



## Short communication

# A novel, sensitive, reusable and low potential acetylcholinesterase biosensor for chlorpyrifos based on 1-butyl-3-methylimidazolium tetrafluoroborate/multiwalled carbon nanotubes gel

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## ABSTRACT

A novel, low potential and highly sensitive acetylcholinesterase (AChE) biosensor was developed based on 1-butyl-3-methylimidazolium tetrafluoroborate/multiwalled carbon nanotube composite gel thiocholine sensor. Composite gel promoted electron transfer reaction at a lower potential (+50 mV) and catalyzed electrochemical oxidation of thiocholine with high sensitivity. AChE was immobilized in sol–gel matrix that provides a good support for enzyme without any inhibition effect from the ionic liquid. The amount of immobilized enzyme and incubation time with chlorpyrifos were optimized. Chlorpyrifos could be determined in the range of  $10^{-8}$ – $10^{-6}$  M with a detection limit of 4 nM. Fast and efficient enzyme reactivation was obtained at low obidoxime concentration (0.1 mM). Moreover, the biosensor exhibited a good stability and reproducibility and could be used for multiple determinations of pesticide with no loss of the enzyme activity.

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## 1. Introduction

The measurement of pesticides in water and food is of great importance. The major concern about pesticides relates to acute intoxication due to the fact that these compounds are found at low levels in the environment. These compounds inhibit the acetylcholinesterase by phosphorylation of its active site. The current produced by the electrochemical oxidation of enzymatically generated thiocholine can be used as a quantitative measure of the enzyme activity, which is a marker for the biological effect of the pesticides.

Thiocholine (TCh) is oxidized at relative high potential on the surface of classical electrode. Reducing the working potential was achieved by using chemically modified electrodes based on mediators or multiwalled carbon nanotubes (MWCNT). Anyway, the lowest potential achieved was +150 mV (Liu et al., 2005). Recently, ionic liquid (IL)–MWCNT modified carbon paste (CP) electrodes have been shown to improve the detection of thiocholine by catalyzing the oxidation of thiocholine

at very low potentials between 0 and 50 mV (Rotariu et al., 2010).

The immobilization of the enzyme onto the electrode surface represents a crucial step in the development of the biosensor (Arduini et al., 2010). Various methods have been used, from direct adsorption (Marty et al., 2003), cross-linking with glutaraldehyde on chitosan–MWCNT composite (Du et al., 2007b) to mixing the enzyme in polymers (Du et al., 2010), MWCNT/sol–gel (Du et al., 2007a) or use of chelate-functionalized magnetic microbeads (Istamboulie et al., 2007). The sol–gel silicate network provides a biocompatible microenvironment around the enzyme that stabilizes its biological activity and prevents it from leaking out of the catalytic layer (Andreescu et al., 2002).

Chlorpyrifos is a common pesticide widely used in agriculture but few groups focused their research on this compound probably because it is a weak AChE inhibitor (Cetinkaya and Baydan, 2010). Detection limit achieved are comprised between 1  $\mu$ M (Halamek et al., 2005) and 1 pM (Viswanathan et al., 2009).

There are few papers about use of ionic liquids in development of pesticide biosensors. Direct detection of methylparathion was performed with 1-butyl-3-methylimidazolium hexafluorophosphate–single-walled carbon nanotube paste coated glassy carbon electrode (Fan et al., 2008). A biosensor for detection of paraoxon that use immobilized organophosphate hydrolase on MWCNT/IL-modified Au electrodes has also been described (Choi

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et al., 2009). Anyway, no biosensor based on AChE and IL–carbon nanotubes gels was reported in the literature.

Pyridine-2-aldoxime methochloride (PAM) was exclusively used for AChE biosensor regeneration achieving a reactivation degree between 90 and 95% (Du et al., 2007b, 2010). The reactivation potency of various pyridinium oximes was also investigated and obidoxime was found to be more efficient than PAM in the recovery of AChE activity (Jokanovic and Maksimović, 1995; Kuca et al., 2004).

In this paper a new AChE biosensor for chlorpyrifos was developed based on IL–MWCNT gel thiocholine sensor previously reported. Due to the highest sensitivity presented at a low applied potential (+50 mV) 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]) ionic liquid was chosen for this study (Rotariu et al., 2010).

Sol–gel immobilization of AChE was used in this work providing an excellent protection of the AChE molecule from the potential inhibitory effect of IL. Obidoxime was tested as reactivator of the enzyme activity and proved to be more effective comparing with pralidoxime. This is the first report about use of obidoxime for an AChE biosensor regeneration.

## 2. Experimental

### 2.1. Materials

Acetylcholinesterase (AChE, from electric eel, 425.94 IU/mg) acetylthiocholine chloride (ATCh, purity  $\geq 99\%$ ), multiwalled carbon nanotubes (MWCNT, purity  $\geq 95\%$ , outside diameter = 40–60 nm, inside diameter = 5–10 nm, length = 0.5–500  $\mu\text{m}$ ), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]), PAM, obidoxime (1,1'-[oxybis(methylene)]bis{4-[(E)-(hydroxyimino)methyl]pyridinium}), acetonitrile (purity  $\geq 99.9\%$ ), tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMOS), polyethylene glycol (PEG600) were purchased from Sigma–Aldrich. Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate, purity  $\geq 99.9\%$ ) were purchased from Riedel-de Haën. The carbon paste (66% graphite, 34% paraffin) was supplied by Metrohm. The AChE stock solution (66 IU/mL) was prepared in 0.1 M PBS, pH 8. The chlorpyrifos dilutions were prepared in ultrapure water from a  $10^{-4}$  M stock solution, prepared in acetonitrile.

### 2.2. Instruments

Electrochemical measurements were carried out using a potentiostat AUTOLAB PGSTAT12 (Eco Chemie BV, Utrecht, Netherlands). A three-electrode cell was used: mini carbon paste electrodes (diameter 3.0 mm) as working electrode, an Ag/AgCl 3 M KCl reference electrode and a Pt auxiliary electrode, all produced by Metrohm. All determinations were performed at +50 mV vs. Ag/AgCl reference electrode. All experiments were carried out at room temperature (22 °C), using a 5 mL cell.

### 2.3. Preparation of AChE biosensor

#### 2.3.1. Preparation of thiocholine sensor

100  $\mu\text{L}$  of [BMIM][BF<sub>4</sub>] was sonicated with 5 mg MWCNT for 45 min in a 1.5 mL tube. The composite gel is centrifuged at 6400 rpm and the excess of ionic liquid was removed. Preparation and modification of the mini-carbon paste electrode with composite gel was performed according to the procedure described by Rotariu et al. (2010).

**Table 1**

Response characteristics of AChE biosensors for different enzyme loading.

| AChE (IU/electrode) | Linear range (mM) | Sensitivity ( $\mu\text{A mM}^{-1}$ ) | Correlation ( $R^2$ ) | LOD <sup>a</sup> (mM) |
|---------------------|-------------------|---------------------------------------|-----------------------|-----------------------|
| 0.96                | 0.3–7             | 2.42                                  | 0.9992                | 0.12                  |
| 0.24                | 0.2–5.5           | 1.92                                  | 0.9988                | 0.10                  |
| 0.13                | 0.2–4             | 1.12                                  | 0.9972                | 0.10                  |
| 0.066               | 0.1–3             | 0.81                                  | 0.9890                | 0.08                  |
| 0.033               | 0.1–3             | 0.55                                  | 0.9735                | 0.07                  |
| 0.017               | 0.05–4            | 0.17                                  | 0.9946                | 0.03                  |

<sup>a</sup> LOD was calculated for S/N = 3.

### 2.3.2. AChE immobilization procedure

The sol–gel matrix was prepared following a protocol previously published (Andreescu et al., 2002). 100  $\mu\text{L}$  TMOS and 100  $\mu\text{L}$  MTMOS were mixed with 400  $\mu\text{L}$  of ultrapure water, 440  $\mu\text{L}$  of HCl 1 mM and 40  $\mu\text{L}$  PEG600 in order to hydrolyze the monomers and to obtain the “sol”. After sonication for 5 min, the mixture was stored overnight at 4 °C. The AChE solution was mixed with “sol” in a ratio of 50:50 (vol.) and 5  $\mu\text{L}$  of this mixture are dropped on the modified CP electrode. The biosensor is left to dry for 3–4 h at 4 °C and kept afterward in phosphate buffer pH = 8 at 4 °C.

## 3. Result and discussion

### 3.1. Study of the effect of ionic liquid on the enzyme kinetic

Arning et al. (2008) reported the ILs potential inhibitory effect on the activity of AChE. ILs have also the capability to disperse the carbon nanotubes and form composite gels (Fukushima and Aida, 2007). Consequently, the mobility of the [BMIM][BF<sub>4</sub>] is reduced in the composite gel and it is expected to have a low accessibility to the enzyme catalytic site.

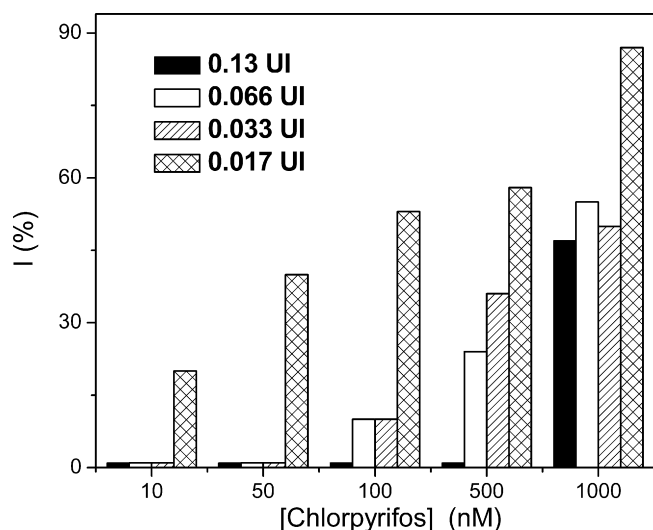
Immobilization of the AChE in sol–gel matrix intended to prevent the loss of the enzyme from the catalytic layer and to protect it from the potential inhibitor effect of the IL. In order to verify the efficiency of the immobilization procedure two biosensors were prepared: one based on a CP electrode and another one based on [BMIM][BF<sub>4</sub>]-MWCNT gel-modified CP electrode. 0.96 IU AChE were immobilized on both sensors. Enzyme reaction rate was determined amperometrically. The Lineweaver–Burk linear representation of the kinetic data is presented in Fig. S1 and the calculation of the apparent Michaelis–Menten constant gives similar results, 2.1 mM in the absence of IL and 6.6 mM in the presence of [BMIM][BF<sub>4</sub>] gel. These results lead us to the conclusion that no inhibition from IL could be distinguished and AChE immobilized in sol–gel matrix could be successfully used for determination of pesticide without any interference from the IL.

### 3.2. Optimization of the amount of immobilized AChE

The amount of AChE immobilized onto the electrode surface is crucial for pesticide determination. A compromise between a high enough analytical signal and a highest possible inhibition degree should be realized.

Amounts of AChE between 0.96 and 0.017 IU were immobilized onto the thiocholine sensor. The optimum ATCh concentration and biosensors sensitivity were determined from calibration curves. To determine the enzyme activity, an excess of substrate is necessary in order to ensure a saturation of the enzyme catalytic sites but it has to be avoided the inhibition by excess of substrate. This is why the ATCh concentration should be very carefully settled.

For 17 mIU AChE the plateau is reached at about 5 mM ATCh while for higher amount of enzyme the saturation is observed at approximately 10 mM ATCh. Table 1 presents the main response



**Fig. 1.** Effect of enzyme loading on the inhibition degree of AChE biosensor. (AChE 17 mIU,  $E = 50$  mV, ATCh 5 mM, pH = 8, incubation time 15 min.)

characteristics for each AChE loading. Despite the fact that the sensitivity decreases dramatically from 0.96 to 0.017 IU/electrode, it should be noticed that pesticide detection is performed in enzyme saturation conditions.

Effect of enzyme loading on the inhibition degree was studied using chlorpyrifos concentrations in the range  $10^{-8}$ – $10^{-6}$  M and the results are presented in Fig. 1. Amperometric signal is recorded after addition of ATCh 5 mM. After incubation with chlorpyrifos solution for 15 min a new amperometric measurement is realized in the presence of ATCh 5 mM. The inhibition degree is calculated according to the relation:

$$\text{Inhibition (\%)} = \frac{i_0 - i_1}{i_0} \times 100 \quad (1)$$

where  $i_0$  and  $i_1$  are the currents recorded before and after pesticide inhibition, respectively.

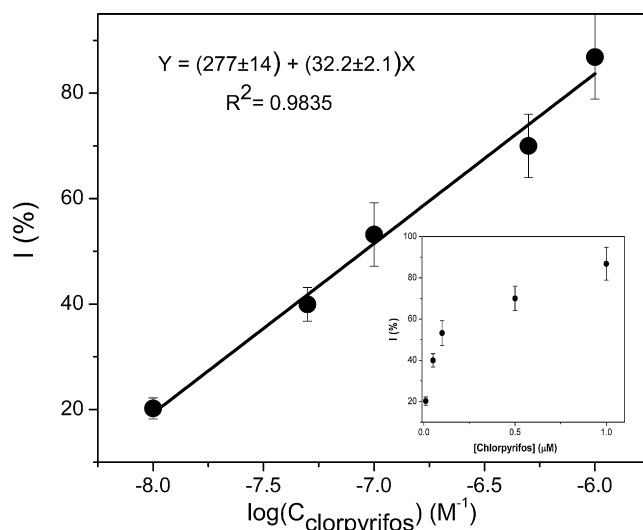
Fig. 1 reveals that only the biosensor with 17 mIU AChE gives a measurable inhibition for 10 nM chlorpyrifos. The highest inhibition percentage was also calculated for other pesticide concentration using the same biosensor. All further chlorpyrifos determinations were performed with 17 mIU AChE/electrode and a substrate concentration of 5 mM.

### 3.3. Storage stability of AChE biosensor

Biosensors with immobilized AChE were stored for 24 h, at  $4^\circ\text{C}$  in different conditions: air, desiccator and buffer. Biosensor stored in buffer presented, for ATCh 5 mM, a signal for more than 2 times higher comparing with other biosensors (data not shown). Buffer solution maintains an appropriate environment for enzyme and ensures a hydration of the sol–gel structure. After 1 week the signal reaches 95% from the initial value, indicating a good stability of the biosensor.

### 3.4. Optimization of the incubation time with pesticide

$10^{-8}$  M chlorpyrifos was tested and the pesticide was incubated with immobilized enzyme between 5 and 30 min. A major increase of inhibition degree is observed from 5 to 15 min from 5 to 20% inhibition degree. No significant increase is observed for incubation time higher than 15 min. Therefore, all inhibition experiments were performed with an incubation time of 15 min. This value is similar with data reported by other research groups (Du et al., 2010).



**Fig. 2.** Calibration curve of chlorpyrifos biosensor. (AChE 17 mIU,  $E = 50$  mV, ATCh 5 mM, pH = 8,  $n = 5$ ).

### 3.5. Calibration curve for chlorpyrifos

Biosensor calibration was performed using chlorpyrifos as reference pesticide. For each concentration, 5 replicates were realized and the mean value of the inhibition degree was represented against chlorpyrifos concentration. Calibration curve presented in Fig. 2 (inset) was linearized using a semi-logarithmic representation. Chlorpyrifos concentration could be determined in the range  $10^{-8}$ – $10^{-6}$  M and the RSD was no more than 6%.

Detection limit was calculated to be 4 nM (approximately 1 ppb) for an inhibition degree of 10%. This is one of the lowest detection limit reported for this pesticide using commercial AChE from *Electric eel* and amperometric detection (Hildebrandt et al., 2008; Prabhakar et al., 2007). A lower detection limit of  $10^{-12}$  M chlorpyrifos was achieved by Viswanathan et al. (2009).

### 3.6. Biosensor regeneration

AChE inhibition by organophosphate is caused by an interaction between phosphate group and serine hydroxyl group from catalytic site. Oximes like PAM, obidoxime (Fig. S2) could reverse the binding of cholinesterase inhibitors with acetylcholinesterase.

Some papers showed that obidoxime is more effective in AChE regeneration and it presents higher kinetic constants of reactivation comparing with PAM (Kuca et al., 2004). Therefore, we decided to use obidoxime for biosensor regeneration.

Effect of obidoxime on the biosensor regeneration was studied using 10 nM chlorpyrifos. Initial measured inhibition degree was  $20 \pm 3\%$ . Then the biosensor was incubated with obidoxime solution under stirring. Regeneration degree was calculated with formula:

$$\text{Regeneration (\%)} = \frac{i_R - i_1}{i_0 - i_1} \times 100 \quad (2)$$

where  $i_R$  is the current recorded after reactivation with oxime,  $i_0$ ,  $i_1$  have the meaning from Eq. (1).

Fig. S3A shows the regeneration degree of the biosensor after incubation with obidoxime 0.1, 2.5 and 5 mM with an incubation time from 1 to 15 min. In all cases 5 min were enough to completely regenerate the biosensor. Obidoxime proved to be a fast reactivator of the AChE and for all experiments a regeneration time of 5 min was used.

Fig. S3B shows the variation of the regeneration degree function of the obidoxime concentration and the inhibition degree for a second incubation with chlorpyrifos 10 nM. At higher concentrations of obidoxime, apparently the regeneration is sometimes more than 100%. Some studies showed that hydrolysis of AChE can occur in the presence of oximes (Kuca et al., 2004). The hydrolysis velocity is higher in the presence of obidoxime. Basic pH also favors the hydrolysis reaction. The presence of two positive charges onto the obidoxime molecule leads to a stronger reactivator–enzyme bond. This could explain the apparent higher regeneration degree of biosensor.

A lower inhibition degree was obtained after regeneration with higher concentration of obidoxime probably also due to the presence of obidoxime traces, which prevent the enzyme inhibition. A final washing step with buffer will reduce considerably this potential effect.

Concentration of 0.1 mM obidoxime was considered the optimal concentration, which gives reproducible regeneration and inhibition degree in repetitive cycles.

To compare the regeneration effect of pralidoxime and obidoxime two biosensors were incubated with chlorpyrifos 10 nM for 15 min. Then one biosensor was incubated with obidoxime 0.1 mM and the other with pralidoxime 0.1 mM for 5 min. The results showed that PAM regenerates only 65% from the enzyme activity comparing with 100% for obidoxime.

The same regeneration effect is obtained with 5 mM PAM in 30 min and with 0.1 mM obidoxime in 5 min. Moreover, PAM is less effective for the second and third regeneration while obidoxime could be used repeatedly with very good reproducibility in both directions inhibition and regeneration (Fig. S3C). The successive inhibition–regeneration cycles showed a variation of the analytical signal of no more than 5% ( $n=8$ ), indicating a very good precision of the measurements.

#### 4. Conclusions

A low potential (+50 mV), highly sensitive amperometric biosensor for chlorpyrifos detection has been developed based on [BMIM][BF<sub>4</sub>]/MWCNT-CP electrode. AChE immobilization in sol–gel matrix conducted to a very stable biosensor. The amount of immobilized AChE was optimized in order to have the highest possible sensitivity for chlorpyrifos detection. A low detection limit of 4 nM was achieved using 17 mIU AChE. An extended two decades response range from 10 nM to 1  $\mu$ M chlorpyrifos was obtained with RSD = 6%.

The capacity of obidoxime on the biosensor regeneration was studied. Complete AChE reactivation is achieved after 5 min incubation with 0.1 mM obidoxime. A comparison between pralidoxime and obidoxime reveals the fact that obidoxime is more effective than pralidoxime in removing the inhibitor from the enzyme catalytic site. The use of obidoxime as regeneration agent permitted multiple and fast analysis of chlorpyrifos.

The resulting AChE biosensor exhibited high sensitivity, good reproducibility and stability, very simple and low cost construction, which provided a new promising tool for pesticide analysis.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.02.001.

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