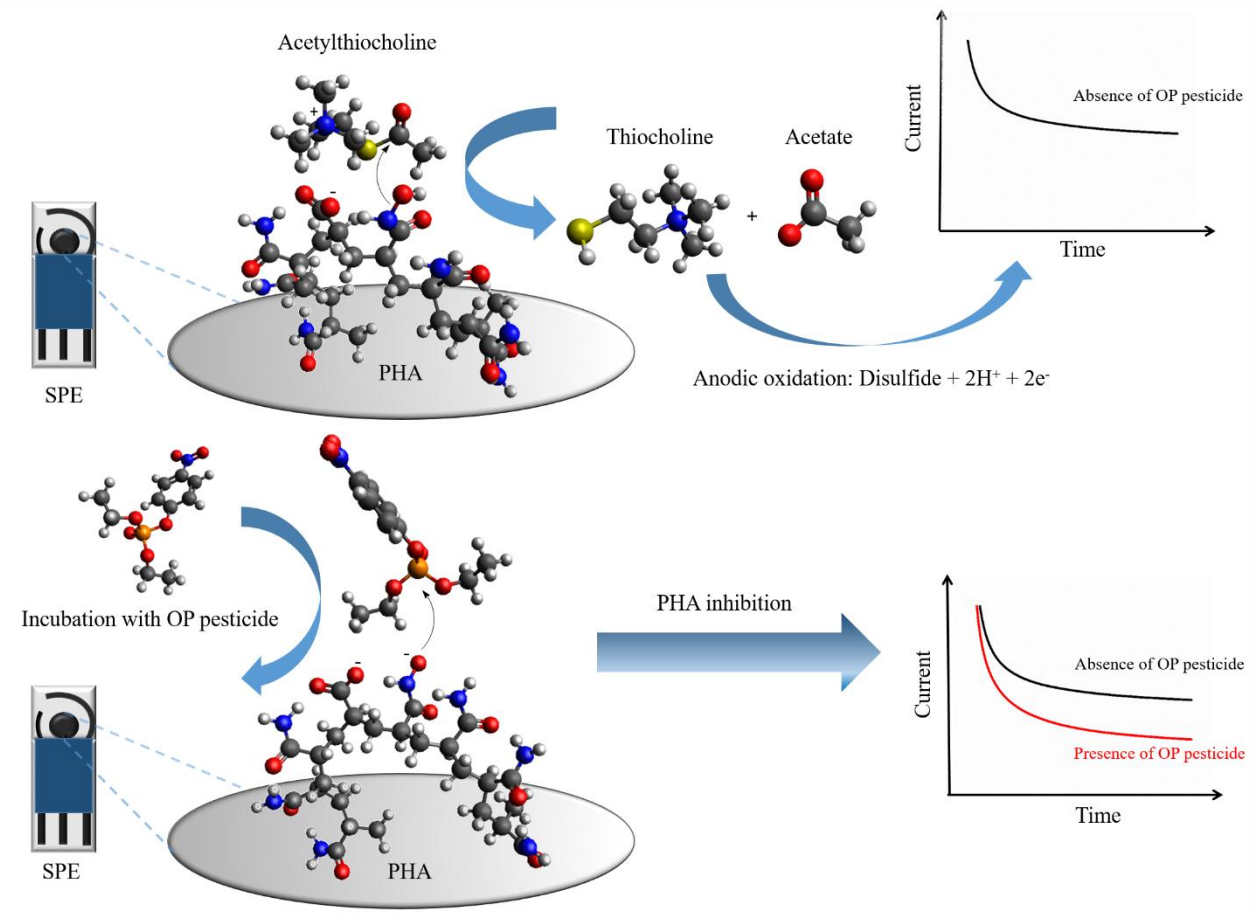

Figure 3. Scheme of sensing principle for the biomimetic electrochemical SPE/PHA/mPEG sensor, where PHA is represented by a polymeric fragment.

Initially, we investigated the real catalytic performance of PHA after its immobilization on the SPE surface for eliminating the possibility of spontaneous hydrolysis. The first step consisted of performing DPV in the blank (0.1 mol L^{-1} BR buffer pH 8.0), and no detectable peak was observed due to the absence of ATCh (Fig. 4, line a). Thus, 50 µL of a freshly prepared 4.0 mmol L^{-1} ATCh solution in 0.1 mol L^{-1} BR buffer at pH 8.0 was dropped on the bare SPE surface followed immediately by DPV measurement, and no faradaic response was detected (Fig. 4, line b). After 20 min, DPV was again performed with no evidence of oxidation peak (Fig. 4, line c).

The results revealed that there was no formation of thiocholine on the bare SPE surface due to spontaneous hydrolysis of ATCh.

Subsequently, the SPE/PHA/mPEG sensor was evaluated in relation to the catalytic behavior of PHA through ATCh hydrolysis. Then, 50 μL of a new freshly prepared 4.0 mmol L^{-1} ATCh solution in 0.1 mol L^{-1} BR buffer at pH 8.0 was dropped on the SPE/PHA/mPEG surface, followed by DPV. Fig. 4 (line d) exhibits a peak formation at approximately +0.46 V indicating the oxidation of thiocholine generated towards the catalysis of ATCh in the hydrolysis reaction, promoted by the biomimetic PHA. After 20 min, the acquisition of new DPV profile was obtained and a significant increase in the peak current at +0.46 V was observed (Fig. 4, line e). As it can be seen, the increase in the peak current is associated with the hydrolysis of more ATCh molecules accomplished by PHA polymer immobilized on SPE surface. Thereby, those outcomes revealed the efficiency of PHA in the catalysis of ATCh.

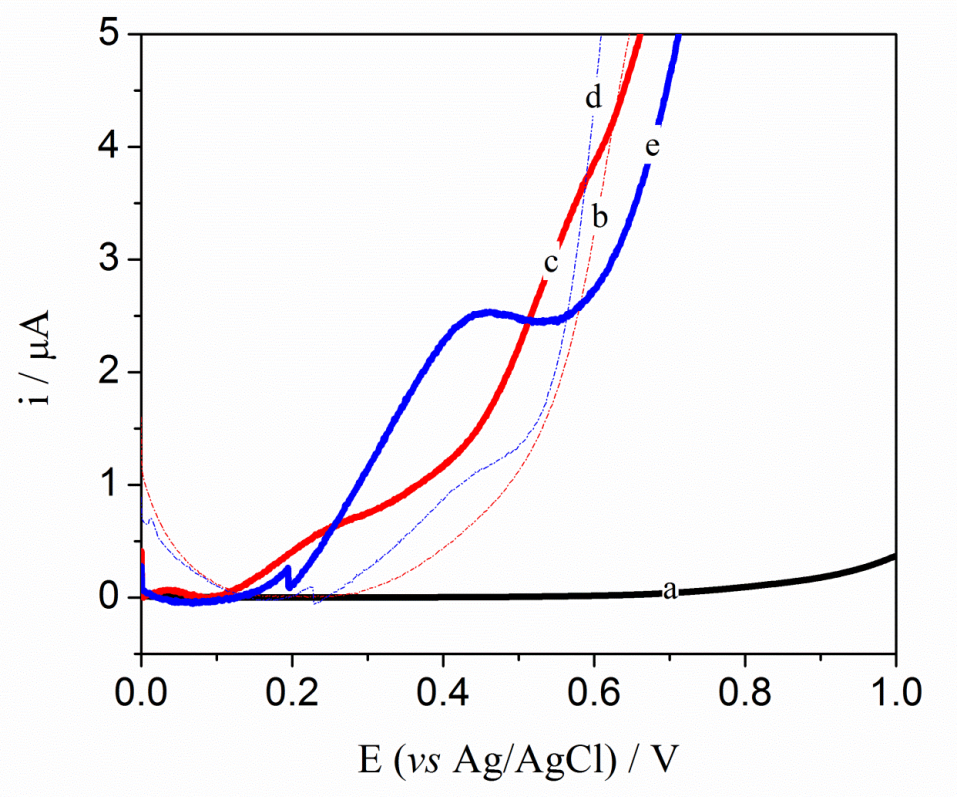

Figure 4. DPV responses at SPE/PHA/mPEG sensor in the absence of ATCh (line a); in the presence of 4 mmol L^{-1} ATCh immediately after the addition (line d); and after 20 min of reaction (line e). DPV responses using bare SPE containing 4 mmol L^{-1} ATCh right after its

addition (line b), and after 20 min of reaction (line c). All the DPV measurements were performed in 0.1 mol L^{-1} BR buffer solution at pH 8.0 as the supporting electrolyte.

The effect of the catalysis time was evaluated considering the instant right after the ATCh solution was dropped on the SPE/PHA/mPEG surface (0 min). Besides, different periods of time (10 min, 20 min, 30 min and 40 min) after dropping ATCh solution on the modified electrode surface were studied regarding the reaction between PHA and ATCh. Fig. S1 shows the DPV responses considering the aforementioned times for different values of ATCh concentration of 1.0, 2.0, 5.0 and 8.0 mmol L^{-1}. It can be observed that from 10 min, there is a peak current in +0.46 V which increases after more 10 min (total reaction time of 20 min) which is related to the oxidation of thiocholine formation after ATCh hydrolysis catalyzed by PHA. After longer period of time than 20 min, the peak current stabilized which is associated with the completed hydrolysis of all ATCh present in the solution by PHA. These behavior remains for every ATCh concentration, thereby we chose 20 min as an optimal catalysis time using 8.0 mmol L^{-1} ATCh as the fixed concentration for the inhibition assay in presence of OP pesticides.

3.5. Amperometric performance of the biomimetic SPE/PHA/mPEG sensor towards ATCh hydrolysis and thiocholine detection

The amperometric responses of the SPE/PHA/mPEG sensor to successive additions of ATCh in 0.1 mol L^{-1} BR buffer pH 8.0 at the applied potential of +0.5 V during 60 s were investigated and the results are presented in Fig. 5A. The current responses were proportional to ATCh concentrations in the range from 1.0 to 10.0 mmol L^{-1} (Fig. 5B). The linear equation of the minimum squares fitting was obtained as I (A) = 5.50×10^{-4} c(mol L^{-1})+2.64×10^{-7} (r = 0.9967) where c is the concentration of ATCh. The corresponding sensitivity, determined as the slope of the linear calibration curve (dI/dc), was 5.50×10^{-4} A M^{-1}. The detection limit (LOD) was estimated to be 0.26 μmol L^{-1} according to the statistical method described by Miller and Miller (2005). Taking into account the simplicity of the proposed biosensing platform, without the incorporation of any electron transfer promoter element (as carbon nanotubes, graphene or

nanoparticles), the obtained LOD was lower or comparable to those previously reported shown in Table S1 (Du et al., 2007; Du et al., 2010; Li et al., 2006; Zamfir et al., 2011; Zhang et al., 2014a; Zhou et al., 2013). Remarkably, the oxidation peak was relatively low in potential compared with the previous reports as shown in Table S1 (Du et al., 2007; Du et al., 2010; Li et al., 2006; Liu and Lin, 2006; Liu et al., 2011; Yang et al., 2013; Zhai et al., 2013).

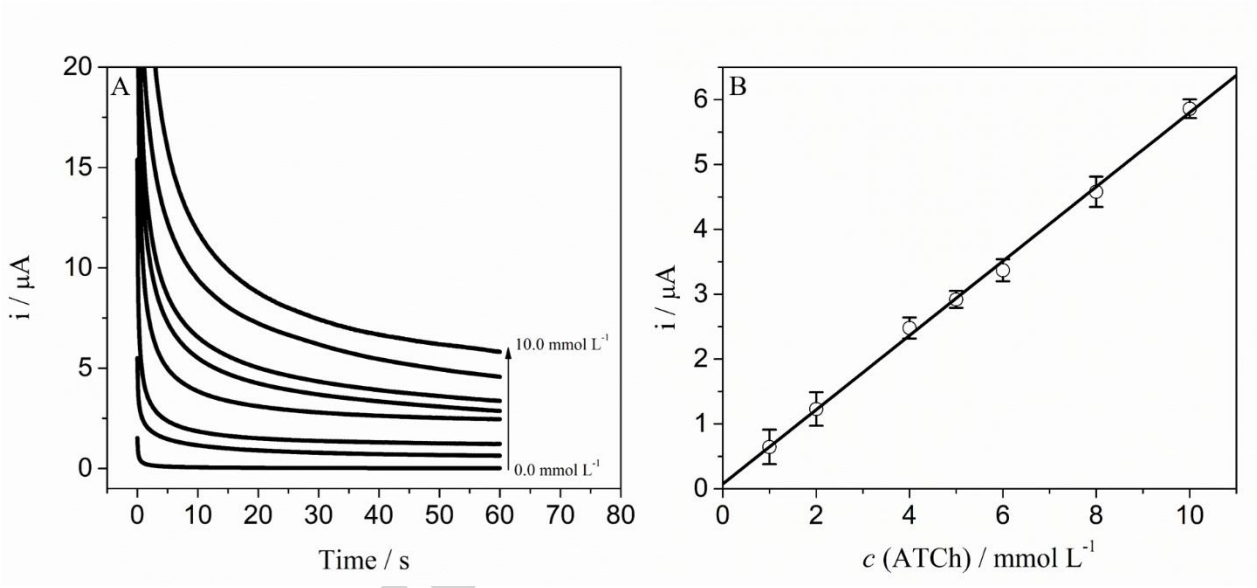

Figure 5. (A) Amperometric responses for the biomimetic SPE/PHA/mPEG sensor in the presence of ATCh ranging from 1.0 to 10.0 mmol L^{-1} at 0.5 V during 60 s. (B) Calibration curve for ATCh determination.

3.6. Calibration curve for OP pesticides detection

Similarly as the AChE-based biosensors, this biomimetic device works in an indirect electrochemical approach, by the inhibition of the artificial enzyme PHA by OP pesticides. Particularly, the PHA polymer promotes the dephosphorylation reaction of OP pesticides being phosphorylated and then, releasing the specific leaving group of each type of OP.

Under the optimized experimental conditions, the biomimetic SPE/PHA/mPEG sensor was explored through chronoamperometric measurements to detect OP pesticides at fixed

concentration of 8.0 mmol L^{-1} ATCh (Fig. 6A). Paraoxon-ethyl was chosen as reference pesticide with a concentration range from 1.0 to 10.0 μmol L^{-1}. The incubation step was performed adding 50 μL of paraoxon-ethyl solution prepared in 0.1 mol L^{-1} Borax buffer at pH 10.0 on SPE/PHA/mPEG surface. Detailed studies by Sgobbi et al. (2017) indicated the superior rate regarding the dephosphorylation reaction promoted by PHA towards paraoxon-ethyl in pH 10.0. Subsequent to the incubation time of 20 min, the ATCh hydrolysis was carried out in BR buffer at pH 8, after allowing the reaction between phosphorylated-PHA and ATCh for 20 min. The calibration curve for paraoxon-ethyl was obtained by plotting the percent relative inhibition (*I*%) (Eq. 1) against paraoxon-ethyl concentrations (Fig. 6B). Linear equation of paraoxon-ethyl was I(%) = [4.8×10^{6} *c* (mol L^{-1})+0.8169]×100, with correlation coefficient of 0.9997. The obtained LOD was 0.36 μmol L^{-1}.

Additionally, the dephosphorylation reaction towards two different types of OP pesticides was also evaluated. Fenitrothion and chlorpyrifos were chosen based on their dissimilar chemical structure contrasting with paraoxon-ethyl. Fig. S2 exhibits the calibration plots of inhibition percentage versus pesticide concentration. The linear equation for fenitrothion is (I%) = [2.84×10^{6} *c* (mol L^{-1})+0.5616]×100, with correlation coefficient of 0.9965 and limit of detection of 0.61 μmol L^{-1}. For chlorpyrifos, the linear equation is (I%) = 1.94×10^{6} *c* (mol L^{-1})+0.7184]×100, with correlation coefficient of 0.9968 and limit of detection of 0.83 μmol L^{-1}. The increase in the limit of detection is related with the structure of the chosen OPs. Most of OP pesticides have the phosphorothionate group (P=S) (e.g. fenitrothion and chlorpyrifos) which confers hydrolytic stability to them. However, those thionate pesticides can be converted to oxons (e.g. paraoxon-ethyl) either in environment or *in vivo*, and then, becoming more reactive (Bharate et al., 2010).

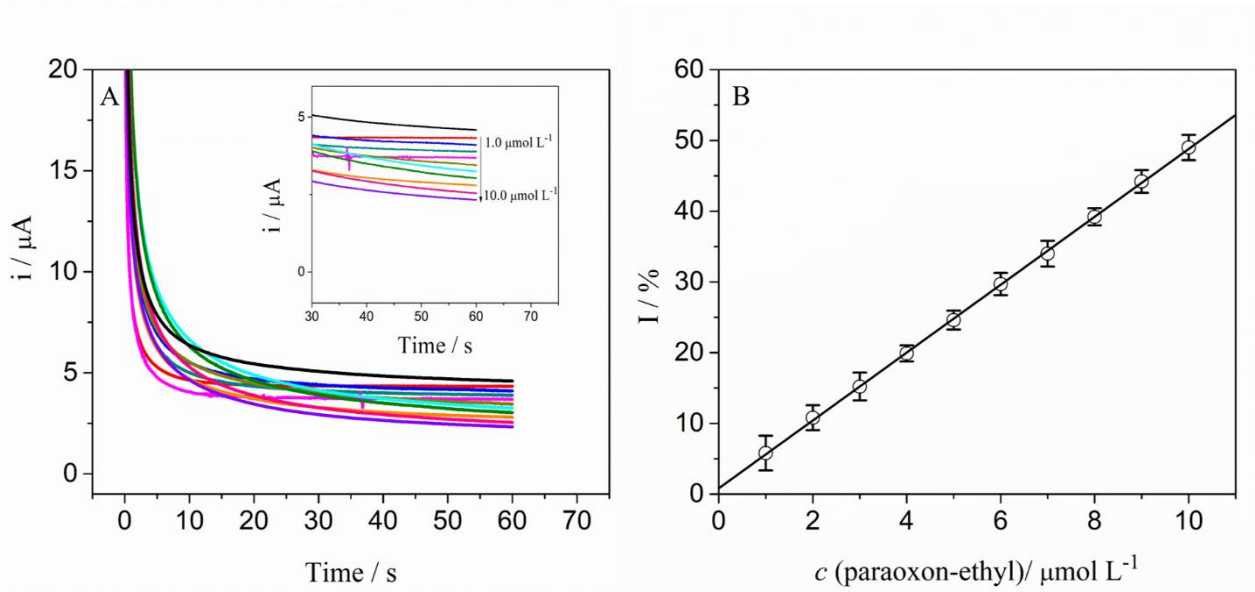

Figure 6. (A) Chronoamperograms obtained from the biomimetic SPE/PHA/mPEG sensor after immersion in different concentration of paraoxon-ethyl solutions performed in 0.1 mol L^{-1} BR buffer at pH 8.0 in the presence of 8.0 mmol L^{-1} ATCh. (B) The inhibition curve of SPE/PHA/mPEG to different paraoxon-ethyl concentrations.

3.7. Repeatability, reproducibility, stability and interference studies

The repeatability of the biosensor was evaluated with the same SPE/PHA/mPEG device by 6 successive amperometric measurements in 8.0 mmol L^{-1} ATCh after being immersed in the 5.0 μmol L^{-1} paraoxon-ethyl during 20 min; and the relative standard deviation (RSD) was found to be 4.7%. The reproducibility was estimated through determining the response of 8.0 mmol L^{-1} ATCh at five different SPE/PHA/mPEG sensors, which were immersed in 5 μmol L^{-1} paraoxon-ethyl during 20 min, then the RSD was found to be 6.5%. These results were indicative of acceptable repeatability and reproducibility for pesticide determination.

The biomimetic PHA polymer can be stored at room temperature, which implies a remarkable stability in comparison to the AChE enzyme that easily denatures. Besides, when the PHA/mPEG blend is immobilized on the SPE surface, the PHA activity is remained and its leaching from the electrodic surface prevented. Concerning the publications listed in Table S1 (Du et al., 2007; Du et al., 2010; Li et al., 2006; Liu and Lin, 2006; Liu et al., 2011; Yang et al., 2013; Zamfir et al., 2011; Zhai et al., 2013; Zhang et al., 2014a; Zhou et al., 2013), all the reported devices need to be

stored at 4 ºC losing about 10% of their activity after 30 days, these conditions indicate the problematic issues of the enzymatic sensors regarding their robustness, stability and shelf-time. On the other hand, the proposed biomimetic SPE/PHA/mPEG sensor can be stored at room temperature due to the superior PHA stability. After 6 months, only 11% of biomimetic sensor activity was lost, which is an extraordinary outcome in the biosensors field, combining low cost scalable material with high stability at room temperature.

We selected typical interference species that inhibit AChE, such as carbaryl (1 μM), Ni^{2+} (0.1 mmol L^{-1}) and Zn^{2+} (0.1 mmol L^{-1}) to investigate their effect in the catalytic performance of PHA through ATCh hydrolysis. We followed spectrophotometrically the ATCh hydrolysis catalyzed by PHA in the absence and presence of those interference species. The reaction rate remained constant with differences below 3% (Table S2). The chosen interference species do not affect the catalytic performance of the biomimetic polymer.

3.8. Real sample analysis

In order to investigate the possible application of the developed biosensor in real samples analysis, the biomimetic SPE/PHA/mPEG sensor was employed in the recovery tests, performed by adding different amounts of pesticides into tap water, with recovering of 115%, 106% and 94.5% for 3.0 μmol L^{-1}, 6.0 μmol L^{-1} and 8.0 μmol L^{-1}, respectively (Fig. S3). Thereby, those results demonstrated that the biosensor exhibited an excellent accuracy for the pesticides sensing in real samples and a great potential for practical application.

4. Conclusions

In this study, a disposable biomimetic sensor based on the performance of functionalized polyacrylamide was proposed by the first time, which provides a new promising platform for OP pesticides quantification. The biomimetic PHA polymer exhibited remarkable catalytic behavior towards ATCh hydrolysis and OP dephosphorylation. Our proposition is based on the tridimensional characteristic of the PHA, which contains active sites working similarly as those

of the natural enzyme. The significant advance mostly comprises the use of a cost-effective scalable material, which behaves similarly as AChE with superior stability. Fundamentally, the biomimetic PHA overcomes the problematic issues of the enzymatic systems in biosensing.

The resulting biomimetic biosensor exhibited excellent stability, fast electrochemical response and good reproducibility. The biosensor performance could be further improved modifying the electrode surface with different kinds of active materials (nanostructured carbon structures, for example) aiming the enhancement of the electron transfer rate of the hydrolysis product thiocholine. Additionally, the catalytic properties of biomimetic polymer can be tailored by the replacement of different functional groups.

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Highlights

- For the first time, a biomimetic acetylcholinesterase-based molecule was applied as biological recognition element in electrochemical biosensing.

- A functionalized polyacrylamide containing carboxylic acid and hydroxamic acid moieties acting as active sites mimicked the acetylcholinesterase behavior.

- A disposable droplet-based biosensor required only 50 μL of sample volume to detect organophosphorus pesticide based on the inhibition of the functionalized polyacrylamide.