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# Acetylcholinesterase biosensor based on self-assembled monolayer-modified gold-screen printed electrodes for organophosphorus insecticide detection

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ABSTRACT

A mono-enzymatic acetylcholinesterase (AChE) amperometric biosensor for organophosphate detection was developed immobilizing the AChE enzyme via glutaraldehyde on a preformed cysteamine self-assembled monolayer (SAM) on gold-screen printed electrodes (Au-SPEs). The enzymatic activity was monitored measuring the enzymatic product, thiocholine, at an applied potential of +400 mV vs. Ag/AgCl using ferricyanide in solution as electrochemical mediator. The electrocatalytic activity of ferricyanide towards thiocholine was investigated by using Nicholson–Shain method and finding a second order homogenous rate constant $k_s$ equal to $(5.26 \pm 0.65) \times 10^4\ M^{-1}\ s^{-1}$. In order to develop a sensitive biosensor, the effect of cysteamine concentration, duration of SAM deposition and AChE concentration were optimized. Using paraoxon as model compound, the biosensor showed a linear range up to 40 ppb with a detection limit of 2 ppb (10% of inhibition). The biosensor was successfully challenged with drinking water sample demonstrating to be a useful analytical tool for organophosphorus insecticide detection.


## 1. Introduction

Pesticides are among the most important environmental pollutants because of their significant presence in the environment [1]. The organophosphorus insecticides are one of the most used insecticides due their high toxicity but low persistence in the environment when compared with the organochlorine pesticides. Their toxicity is based on the ability to irreversibly inhibit AChE which is a key enzyme of nervous transmission. AChE rapidly converts the neurotransmitter acetylcholine to choline and acetic acid after the transmission of a nerve impulse. As part of normal cholinergic neurotransmission, a properly functioning of AChE is a critical step; in fact, the inhibition of this enzyme leads to cholinergic dysfunction and death [2,3]. The detection of organophosphorus insecticides is generally carried out using gas chromatography or high performance liquid chromatography that require skilled personnel and laboratory set-up [4,5]. Simple and sensitive strategies for detecting organophosphorus compounds are therefore critically important in order to perform the measurement "in situ" using miniaturized, cost-effective and easy to use analytical systems. In this context, the use of cholinesterase enzymes has shown great promise to assemble enzyme sensors for environmental screening analysis [6–9]. By measuring the AChE activity before and after exposure of the biosensor to environmental samples, it is possible to quantify the amount of organophosphorus insecticides present in the sample.

The development of an AChE biosensor requires the immobilization of AChE on the transducer and the immobilization is a key point to obtain a sensitive biosensor. In fact, the enzyme should retain its quaternary structure, should be close to the transducer and the film of the membrane should be thin in order to avoid a limited diffusion of the enzymatic substrate. In literature several techniques for immobilizing enzymes on transducers are reported, such as adsorption [10], entrapment by means of sol–gels [11,12] and cross-linking [13,14]. Unfortunately, usually using these types of immobilization no control over the orientation of the enzyme is achieved [15]. In order to avoid this drawback, the immobilization by using SAM has been reported as a successfully alternative to fabricate biosensors [16]. SAMs were usually prepared using the affinity of thiols such as alkanethiols for some metal surfaces, particularly gold. In this case, the main advantage consists in the immobilization of the enzyme close to the electrode surface with a high degree of control over the molecular architecture of the recognition interface [17,18]. Few papers in literature report the assembling of a AChE-SAM biosensor. Somerset et al. have developed a gold electrode modified with mercaptobenzothiazole and either poly(o-methoxyaniline) or poly(2,5-dimethoxyaniline) [19,20]. An AChE based amperometric biosensor was developed by immobilizing the enzyme onto a self assembled modified gold electrode using 3-mercaptopropionic acid, glutaraldehyde or

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(N″-cyclohexy-N″-(2-morpholinoethyl)carbodiimide)methyl-p-toluenesulphonate by Pedrosa et al. [21,22]. An AChE biosensor was also constructed by means of gold nanoparticles and cysteamine assembled on glassy carbon paste [23] or by single walled carbon nanotubes wrapped by thiol terminated single strand oligonucleotide (ssDNA) on gold [24].

In all these cases, however, keeping in mind that the organophosphorus insecticides are able to irreversibly inhibit the AChE, after each measurement the biosensor need to be re-prepared or reactivated. The AChE biosensor, in fact, can be reactivated putting in contact the biosensor, after the measurement, for several minutes with 2-PAM (pyridine-2-aldoxime methyliodide) solution [25] or, for example, obidoxime solution [26]. The reactivation should be performed immediately after the measurement, in order to avoid the enzyme phenomenon called "ageing" that renders the inhibited enzyme more resistant to the reactivation becoming permanently inhibited [27]. In order to avoid these steps with drawbacks in term of time of analysis, the use of screen printed electrodes (SPEs) can be a successfully alternative.

The gold SPEs have the advantages to be miniaturized, mass produced, and cost-effective, thus suitable for a single measurement, properties very useful in the case of irreversible inhibition based biosensors. In the present work we report the development of a novel amperometric mono-enzymatic AChE biosensor in which the enzyme is immobilized via glutaraldehyde on a preformed SAM of cysteamine onto Au-SPE. In order to have a mono-enzymatic biosensor, the acetylthiocholine was used as substrate. The enzymatic product thiocholine was detected by using ferricyanide in solution as electrochemical mediator.

## 2. Materials and methods

### 2.1. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to an X-t recorder (L250E, Linseis, Selb, Germany).

Cyclic voltammetry (CV) was performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands). Electrochemical impedance spectroscopy (EIS) measurements were carried out in the same cell with a PC-controlled Autolab. A sinusoidal voltage perturbation of 10 mV amplitude was applied over the frequency range of 100 kHz to 0.1 Hz with 10 measurement points per frequency decade. For the fitting of the data obtained by EIS, Z-views software (Scribner Associates, Inc.) was used.

### 2.2. Electrodes

Au-SPEs were bought from Ecobioservice (Florence, Italy). The diameter of the working electrode was 0.3 cm resulting in an apparent geometric area of 0.07 $cm^2$. Before thiol measurements, the reference electrode was chlorinated by applying a potential of 0.6 V between the silver and an external Ag/AgCl electrode for 20 s in a phosphate buffer solution in the presence of 0.1 M KCl [28].

### 2.3. Reagents

All chemicals from commercial sources were of analytical grade. Potassium ferricyanide from Carlo Erba (Milano, Italy), acetylcholinesterase (AChE) from electric eel, acetylthiocholine chloride, cysteamine, glutaraldehyde and paraoxon were purchased from Sigma Chemical Company (St. Louis, USA).

### 2.4. Thiocholine measurements

Thiocholine was produced enzymatically by AChE using acetylthiocholine as substrate (because thiocholine is not commercially available). For this purpose, 1 mL of 1 M acetylthiocholine solution was prepared in phosphate buffer 0.1 M (pH = 8), and 100 units of AChE were added to this solution. After 1 h, the concentration of thiocholine produced by AChE was estimated spectrophotometrically by Ellman's method. For this purpose, 900 μL of phosphate buffer solution (0.1 M, pH = 8), 100 μL of 0.1 M DTNB, and 5 μL thiocholine solution (diluted 1:100 in water) were put in a spectrophotometric cells. The absorbance was measured, and the real concentration was evaluated by using the Lambert–Beer law with the known molar extinction coefficient of TNB ($\varepsilon$ = 13,600 $M^{-1}$ $cm^{-1}$) [29]. After 1 h, the acetylthiocholine hydrolysis is completed, and 1 mL solution of 1 M thiocholine is obtained. The solution is stable for 1 day at 4 °C.

Thiocholine measurements were carried out using amperometric batch analysis at Au-SPE in a stirred 0.05 M phosphate buffer solution + 0.1 M KCl, pH 7.4 (10 mL) containing 1 mM of ferricyanide ions at an applied potential of +400 mV vs. Ag/AgCl. After around 7 min, time required for baseline stabilization, the thiocholine was added and the response was recorded.

### 2.5. AChE biosensor

The Au-SPE, as received by Ecobioservice, was immersed in 100 mM cysteamine aqueous solution for 15 h at room temperature in darkness to form cysteamine monolayer. Then, it was thoroughly washed with double distilled water to remove the physically adsorbed cysteamine. After that, the sensor was covered with an aqueous solution of glutaraldehyde 5% (v/v) for 30 min, then, it was thoroughly washed with double distilled water to remove the unreacted glutaraldehyde. Finally the resulting electrode was incubated with AChE solution (10 U/mL) for 15 h; after that, the biosensor was washed and maintained in phosphate buffer at 4 °C (Scheme 1).

### 2.6. Acetylthiocholine measurement

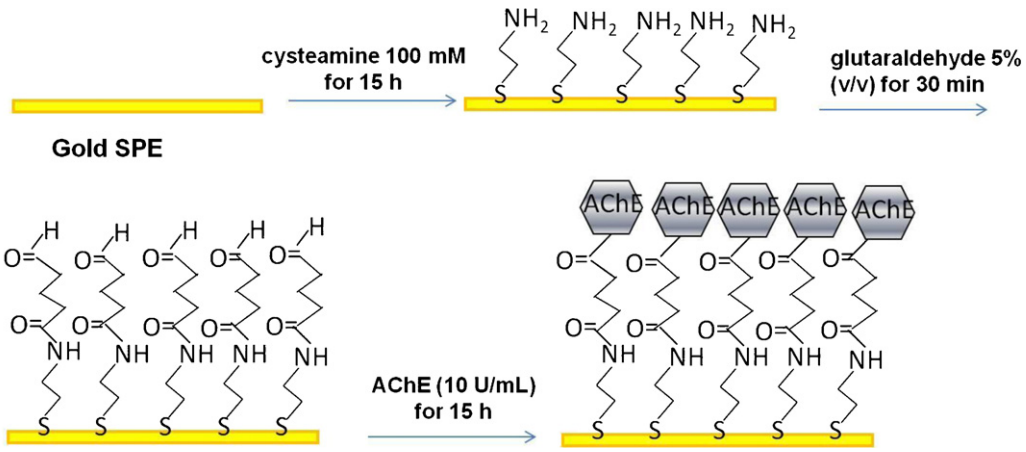
The acetylthiocholine was measured using AChE biosensor at an applied potential of +400 mV vs. Ag/AgCl in stirred 0.05 M phosphate buffer solution + 0.1 M KCl, pH 7.4 (10 mL) containing 1 mM of ferricyanide ions. When a stable baseline current was reached, the acetylthiocholine was added and the analyte response at the steady state was recorded.

### 2.7. Inhibition measurement using AChE biosensors

Paraoxon in aqueous solutions was used as standard insecticide for the AChE inhibition measurements. The enzymatic activity of the biosensor was measured before ($i_0$) and after ($i_i$) its exposure to paraoxon for a certain time (incubation time) at 3 mM substrate (acetylthiocholine) concentration. After the incubation time, the biosensor was rinsed three times with distilled water and the response towards the substrate was measured as described above. The degree of inhibition was calculated as a relative decay of the biosensor response.

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

where $i_0$ and $i_i$ represent the biosensor response before and after the incubation procedure, respectively.




**Scheme 1.** The scheme of the assembling of the AChE biosensor.

### 2.8. Sample collection

The drinking water sample was collected at Tor Vergata University Laboratory, the Sacco river water sample was collected near Ceccano; real samples were diluted 1:2 with 0.1 M phosphate buffer solution + 0.2 M KCl, pH 7.4 and directly analysed.

## 3. Results and discussion

### 3.1. Amperometric thiocholine detection at Au-SPE by means of ferricyanide

The mono-enzymatic AChE biosensor uses the acetylthiocholine as substrate, thus the first goal was the development of a highly sensitive enzymatic product thiocholine (RSH) sensor. The major drawback relative to the electrochemical detection of thiocholine is the high overpotential required at most conventional electrode surfaces (gold, platinum and carbon paste) and the fouling of the working electrode surface. To overcome these problems, the electrochemical detection of thiocholine could be performed using nanostructured materials or redox mediators [30–35]. In the last case, the electrochemical mediator can be adsorbed on the working electrode surface such as in the case of Prussian Blue [33], cobalt hexacyanoferrate [34] or it can be directly mixed to the ink used to print the working electrode, such as in the case of cobalt phthalocyanine (CoPc) [35]. In the case of Au-SPEs modified with SAM, our choice was to use the electrochemical mediator ferricyanide in solution because it is able to electrocatalyse the oxidation of thiocholine [36].

The electrochemical behaviour of ferricyanide towards thiocholine oxidation was firstly investigated using a CV technique and a Au-SPE. Before the thiocholine measurement, the Au-SPE was conditioned by means of 30 CVs using ferricyanide 10 mM prepared in 0.05 M phosphate buffer plus 0.1 M KCl, pH 7.4 at scan rate of 50 mV/s. This condition step was necessary in order to decrease the peak to peak separation from 314 mV to 93 mV after 30 scans as we can see in Fig. 1, this behaviour can be ascribed to the enhanced kinetic rate after electrochemical pretreatment as reported in literature using carbon SPE [37]. Next, we have investigated the response of the so conditioned Au-SPE towards ferricyanide by CV over a scan range of 0.010–0.200 V/s. The current of both anodic and cathodic peaks increases linearly with the square root of the scan rate (data not shown), indicating a semi-infinite linear diffusion-controlled current. After that, the Au-SPE was studied to evaluate the electrocatalytic effect of ferricyanide towards the thiocholine oxidation.

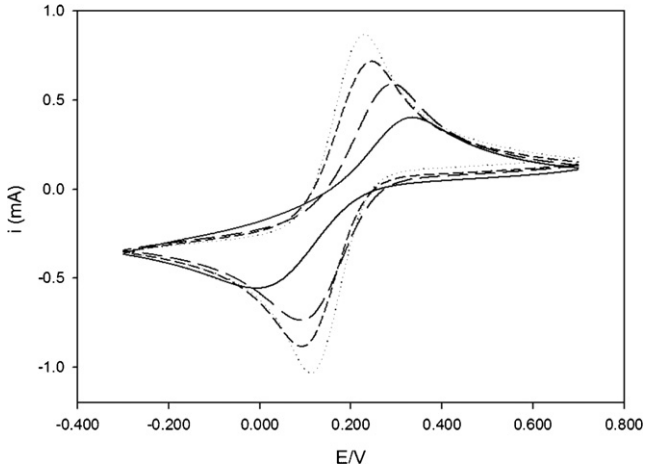



**Fig. 1.** CVs of Au-SPE in 10 mM ferricyanide in 0.05 M phosphate buffer + 0.1 M KCl, pH = 7.4, scan rate 50 mV/s (continuous line = first scan, long dashed line = 10th scan, short dashed line = 20th scan and dotted line = 30th scan).

Fig. 2 shows a typical response of $i_k$ (kinetically controlled current) obtained in presence of thiocholine and $i_d$ (diffusion controlled current) obtained in absence of thiocholine. The generic reaction can be described by the following equations:

$$\text{ferricyanide} + \text{RSH} \rightarrow \text{ferrocyanide} + \text{RSSR} \quad (2)$$

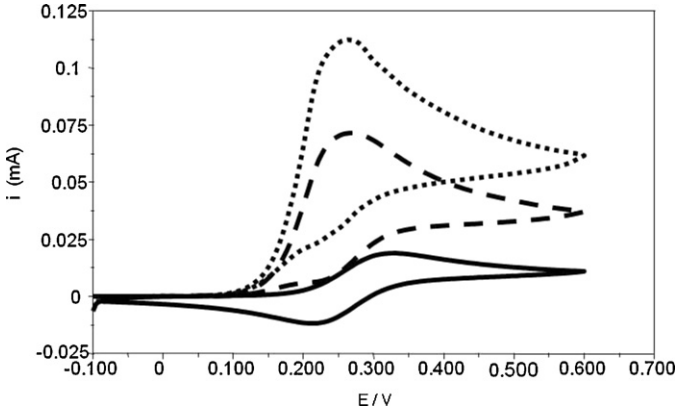



**Fig. 2.** CVs of ferricyanide 4 mM in 0.05 M phosphate buffer + 0.1 M KCl, pH = 7.4, scan rate 20 mV/s in absence (continuous line) and in presence of thiocholine 10 mM (dashed line) or 20 mM (dotted line).

$$\text{ferrocyanide} \overset{\text{electrode}}{\longleftrightarrow} \text{ferricyanide} \quad (3)$$

According to the above equations, the addition of thiocholine causes an increase in concentration of the ferrocyanide, resulting in an increase of the anodic peak current. On the contrary, the cathodic peak current is proportional to the amount of ferricyanide that decreases after the addition of RSH (thiocholine). Moreover, the increase of thiocholine concentration leads to a further increase of the anodic peak current and a decrease of the cathodic peak, confirming the property of ferricyanide as electrocatalyst for thiocholine oxidation.

In order to calculate the second-order homogeneous rate constant $k_s$ for the reaction between ferricyanide and thiocholine, the theory of stationary electrode voltammetry developed by Nicholson and Shain [38] was used. Calculation of $k_s$ has been performed by varying scan rates in the range of 5–100 mV/s and thiocholine concentration in the range of 10–20 mM, fixing the concentration of ferricyanide at 4 mM. The reaction scheme can be described as:

$$\text{ferrocyanide} - e^- \rightarrow \text{ferricyanide} \quad (4)$$

$$\text{thiocholine} + \text{ferricyanide} \overset{kf}{\longleftrightarrow} \text{ferrocyanide} \quad (5)$$

where $k_f$ is the pseudo-first order rate constant. Using the experimental values of $i_k/i_d$ a value of ($k_f RT/n$Fv) can be obtained from the working curve reported in Nicholson and Shain paper [38]. Plotting ($k_f RT/n$Fv) vs. $1/\nu$ at each concentration of thiocholine, it is possible to calculate $k_f$. Then, a plot of $k_f$ vs. thiocholine concentration was carried out obtaining a linear behaviour whose slope was equal to the second-order homogenous rate constant $k_s$ for reaction between ferricyanide and thiocholine. It was found equal to $(5.26 \pm 0.65) \times 10^4\ M^{-1}\ s^{-1}$, demonstrating the good electron transfer between thiocholine as thiol and ferricyanide [39]. This value is, for example, higher than the one found for the electrocatalytic reduction of nitrite using ferricyanide ($2.75 \times 10^3\ M^{-1}\ s^{-1}$) [40].

### 3.2. Amperometric thiocholine detection at Au-SPE modified by means of ferricyanide

#### 3.2.1. Choice of applied potential

A plot of current/applied potential using a thiocholine and ferricyanide concentration of $3 \times 10^{-4}$ M and 1 mM respectively [36], was constructed in the range of the applied potential 0–800 mV vs. Ag/AgCl (data not shown). As expected, the amperometric response followed the behaviour of the oxidation current in the CV. The oxidation current increased from 0 to +100 mV vs. Ag/AgCl reaching a plateau at around +200 mV vs. Ag/AgCl. However, we observed the highest ratio signal/noise at +400 mV vs. Ag/AgCl, thus this applied potential was selected for the rest of work.

#### 3.2.2. Analytical features of thiocholine measurement

The good electroanalytical performances of the developed system were then confirmed by performing amperometric batch measurements. In fact, our purpose was the development of a sensor for thiocholine detection as a platform for an amperometric biosensor for insecticide detection.

Using the previous selected applied potential (+400 mV vs. Ag/AgCl) and a ferricyanide concentration equal to 1 mM [36], calibration curves were carried out obtaining a detection limit (S/N = 3) of $3 \times 10^{-6}$ M together with a linear range up to $1 \times 10^{-3}$ M ($R^2 = 0.9964$). The sensor showed also a sensitivity equal to 113 mA $M^{-1}$ $cm^{-2}$, which is higher than the one shown by the SPE modified with CoPc (24 mA $M^{-1}$ $cm^{-2}$) and comparable with that obtained in the case of SPE modified with Prussian Blue (143 mA $M^{-1}$ $cm^{-2}$) [13]. Moreover, the reproducibility was evaluated by studying the response of $5 \times 10^{-5}$ M thiocholine, founding a RSD% equal to 5% ($n = 4$).

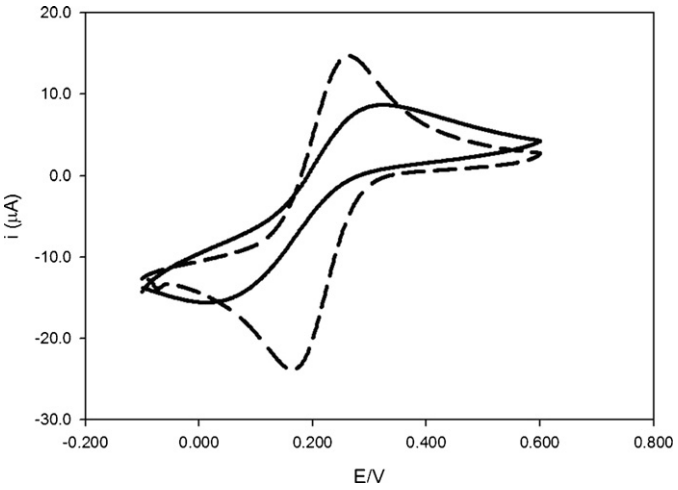



**Fig. 3.** CVs of ferricyanide 4 mM in 0.05 M phosphate buffer + KCl 0.1 M, pH = 7.4, scan rate 20 mV/s using a bare gold SPE (continuous line) and a cysteamine modified gold SPE (dashed line).

### 3.3. Ferricyanide behaviour at Au-SPE modified with a SAM of cysteamine

Our goal was the development of biosensor for insecticide detection immobilizing the AChE by SAM. In this case, cysteamine was selected as alkanethiol taking in consideration that glutaraldehyde will be used to link the AChE to cysteamine. Firstly, the effect of cysteamine coverage of Au-SPE on ferricyanide response was investigated, because the electron transfer for redox reactions of different molecules at SAM can be controlled by electrostatic and hydrophobic effects [41].

A CV study was performed using ferricyanide at bare Au-SPE and Au-SPE modified with a SAM of cysteamine, prepared by dipping the Au-SPE in a 100 mM cysteamine solution overnight (Cyst–Au-SPE). We observed that in the case of bare Au-SPE, the peak to peak separation was 314 mV; in the case of cysteamine modified Au-SPE, instead, it was 89 mV (Fig. 3); it seems that the presence of cysteamine on the surface of the working electrode can improve its electrochemical performance. This behaviour should be ascribed to the electrostatic interaction between cysteamine and ferricyanide. The p$K_a$ of the cysteamine immobilized on the gold electrode was estimated by Shervedani et al. to be equal to 7.6, thus in the pH region lower than 7.6, the Au surface is positively charged [42].

In our case, we worked at pH = 7.4, thus the surface of gold electrode modified by SAM of cysteamine should be characterized by positive charge, reason for that probably the CV of ferricyanide, which is negatively charged, is characterized by a peak to peak separation lower than at the bare Au-SPE.

In conclusion, in our case the cysteamine will be used to immobilize the AChE by cross-linking with glutaraldehyde, but we have also demonstrated that a SAM of cysteamine can facilitate the electron transfer of ferricyanide at working electrode surface of Au-SPE.

### 3.4. AChE biosensor

#### 3.4.1. Optimization of bioactive layer

In order to develop a sensitive AChE biosensor, the AChE and the cysteamine concentrations and the deposition time of cysteamine on the gold electrode surface were investigated and optimized.

*3.4.2. Effect of AChE concentration*

The effect of AChE concentration on the biosensor response was investigated. The AChE concentration was varied in a range comprised between 0.1 and 10 U/mL using a SAM prepared with cysteamine 0.1 mM and a deposition time of 15 h, and a glutaraldehyde solution at 5% (v/v). The response of the biosensors was tested using acetylthiocholine $3 \times 10^{-4}$ M. We found that in the case of AChE 0.1 U/mL, 1 U/mL and 10 U/mL, a current equal to 0, 116 and 209 nA was observed, respectively. The results showed that the response of the biosensor increased with the increase of the enzyme amount as expected, but also the working stability (successively measured) increased with the increase of the enzyme amount. Keeping in mind that: (i) the AChE inhibition by organophosphorus insecticides is a irreversible, the lower possible amount of enzyme should be used [6] and (ii) an enzyme loading lower than 10 U/mL does not allow a satisfactory working stability, thus an enzyme amount of 10 U/mL was finally selected for further work.

*3.4.3. Effect of cysteamine concentration and deposition time on gold electrode surface*

The density of hydrocarbon chains on gold electrode surface was due to the concentration of alkanethiols used and the duration of SAM deposition. For the investigation of SAM time deposition, Au-SPE was immersed for 1 and 15 h in cysteamine solution at concentration of 0.1 mM, using AChE at concentration of 10 U/mL and glutaraldehyde at 5% (v/v). The response of the biosensors was tested using acetylthiocholine $3 \times 10^{-4}$ M. We have observed a response towards the substrate only in the case of biosensor preapared with 15 h as deposition time, thus this time was selected for further experiments. In order to evaluate the effect of cysteamine concentration, the Au-SPE was immersed in cysteamine solution at different concentrations: 0.1, 1, 10 and 100 mM, using AChE at concentration of 10 U/mL and glutaraldehyde at 5% (v/v). The response of the biosensors was tested using acetylthiocholine $3 \times 10^{-4}$ M. The biosensors prepared using cysteamine 100 mM had the highest response (around 200 nA) and the highest working stability. For electrodes immersed in cysteamine 0.1, 1 and 10 mM the biosensors allowed results characterized by low working stability. Looking at these results, we selected as most suitable biosensor in terms of working stability, reproducibility and sensitivity towards acetythiocholine, the one developed using SAM prepared with cysteamine 100 mM, a deposition time of 15 h, AChE at 10 U/mL and glutaraldehyde at 5% (v/v). This biosensor was characterized by a reproducibility (RSD%) intra- and inter-electrode of 2.3% and 16%, respectively. The high value of RSD% in the case of inter-electrode reproducibility does not affect the accuracy of insecticide measurements because they were carried out measuring the response before and after the exposure to the organophosphate, thus measuring the relative decay of enzymatic activity of a single biosensor.

*3.5. Electrochemical impedance spectroscopy measurements*

Electrochemical impedance spectroscopy can provide useful information on the impedance changes of the electrode surface during the fabrication process of biosensors, giving information about how the interfacial region of the gold electrode surface in presence of SAM of cysteamine changes before and after the AChE immobilization, measuring the value of electron transfer resistance ($R_{ct}$). The $R_{ct}$, estimated according to the diameter of the semicircle present at the high frequency region, represents, in fact, the difficulty of electron transfer of ferro/ferricyanide redox probe between the solution and the electrode. Fig. 4 shows the typical Nyquist plot obtained for cysteamine modified Au-SPE (Cyst–Au-SPE), glutaraldehyde–Cyst–Au-SPE and AChE–glutaraldehyde–Cyst–Au-SPE (biosensor). In this figure the Nyquist plot of the bare gold SPE was omitted because characterized by a much higher $R_{ct}$

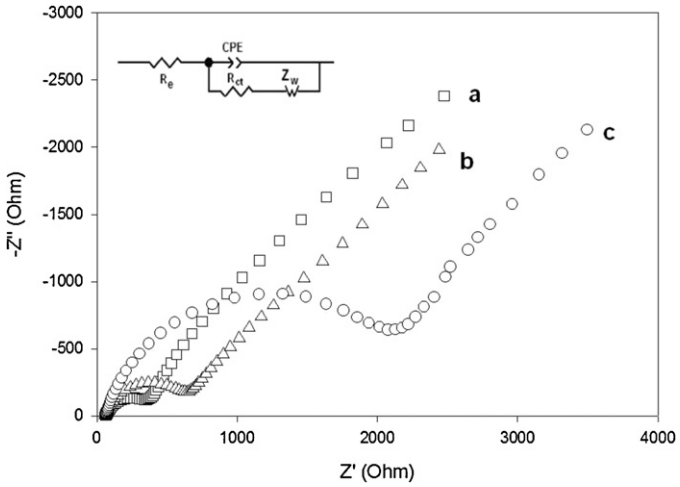



**Fig. 4.** Complex plane impedance plots at open circuit potential for Cyst–Au-SPE (a), glutaraldehyde–Cyst–Au-SPE (b) and AChE-glutaraldehyde–Cyst–Au-SPE (biosensor) (c) using 5 mM ferro/ferricyanide in 0.1 M KCl. Inset: Randles circuit.

(72,187 ± 315 Ω) than the one obtained in the case of Cyst–Au-SPE (407 ± 9 Ω), confirming the data obtained using the CV technique that showed a better electron transfer of ferro/ferricyanide at Cyst–Au-SPE than the at bare Au-SPE.

The fabrication process of biosensors was followed measuring the $R_{ct}$ values, observing that the $R_{ct}$ increases in the following order: $R_{ct}$ AChE–glutaraldehyde–Cyst–Au-SPE (2431 ± 30 Ω) > glutaraldehyde–Cyst–Au-SPE (681 ± 9 Ω) > $R_{ct}$ Cyst–Au-SPE (407 ± 9 Ω), confirming also the deposition of a glutaraldehyde layer and of the AChE enzyme on the Cyst–Au-SPE.

*3.6. AChE biosensor for insecticide detection*

The optimized biosensor was challenged with the enzymatic substrate acetylthiocholine. Fig. 5 shows the calibration curve obtained for different substrate concentrations described by Michaelis Menten equation. It was possible to calculate the apparent Michaelis Menten constant ($K_M^{app}$), found equal to 1.1 ± 0.2 mM. A substrate concentration of 3.0 mM was chosen for the inhibition measurements.

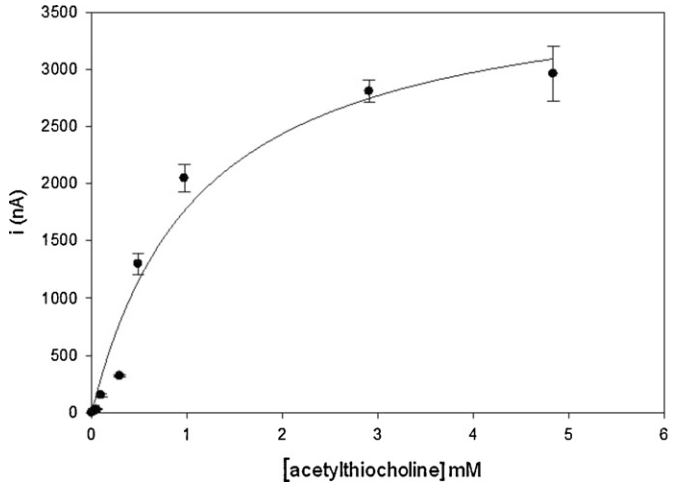



**Fig. 5.** Calibration plot of acetylthiocholine chloride using the AChE biosensor. Applied potential: +400 mV vs. Ag/AgCl. 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4 + 1 mM ferricyanide.

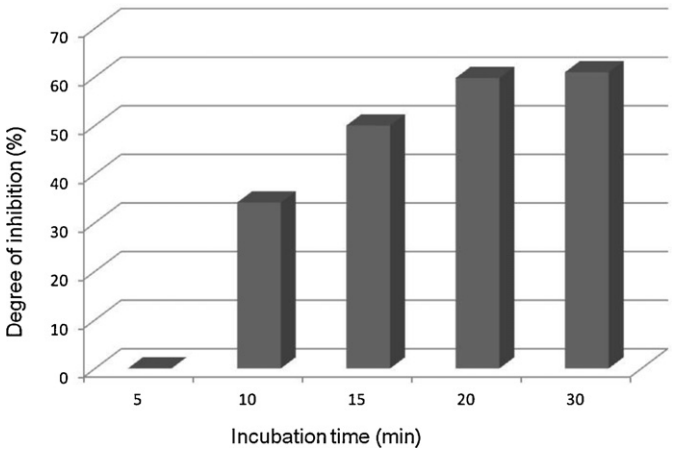



**Fig. 6.** Study of incubation time. Applied potential: +400 mV vs. Ag/AgCl, 0.05 phosphate buffer + 0.1 M KCl, pH 7.4 + 1 mM ferricyanide, 3 mM acetylthiocholine as substrate concentration, 40 ppb of paraoxon as inhibitor.

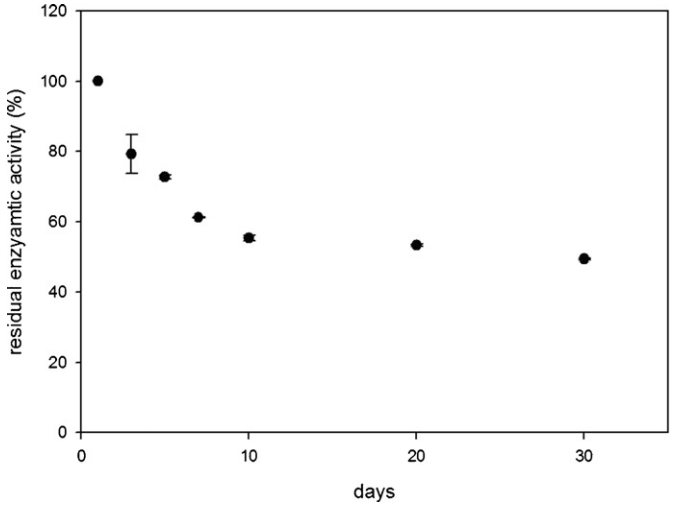



**Fig. 7.** Storage stability as percentage of residual activity. Applied potential: +400 mV vs. Ag/AgCl, 0.05 phosphate buffer + 0.1 M KCl, pH 7.4 + 1 mM ferricyanide, 3 mM acetylthiocholine as substrate.

### 3.6.1. Study of incubation time

The incubation time is the reaction time of the enzyme with the inhibitor. For irreversible inhibition such as the one of AChE by organophosphorus insecticides, it is possible to achieve lower detection limits using longer incubation times; in fact, the degree of the enzyme inhibition usually increases with the incubation time until reaching a plateau [6]. In our case (Fig. 6) a rapid increase of the inhibition degree up to 15 min, using a concentration of paraoxon of 40 ppb, was found, reaching a plateau after 20 min. Thus, a time of 15 min was selected as a compromise between a sensitive measurement and a reasonable measurement time.

### 3.6.2. Paraoxon detection in standard solutions and in water real samples

We selected paraoxon as organophosphorus compound for insecticide detection using the developed biosensor. The paraoxon measurement was performed using the procedure called "medium exchange" in which it is possible to avoid electrochemical interferences from electroactive species and from reversible inhibitors during the pesticide measurement [6,13]. Briefly, the enzymatic activity measurement before inhibition is carried out in buffer solution in presence of the enzymatic substrate. After, the biosensor is put in contact with the sample contaminated with insecticides for a selected time; then, the biosensor is rinsed several times with distilled water and the enzyme residual activity is finally measured in a new buffer aliquot in presence of the enzymatic substrate but in absence of any interfering species. In this way, it was possible to avoid both electrochemical interferences and the possible presence of reversible AChE inhibitors such as fluoride, $Cd^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Mn^{2+}$ and glycoalkaloids [43]. The measurements were performed using the selected incubation time of 15 min, obtaining a calibration curve described by the following equation: $y = (1.07 \pm 0.03)x + (8.09 \pm 0.51)$, $R^2 = 0.9868$ with a linear range up to 40 ppb and a detection limit (LOD), calculated as the amount of paraoxon for obtaining a 10% of inhibition, equal to 2 ppb. The analytical performances obtained are competitive with the ones reported in literature using, for instance, cobalt phthalocyanine modified carbon epoxy composite with AChE immobilized on nylon net (LOD equal to 12 ppb for paraoxon) [44], AChE biosensor based on a polishable 7,7,8,8-tetracyanoquinodimethane-modified graphite-epoxy biocomposite (LOD equal to 27.5 for paraoxon) [45], AChE captured in a gelatin membrane coupled with carbon screen-printed electrode (LOD equal to 2.5 ppb for paraoxon) [46], AChE immobilized by glutaraldeyde onto carbon screen-printed electrode modified with Prussian Blue (50% of degree of inhibition for paraoxon 25 ppb) [13]. The results found in this work are also satisfactory when compared with AChE immobilized onto a SAM gold electrode (LOD equal to 9.3 ppb for parathion) [22] with the advantage in the case of gold screen-printed electrodes that they are mass produced and cost-effective thus suitable for a single measurement, property very useful in the case of irreversible inhibition based biosensors.

In order to evaluate the accuracy of the method, the biosensor was challenged in spiked drinking water sample. Drinking water sample collected and tested in our laboratory gave no degree of inhibition. When, the sample was fortified with 10 ppb of paraoxon, a recovery of $103 \pm 3\%$ ($n = 3$) was obtained. In addition, a sample collected from the Sacco river was analysed, obtaining in this case a degree of inhibition equal to $10.9 \pm 0.4\%$ ($n = 3$), corresponding to $5.2 \pm 0.7$ ppb of paraoxon in the sample, keeping in mind that the real sample was diluted 1:2 with phosphate buffer (see Section 2.8). In order to evaluate the accuracy of the biosensor in the Sacco river sample, the sample was also fortified with 10 ppb of paraoxon, obtaining a recovery equal to $97 \pm 5\%$ ($n = 3$).

### 3.6.3. Storage stability of biosensor

In order to test the practicability of the developed biosensor, the storage stability was tested. When the biosensor was not in use, it was stored at 4 °C in phosphate buffer solution. The stability was tested measuring the biosensor response in a 3 mM acetylthiocholine solution. We observed a rapid decrease of enzymatic activity during the first week up to around 60% of residual enzymatic activity (Fig. 7), after that it remained almost stable up to 1 month.

## 4. Conclusions

In this work, an AChE biosensor for organophosphorus insecticides based on enzyme inhibition was developed. The AChE enzyme was immobilized via glutaraldehyde on a preformed cysteamine SAM on Au-SPEs. As reported in literature, the immobilization using a self assembled monolayer allows obtaining a highly orientated enzyme immobilization, leading to a low detection limit. The strategy of using ferricyanide in solution as electrochemical mediator allowed to obtain a sensitive sensor for the enzymatic product detection. Moreover, the electrochemical and enzymatic interferences were avoided because the "medium exchange" measurement method was used. The biosensor was challenged in

drinking water sample obtaining satisfactory results. In addition, the use of Au-SPEs allows a single insecticide measurement which is a great advantage since these compounds inhibit the AChE enzyme in irreversible way. Multiple insecticide measurements with the same biosensor require, in fact, a reactivation of the immobilized enzyme or the use of a renewable enzymatic membrane, both time-consuming procedures. The developed biosensor has thus demonstrated to be an useful analytical tool for screening analysis of organophosphorus insecticides.

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