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A New Label-Free Impedimetric Affinity Sensor Based on Cholinesterases for Detection of Organophosphorous and Carbamic Pesticides in Food Samples: Impedimetric Versus Amperometric Detection

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Abstract Due to their increasing use in agriculture, the presence of pesticide residues in food and water currently represents one of the major issues for the food safety. Among the pesticides, organophosphate and carbamate species are the most used, and their toxicity is mainly due to their inhibitory effect on acetylcholinesterase (AChE). For this reason, a monoenzymatic acetylcholinesterase impedimetric biosensor was developed in order to sensitively detect carbamate and organophosphate compounds with a very fast response. The working principle of the AChE biosensor exploits the capability of carbamate and organophosphate pesticides to form a stable complex with the enzyme, which causes an impedimetric change. The impedimetric biosensor showed a linearity between 5 and 170 ppb for carbamates and 2.5–170 ppb for organophosphate compounds, with a reproducibility (RSD%) interelectrode equal to 4.8 and 3.1% for organophosphates and carbamates, respectively. Moreover, the common amperometric evaluation of AChE inhibition degree was correlated to the impedimetric changes of the electrode surface, showing a good correlation ($R^2 = 0.99$ for carbamates and $R^2 = 0.98$ for

organophosphates) between the two methods. In contrast to amperometric evaluation that needs a response time of 20 min, impedimetric detection requires only 4 min. Finally, the impedimetric biosensor was used to measure carbaryl and dichlorvos spiked in different concentrations in tap water and lettuce samples, showing a recovery near to 100% for all concentrations and for both pesticides.

Keywords Pesticides · Electrochemical impedance spectroscopy · Affinity biosensors · Acetylcholinesterase

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Introduction

Pesticide is a term associated to organic toxic compounds used to protect crops and seeds by destroying insects, bacteria, weeds, rodents and other pests (Sassolas et al. 2012).

The presence of pesticide residues in food, water and soil currently represents one of the major issues for the environmental chemistry; they are, in fact, among the most abundant environmental pollutants due to their increasing use in agriculture. Among the pesticides, organophosphate and carbamate species are the most used because of their high insecticidal activity and relatively low persistence with respect to organochlorine pesticides as well as aldrin or lindane (Arduini et al. 2006).

Carbamates (CBs) and organophosphates (OPs) toxicity is mainly due to their inhibitory effect on acetylcholinesterase (AChE), a key enzyme for the nerve transmission: the inhibition of this enzyme leads to muscle weakness, miosis, respiratory failure, unconsciousness, convulsion and, eventually, death (Storm et al. 2000).

For these reasons, there is a general concern on pesticide food contamination and, consequently, the development of

simple and sensitive strategies for detecting toxic compounds is critically important in order to carry out the measurement "in situ" using miniaturized, cost-effective and easy to use analytical system.

In particular, the biosensors based on AChE inhibition are reliable tools for the detection of OP and CB compounds (Andreescu and Marty 2006, Vakurov et al. 2004). These pesticides are able to form a covalent link (irreversible for OPs and pseudo-irreversible for CBs) with the serine residue present in the catalytic site of the enzyme, blocking the hydrolysis of the acetylcholine in choline and acetic acid.

The most common biosensors developed for pesticides detection are based on AChE inhibition. In particular, the enzymatic activity of AChE is measured before and after the exposure to pesticide samples by different transduction techniques such as amperometric, piezoelectric and optical transducers (Arduini et al. 2013, Albanese et al. 2012, Valdes -Ramirez et al. 2008, Caetano and Machado 2008).

Because the AChE inhibition occurs for both pesticide groups is not specific, AChE-based biosensors give information about the toxicity level of a sample; thus, they can be used as a warning system for this class of toxic compounds, followed, in the case of positive response, by the HPLC or GC-MS analyses to exactly detect the type and the amount of pesticide in the sample (Moscone et al. 2016).

The electrochemical impedance spectroscopy (EIS) is an interesting transduction technology, which enables the direct analyte detection by studying the electrical properties of the sensing device interface: the EIS measurements involve the analysis of impedimetric changes, at the electrode interface when a biological or chemical element interacts with the electrode surface functionalized with a bioreceptor. In particular, EIS combines the analysis of both resistive and capacitive properties of the electrode surface, based on the small amplitude perturbation of a system from steady state, which makes it a non-destructive technique (Guan et al. 2004; Bahadir and Sezginturk 2016).

This type of transduction allows the development of affinity-binding biosensors with noteworthy advantages due to their direct and label-free detection for the analyte of interest.

Exploiting the inhibition mechanism of pesticides versus AChE, and the capability of EIS to measure interaction between the analyte and the bioreceptor, we considered applying the EIS as transduction method for the AChE-based biosensors for the direct measurement of pesticides inhibition compounds in real matrix.

For this study, AChE was immobilized via glutaraldehyde on an electrochemical-deposed multilayer of cysteamine on printed gold electrode. EIS and cyclic voltammetry (CV) were used to characterize each step of electrode modification and the analytical performance of the biosensor

versus CB and OP compounds. Comparison between the impedimetric characterization and the amperometric ones was also reported. The influence of lead and mercury on biosensor response was examined, and finally, it was used to measure carbaryl and dichlorvos spiked in tap water and lettuce samples.

Materials and Methods

Reagents

Acetylcholinesterase (AChE 2 KU, 149 U/mg$_{solid}$), cysteamine (95%), glutaraldehyde solution (50% in H$_2$O), potassium hexacyanoferrate (III) ([Fe(CN)$_6$]$^{3-}$, >99%), hexane (CH$_3$(CH$_2$)$_4$CH$_3$, 99%), carbaryl (C$_{10}$H$_7$OCONHCH$_3$), paraquat dichloride hydrate (C$_{12}$H$_{14}$C$_{12}$N$_2$·H$_2$O), dichlorvos (C$_4$H$_7$C$_{12}$O$_4$P), chlorpyrifos-methyl (C$_9$H$_{11}$C$_{13}$NO$_3$PS), phosmet (C$_{11}$H$_{12}$NO$_4$PS$_2$) and kresoxim-methyl (C$_{18}$H$_{19}$NO$_4$) were purchased from Sigma-Aldrich (Milano, Italy). Potassium ferrocyanide ([Fe(CN)$_6$]$^{4-}$), lead acetate trihydrate (99.9%) and mercuric (II) chloride were obtained from Carlo Erba reagent (Milano, Italy). Acetylthiocholine chloride was purchased from Molekula (Rimini, Italia). Sodium phosphate monobasic (NaH$_2$PO$_4$), sodium phosphate dibasic anhydrous (Na$_2$HPO$_4$) and potassium chloride (KCl) used in the preparation of phosphate buffered (PB 0.1 M KCl, pH 7) were received from Sigma-Aldrich (Milano, Italy).

Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat and NOVA software. Gold thin-film single electrodes and the all-in-one electrochemical cell were obtained from Micrux Technologies (Oviedo, Spain). The electrodes incorporate a conventional three-electrode configuration, with an Au working (diameter 1 mm), reference and counter electrodes.

Acetylcholinesterase Immobilization Procedure

Before enzyme immobilization, gold electrode was cleaned by applying ten potential cycles between −1.0 and +1.3 V versus reference electrode with 100 mV/s scan rate in 0.05 M sulfuric acid. Then, 20 mM cysteamine water solution was electrodeposited on the electrode surface under a constant potential of 1.2 V versus reference electrode for 10 min. After that, the electrode was covered with an aqueous solution of glutaraldehyde 2.5% (v/v) for 30 min; then, it was thoroughly washed with bi-distilled water to remove the unreacted glutaraldehyde. Finally, the resulting modified electrode surface was incubated with 2.5 enzyme units (U) of AChE overnight at 4 °C.

Electrochemical Measurement

Impedimetric Measurement

The electrochemical impedance spectroscopy (EIS) measurements were carried out over imposing an excitation voltage of 10 mV (versus reference electrode) DC potential, in the frequency range from 0.1 to 10^5 Hz. The measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$, 1:1) in PB, 0.1 M pH 7, as background electrolyte, at room temperature.

Each step, of the biosensor build-up, was interrogated also by cyclic voltammetry (CV): the measurements were performed from −0.6 to 0.6 V versus reference electrode with a scan rate of 0.05 V/s; the redox solution used for the CV measurements was the same used for EIS measurements.

For pesticide measurements, 20 μL of CB and OP compounds, respectively, at different concentrations was dropped onto the electrode working area and incubated for 30 min; before the impedance measurement, the biosensor was rinsed with copious amount of bi-distilled water. Single frequency impedance (SFI), at 0.1 Hz, was used for kinetic analysis, in order to calculate the kinetic dissociation constants of OP and CB with AChE.

Amperometric Measurement

The degree of pesticide inhibition was calculated using the following equation:

$$I\% = \frac{i_0 - i_i}{i_0} \times 100, \qquad (1)$$

where (i_0) and (i_i) represent the amperometric biosensor response, before and after the incubation, respectively, at 2 mM substrate (acetylthiocholine) concentration (Albanese et al. 2012). Acetylthiocholine measurements were performed using amperometric analysis in a flow injection analysis (FIA) apparatus, under a constant potential of 0.4 V. Carrier solution (0.1 M phosphate buffer in 0.1 M KCl, pH 7) from a reservoir was pumped with a peristaltic pump (Miniplus 3, Gilson, France) at flow rate of 0.5 mL/min to the injection valve (sample injection valve, Omnifit) equipped with a 100-μL sample loop.

Kinetic Analysis

Kinetics studies of AChE enzymatic reaction were carried out in order to analyse mechanism of enzyme inhibition. Tests were monitored by single frequency impedance (SFI), at 0.1 Hz, obtaining a plot of total impedance of the system versus time.

The inhibition reaction of AChE by a CB or OP compound takes place in two-step process: the first step involves the formation of the enzyme-inhibitor complex with subsequent carbamylation (with CBs) or phosphorylation (with OPs) of the serine hydroxyl resulting in inhibition of the enzyme.

The reaction scheme is the following one (Fukuto 1990):

$$E{-}OH + PX \overset{K_I}{\longleftrightarrow} E \times PX \overset{k_3}{\to} EP + X, \qquad (2)$$

where $E{-}OH$ represents AChE in which the serine hydroxyl moiety (−OH) is emphasized, PX is the pesticide inhibitor, $E \times PX$ is the complex enzyme inhibitor, EP is the carbamylated/phosphorylated enzyme, X is the leaving group, K_I is the dissociation constant for enzyme-inhibitor complex and k_3 is the first-order rate constant for the conversion of the enzyme-inhibitor complex to carbamylated/phosphorylated enzyme.

For the analysis, three different CBs (carbaryl, paraquat, kresoxim-methyl) and three different OPs (dichlorvos, chlorpyrifos-methyl pestanal, phosmet) were used at five different concentrations (5, 10, 32, 48, 80 ppb) and inhibition parameters were evaluated.

Analysis of Food Samples and Interference Study

AChE affinity biosensor was used for the determination of carbaryl and dichlorvos in tap water and lettuce samples.

In order to verify the accuracy of the developed biosensor, three water samples were spiked with three different concentrations of carbaryl (25, 50, 100 ppb) and three different concentrations of dichlorvos (25, 50, 100 ppb).

The preparation of vegetable samples was carried out according to Xavier et al. 2000 and 2.5 g of the homogenate lettuce sample was spiked with known amounts of carbaryl (25, 50, 100 ppb) and dichlorvos (25, 50, 100 ppb). The samples were mixed with 5 mL of pure hexane and sonicated for 15 min. The mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was collected; blank samples were prepared following the previous procedure without pesticide spiking.

Additionally, the influence of two interfering substances, such as lead and mercury, was examined. Two different concentrations of Pb(II) (20, 100 ppb) and Hg(II) (0.5, 1 ppm) were put in contact with AChE enzyme and interference effects were analysed.

Results and Discussion

Electrochemical Characterization of Electrode-Modifying Process

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were widely used as a convenient tools to monitor the various steps of the biosensor build-up.

Figure 1a, b shows the typical cyclic voltammograms and Nyquist plots, respectively, obtained after each step of electrode-modifying process and enzyme immobilization.

The voltammograms of the Au electrode displayed a well-defined anodic and cathodic peaks due to the reversible inter-conversion of $[Fe(CN)_6]^{3-/4-}$. As result of the immobilization on the electrode surface of cysteamine, glutaraldehyde and the final addition of AChE enzyme, the diffusion of the redox probe close to the electrode surface was dramatically reduced, causing a significant decrease of both peaks.

Nyquist plots, in which the imaginary impedance component (Z") is compared against the real impedance one (Z') at different excitation frequencies, showed an increase of total impedance of the system during the immobilization steps due to the layer coating on the electrode surface, which became thicker with the assembly procedure. As expected, the permeability of $Fe(CN)_6^{3-/4-}$ through the immobilization layers was strongly reduced with an increase of the electron transfer resistance.

The CV and EIS results pointed out that the chemical and biomolecular layers act as effective barriers to the charge transfers (Malvano et al. 2016).

Determination of OP and CB Pesticides by Amperometric Detection

Amperometric inhibition biosensors require an initial calibration using the enzyme's substrate; the monoenzymatic AChE biosensor uses acetylthiocholine as substrate, according to the following equation:

$$Acetylthiocholine + H_2O \xrightarrow{AChE} Thiocoline + Acetate\ Acid\ (3)$$
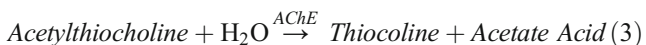
The substrate was enzymatically hydrolysed to thiocoline, which was then subjected to electrocatalytic oxidative dimerization at 0.4 V to give the disulphide compound. This response, at the working electrode, was correlated to the activity of AChE (Chauhan and Pundir 2011).

The optimization of biosensors based on AChE inhibition is closely linked to amperometric signal read by electrochemical transducer. Although a high enzyme activity is required for reproducible and long-term measurements with suitable electrode response on acetylthiocholine, sensitive inhibitor determinations are favoured at low enzyme loading. From preliminary tests and according to our previous studies (Albanese et al. 2012), 2.5 U of AChE was chosen for the construction of AChE biosensor. The calibration curve of the amperometric AChE biosensor (Fig. 2), obtained for different substrate concentrations, showed an high sensitivity 477.34 nA/mM in the range from 0.1 to 2 mM, a limit of detection (LOD) of 0.1 mM, defined as the acetylthiocholine concentration that yields a signal-to-noise (S/N) ratio equal to 3, and a good repeatability of the current response with relative standard deviation (R.S.D.) of 3.78% (calculated on five different injections at 0.5 mM).

For inhibition measurement, with the aim to ensure that the biosensor is working under kinetic-controlled conditions, the concentration of the substrate used must be near the upper limit of the system and within the linear range of response (Liu and Lin 2006). According to above results, 2 mM acetylthiocholine was used to carry out the inhibition tests and the degree of pesticides inhibition was calculated using Eq. (1) mentioned above.

As reported in the official methods for pesticides detection (EPA), the extraction of pesticides is usually carried out using organic solvent; because pesticides are often extracted with pure hexane (Xavier et al. 2000), the effect of this solvent on the AChE activity was evaluated. The influence of this organic solvent, which is completely insoluble in aqueous phase, gave satisfactory results. In fact, according to previous studies (Albanese et al. 2012; Andreescu et al. 2002), after 45 min of biosensor incubation in pure hexane, only a marginal reduction of biosensor response (less than 5%) was detected compared with initial value (data not shown).

Therefore, this solvent was used for the preparation of pesticides standard solutions without effect on enzyme activity
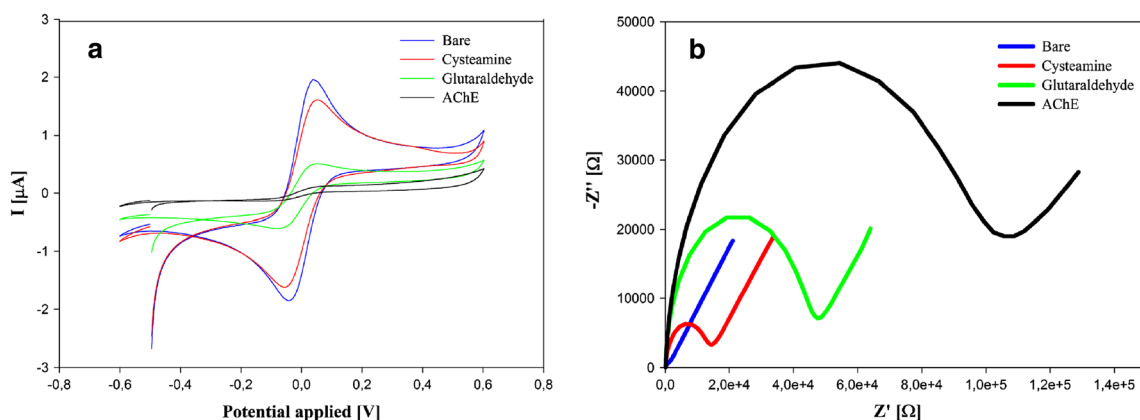
Fig. 1 Cyclic voltammograms in 1 mM $[Fe(CN)_6]^{4-/3-}$ after each step of biosensor construction (**a**). EIS response to all biosensor fabrication steps (**b**)
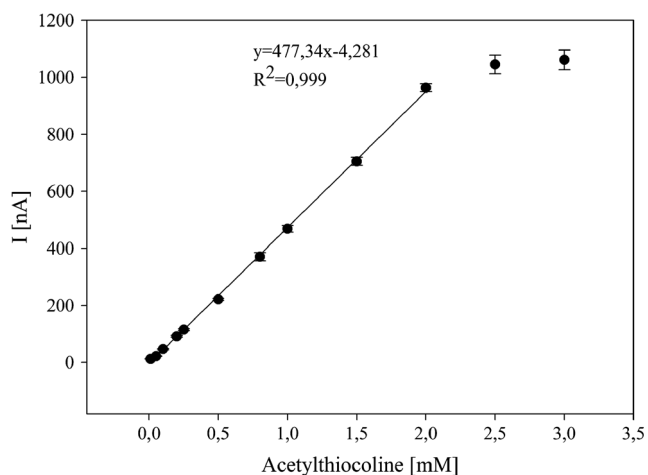
Fig. 2 Calibration curve of AChE biosensors with 2.5 U of AChE enzyme. Data represent the average of three biosensors. Applied potential 400 mV. 0.1 M phosphate buffer 0.1 M KCl, pH 7

and the calibration curves of the AChE biosensor tested with three different CBs (carbaryl, paraquat, kresoxim-methyl) and three different OPs (dichlorvos, chlorpyrifos-methyl pestanal, phosmet) are showed in Fig. 3a, b, respectively.

The AChE biosensor showed a similar sensitivity for CBs and OPs and the same linear range for all analysed compounds: in fact, considering molecular weights of each pesticide, it was able to detect both of compounds in a range of 10–150 ppb.

The detection limits (LOD), calculated as the pesticide concentration that yields the inhibition signal three times higher than noise, were 8 and 10 ppb for CBs and OPs compounds, respectively. These results are competitive with previous studies (Arduini et al. 2006; Arduini et al. 2013; Caetano and Machado 2008; Chauhan and Pundir 2011), who used printed gold electrodes and AChE as monoenzymatic system.

Finally, the biosensor was characterized by a reproducibility (RSD%) interelectrode, calculated on five different AChE biosensors, of 3.1 and 4.8% for OP and CB, respectively.
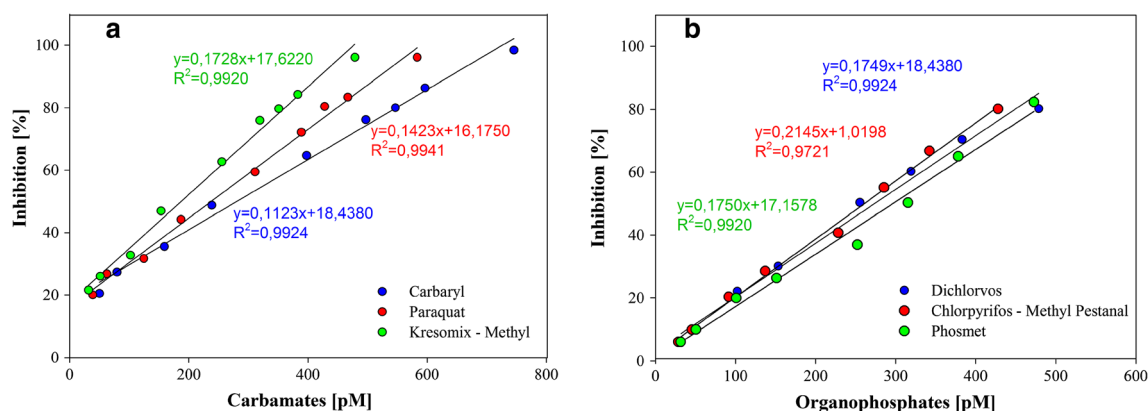
## Impedimetric Detection of Carbamates and Organophosphate Compounds

The capability of EIS to detect the binding between OP and CB compounds, responsible of the enzymatic activity inhibition of AChE, is showed in Fig. 4. The AChE biosensor was put in contact with increasing concentrations of carbaryl (Fig. 4a) and dichlorvos (Fig. 4b) chosen as CB and OP compounds, respectively, and EIS spectra were analysed.

In particular, experimental data were fitted using Nova software by the common Randle's circuit (inset Fig. 4). This equivalent circuit, commonly applied in impedimetric characterizations, consists of resistive, capacitive and diffusive elements: R_s represents the redox solution resistance while CPE (constant phase element) is connected with the capacitance of the complex bioactive layer; R_{ct} is related to the electron transfer resistance through the electrode surface and represent the difficulty of electron transfer of redox probe between the solution and the electrode surface, while the Warburg impedance describes the normal diffusion to the electron surface through the complex layer.

In the given frequency range, for both pesticide classes, a decrease of semicircle diameter of Nyquist plots was observed by increasing pesticide concentration, which correspond to the decrease of R_{ct} values. The ΔR_{ct} value, expressed as the difference between the R_{ct} value before and after the contact enzyme inhibitor, at different pesticide concentrations, was used to characterize the biosensor.

The same behaviour was observed after contact with the other CB and OP pesticides analysed in this work (Fig. S1).

The calibration curves obtained for all tested pesticides (Fig. 5a, b) showed a linear correlation in the range 5–170 ppb for CBs and 2.5–170 ppb for OPs, with a LOD, based on the sum of average blank solution and three times the standard deviation, equal to 5 ppb for carbaryl, paraquat and kresoxim-methyl, and 2.5 ppb for dichlorvos, chlorpyrifos-methyl pestanal and phosmet. AChE biosensor showed a

Fig. 3 Inhibition curves for carbaryl, paraquat, kresoxim-methyl (**a**) and for chlorpyrifos-methyl pestanal, phosmet and dichlorvos (**b**)
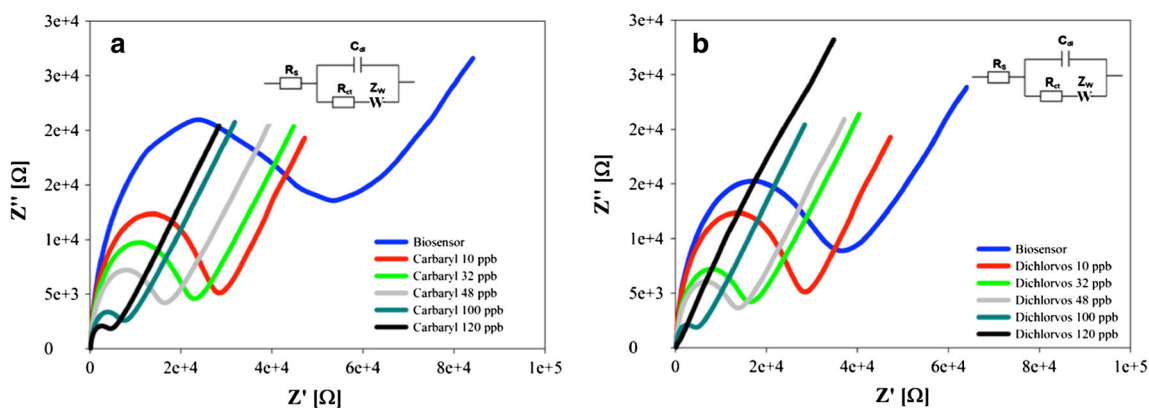
Fig. 4 Nyquist plots of biosensor after incubation of different carbaryl (**a**) and dichlorvos concentrations (**b**). The *inset* corresponds to the equivalent circuit used to fit impedance spectra

reproducibility (RSD%) interelectrode, calculated on five different AChE biosensors, of 3.1 and 4.8% for OP and CB, respectively.

Finally, the storage stability of biosensor was also determined by impedimetric analysis: for this purpose, different AChE biosensors were stored for 3 months at 4 °C without chemical preservatives and characterized at regular interval times. After the investigative storage period, the biosensor showed a negligible loss of activity.

Incubation Time of AChE Biosensor

The incubation time used for the characterization of amperometric and impedimetric AChE inhibition-based biosensors was 40 min. This latter was estimated by single frequency impedance (SFI) that is able to monitor total impedance in a single frequency versus time. In our study, SFI tests were carried out a 0.1 Hz chosen on the basis of the maximum differences among the Bode plots corresponding to different carbaryl and dichlorvos concentrations (Fig. S2 (a) and (b)). A significant change in impedance, for both pesticides, was observed for an incubation time of 40 min, and then no change was registered.

Comparison Between Amperometric and Impedimetric AChE Biosensor

The comparison of the results obtained with the impedimetric and amperometric transduction shows that the first one is able to detect a wider linear range and lower LODs for all pesticides tested (Table 1).

High correlation was found between enzymatic inhibition and change in electrical resistance for all CBs and OPs investigated (Fig. 6a, b) confirming that the binding AChE pesticide observed by impedance transduction is responsible of the decrease of enzymatic activity. It is worth to note that as well as the amperometric detection, the impedimetric technique is not able to discriminate the type of pesticide linked to the enzyme but only the toxicity level given the high relationships between $I\%$ and ΔR_{ct} for both carbamate and organophosphate compounds.

Moreover, the EIS transduction method results to be extremely faster than amperometric one. In fact, at difference of amperometric measurements that require the injection of AChE substrate before and after the exposure with pesticide solution with the impedimetric transduction, only the exposure with the pesticide is required. In our experimental tests, amperometric measurements need of a total
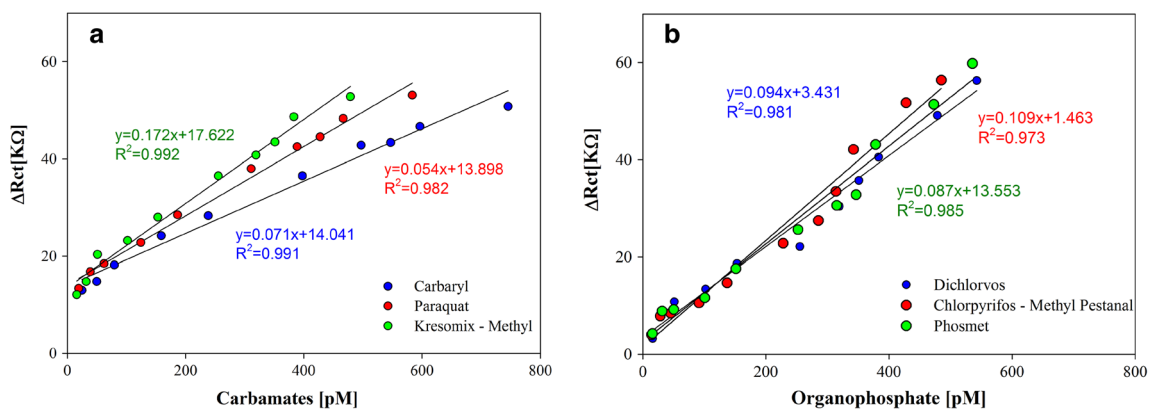
Fig. 5 Calibration curves by impedimetric analysis for all CBs (**a**) and OPs compounds (**b**)

Table 1 Comparison between acetylcholinesterase inhibition-based biosensors developed by amperometric and impedimetric transduction

		Transduction method			
		Amperometry		Electrochemical impedance spectroscopy	
		Linear range (ppb)	Limit of detection (ppb)	Linear range (ppb)	Limit of detection (ppb)
Organophosphates	Dichlorvos	10–150	10	2.5–170	2.5
	Chlorpyrifos-methyl	10–150	10	2.5–170	2.5
	Phosmet	10–150	10	2.5–170	2.5
Carbamates	Paraquat	10–150	8	5–170	5
	Carbaryl	10–150	8	5–170	5
	Kresoxim-methyl	10–150	8	5–170	5

time of 20 min versus the 4 min for impedimetric measurement, excluding the pesticide incubation time.

Kinetic Analysis

Inhibition mechanism of AChE activity by pesticides is presented in the reaction scheme (2), explained above. Time-dependent inhibition is commonly described by the following equation:

$$\ln \frac{E}{E_0} = -\frac{k_3}{1 + K_I / (I)} t, \tag{4}$$

where E/E_0 represents the percentage of the remaining enzyme activity in relation to the initial activity (E_0), K_I is the dissociation constant for the enzyme-inhibitor complex, k_3 is the first-order rate constant for the conversion of the enzyme-inhibitor complex to carbamylated/phosphorylated enzyme, I is inhibitor concentration and t is incubation time (Krstić et al. 2008).

If $I >> E_0$, the slope of Eq. (4), dependent on pesticide concentration, can be expressed in the form (Colovic et al. 2013):

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_3} \frac{1}{(I)} \tag{5}$$

Since the high correlation between the enzymatic activity inhibition and the impedimetric changes for all pesticides studied, we modified Eq. (4) as follows:

$$\ln \frac{R_{ct}}{R_{ct0}} = -\frac{k_3}{1 + K_I / (I)} t, \tag{6}$$

where R_{ct0} and R_{ct} are the impedimetric signals of the AChE biosensor in absence and after the exposure of pesticide solution, respectively.

The values of k_{app} were obtained, for each pesticide at different concentrations, from the slope of the linear dependence of $\ln \frac{R_{ct}}{R_{ct0}}$ versus t. The change of R_{ct} in time was monitored for five different pesticide concentrations by single frequency
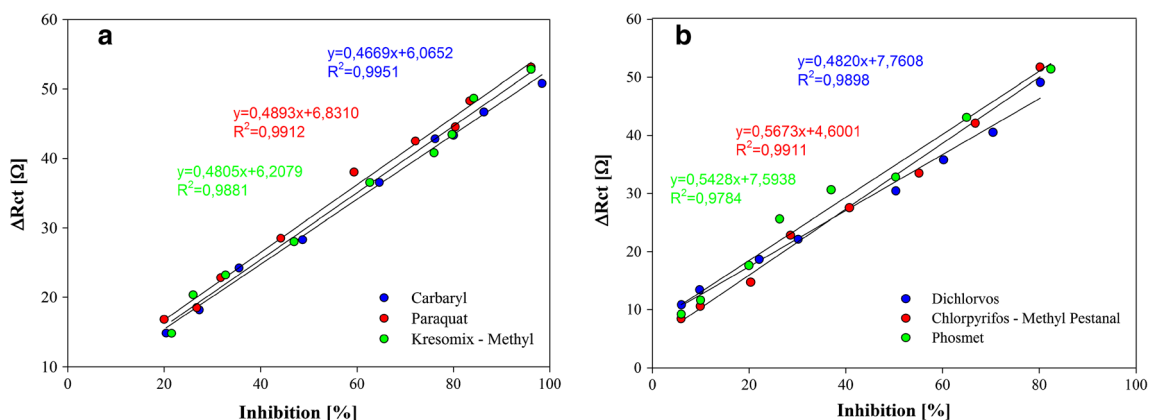
Fig. 6 Linear correlations between AChE inhibition degree and ΔR_{ct} for carbaryl, paraquat, kresoxim-methyl (**a**) and for dichlorvos, chlorpyrifos-methyl pestanal and phosmet (**b**)
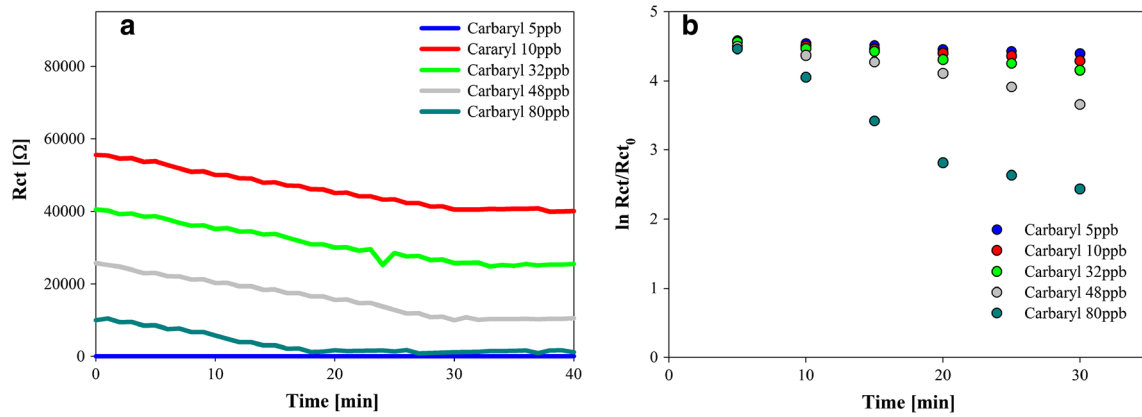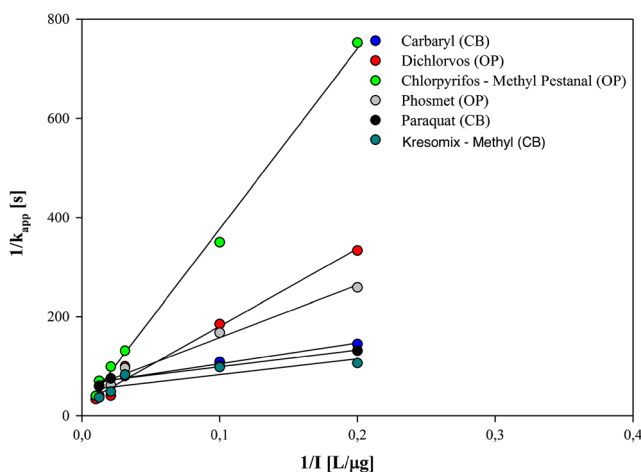
Fig. 7 Single frequency impedance data for carbaryl at different concentrations (**a**). Progressive R_{ct} decrease produced by reaction of AChE with different concentrations of carbaryl plotted as semi-logarithmic curve in accordance with Eq. (5) (**b**)

impedance (SFI): the tests were carried out a 0.1 Hz, as reported above (see the "Incubation Time of AChE Biosensor" section). The change of R_{ct} and $\ln\frac{R_{ct}}{R_{ct0}}$ versus t for carbaryl at five concentrations is shown in Fig. 7a. The slopes of the plots (Fig. 8b) give the k_{app} values. R_{ct} change during the time was studied for all CBs and OPs used in this study (Fig. S3).

Inhibition process by different pesticide concentrations, which progressed with time in accordance with Eq. (5), was analysed also for the other pesticides considered in this work (data not shown).

The values of k_{app}, obtained from the slopes of the dependence of $\ln\frac{R_{ct}}{R_{ct0}}$ versus t for all CBs and OPs tested were then plotted according to Eq. (6) and the results are reported in Fig. 8.

The inhibition parameters K_I and k_3 were calculated from the slope and the intercept of Eq. (6), for all carbamates and organophosphate compounds and the results are showed in Table 2.

Dissociation constants (K_I) calculated for the OPs tested were in accordance with Colovic et al. (2013) and Mehta et al. (2016), who evaluated the inhibition of AChE with diazioxon and chlorpyrifos and parathion, respectively.

No K_I measurements were found in literature about CBs compounds. Since K_I provides a measure of the dissociation of the enzyme-pesticide complex, the data obtained for the investigated CBs and OPs confirm the irreversibility of binding between AChE OP in contrast to the CBs where the acylated intermediate is slowly (about 1 h) hydrolysed to reactivate the enzyme (Darvesh et al. 2008).

Carbamates and Organophosphate Compounds Detection in Real Samples and Interference Studies

The possibility to apply the developed affinity enzymatic biosensor for the detection of pesticide in real food matrices was studied; dichlorvos as organophosphate compound and carbaryl as carbamate were selected. Tap water and lettuce samples were spiked with three different concentrations of carbaryl and dichlorvos and analysed by developed biosensor through impedimetric detection.

Fig. 8 Dependence of K_{app} upon the concentration of chlorpyrifos-methyl pestanal, dichlorvos, phosmet, carbaryl, paraquat and kresoxim-methyl plotted as reciprocal in accordance with Eq. (3)

Table 2 Inhibition parameters K_I and k_3 for carbaryl, paraquat and kresoxim-methyl (as carbamates) and chlorpyrifos-methyl pestanal, dichlorovos and phosmet (as organophosphates)

	Pesticides	K_I [mol/L]	k_3 [1/s]
Carbamates	Carbaryl	3.00×10^{-6}	1.58
	Paraquat	7.92×10^{-6}	1.73
	Kresoxim-methyl	7.44×10^{-6}	1.66
Organophosphates	Chlorpyrifos-methyl pestanal	7.56×10^{-7}	0.73
	Dichlorvos	6.91×10^{-7}	1.15
	Phosmet	2.75×10^{-8}	1.68

Table 3 Carbaryl and dichlorvos results in spiked tap water sample obtained by developed biosensor

Sample	Pesticide	Spiked concentration [ppb]	Biosensor result [ppb]	Recovery [%]
Tap water	Carbaryl	25a	26.53 ± 1.45a	106.12 ± 5.90
		50a	49.69 ± 1.86a	100.62 ± 1.60
		100a	97.50 ± 3.79a	102.49 ± 1.29
Tap water	Dichlorvos	25a	24.87 ± 1.34a	100.50 ± 2.48
		50a	52.48 ± 2.78a	95.02 ± 5.26
		100a	97.92 ± 3.98a	102.07 ± 0.95
Lettuce	Carbaryl	25a	27.12 ± 2.35a	110.00 ± 5.94
		50a	52.14 ± 1.49a	104.28 ± 3.63
		100a	102.10 ± 2.78a	102.10 ± 2.44
Lettuce	Dichlorvos	25a	25.99 ± 1.45a	108.48 ± 4.88
		50a	51.08 ± 2.67a	102.16 ± 3.75
		100a	101.82 ± 0.98b	101.82 ± 1.40

Values are the means of three replicates. The theoretical spiked values and those obtained by impedimetric biosensor were subjected to one-way analysis of variance. Mean values within rows followed by different letters (a, b) are significantly different ($P < 0.05$) by Student–Newman–Keuls multiple comparison test

The results (Table 3) highlight that the developed biosensor exhibited a good recovery percentage for both carbamate and organophosphate compounds.

Heavy metal ions can inhibit the activity of AChE and disturb pesticides detection using the AChE affinity biosensor. Two different concentrations of Pb(II) (20 and 100 ppb) and Hg(II) (500–1000 ppb) were evaluated as inhibitors versus AChE. This interference study was carried out in absence of any pesticides.

An interfering effect lower than 5% was measured at the tested lead concentrations. However, according to literature (Sanllorente-Méndez et al. 2010), the most important interference was caused by Hg(II), which produces an interfering effect of 28 and 42% at mercury concentrations equal to 500 and 1000 ppb, respectively.

This interference study was carried out in absence of any pesticides. No interfering effect was measured at the tested lead concentrations.

Conclusions

A new impedimetric enzyme inhibition-based biosensor for carbamate and organophosphate compounds was proposed, immobilizing acetylcholinesterase enzyme on cysteamine-modified gold electrode.

The high affinity interaction between pesticides and active site of the enzyme was monitored by electrochemical impedance spectroscopy, and the impedimetric changes obtained at different pesticide concentrations allow to go up very fast to the presence of the toxic compounds in tap water or food matrices.

The developed affinity AChE biosensor, with its high number of attractive characteristics associated to the use of EIS transduction, can be considered as promising candidate for pesticide detection on-site applications.

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