

Screen-printed electrode modified with carbon black and chitosan: a novel platform for acetylcholinesterase biosensor development

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Abstract We report a screen-printed electrode (SPE) modified with a dispersion of carbon black (CB) and chitosan by drop casting. A cyclic voltammetry technique towards ferricyanide, caffeic acid, hydroquinone, and thiocholine was performed and an improvement of the electrochemical response with respect to bare SPE as well as SPE modified only with chitosan was observed. The possibility to detect thiocholine at a low applied potential with high sensitivity was exploited and an acetylcholinesterase (AChE) biosensor was developed. A dispersion of CB, chitosan, and AChE was used to fabricate this biosensor in one step by drop casting. The enzymatic activity of the immobilized AChE was determined measuring the enzymatic product thiocholine at +300 mV. Owing to the capability of

organophosphorus pesticides to inhibit AChE, this biosensor was used to detect these pollutants, and paraoxon was taken as model compound. The enzyme inhibition was linearly related to the concentration of paraoxon up to $0.5 \mu\text{g L}^{-1}$, and a low detection limit equal to $0.05 \mu\text{g L}^{-1}$ (calculated as 10% of inhibition) was achieved. This biosensor was challenged for paraoxon detection in drinking waters with satisfactory recovery values. The use of AChE embedded in a dispersion of CB and chitosan allowed an easy and fast production of a sensitive biosensor suitable for paraoxon detection in drinking waters at legal limit levels.

Keywords Acetylcholinesterase biosensor · Carbon black · Chitosan · Pesticides · Amperometric detection · Paraoxon

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Introduction

The efforts in the development of nanomaterials have entailed a new electroanalytical chemistry field with a multitude of applications [1]. Carbon nanomaterials (CNMs), including carbon nanotubes, graphene, and carbon nanofibers, have been extensively studied as modifiers of working electrode surfaces, allowing the assembling of highly sensitive and selective electrochemical sensors [2–5]. One of the main challenges for obtaining highly reproducible modified electrodes involves the achievement of a stable dispersion, since CNMs tend to self-associate into micro-scale aggregates because of their hydrophobic nature and high specific surface area [6]. Consequently, efforts have been devoted to prepare stable dispersions. For instance, the ultrasound-assisted dispersion to obtain a homogenous dispersion of CNMs, such as carbon nanotubes, graphene, and carbon black, has been reported [7–9]. The chemical functionalization with carboxylic, carbonyl, and hydroxyl groups using nitric acid, permanganate, and sulfuric acid was also employed for increasing their hydrophilicity [10]. To improve the stability of

the dispersion, several compounds, such as nafion, chitosan, and sodium dodecylbenzene sulfonate, have been investigated because of their ability to create a link between the hydrophobic CNM surface and the hydrophilic aqueous solvent [11, 12]. In addition, in the case of biocompatible polymers like chitosan, their use provides a favorable environment for the biocomponents, rendering the modified electrode a suitable platform for biosensor development [13–15].

In particular, chitosan is a nontoxic and biodegradable polysaccharide [16], and is used in several fields like gene delivery [17], tissue engineering [18], and food sector [19]. Chitosan is also widely employed for electrochemical (bio)sensor fabrication since furnish a biocompatible and favorable microenvironment for enzymatic immobilization before [20, 21]. In addition, the use of chitosan is often associated with the use of nanomaterials, being able to improve the stability of nanomaterial-based dispersions [22].

To this regards, a glassy carbon electrode modified with a dispersion of multiwalled carbon nanotubes and chitosan was used to detect NADH, reaching a sensitivity of $130 \text{ mA M}^{-1} \text{ cm}^{-2}$. In addition, this sensor was employed as a platform for glucose biosensor by incorporating glucose dehydrogenase, and reaching a sensitivity of $80 \text{ mA M}^{-1} \text{ cm}^{-2}$ for glucose detection [23]. Besides CNTs, colloidal carbon microspheres were also used together with chitosan to form an organic-inorganic hybrid for sensing applications. In this case, the dispersion was cast onto a glassy carbon electrode, and the enzyme horseradish peroxidase was entrapped in this nanohybrid film for hydrogen peroxide determination, reaching a sensitivity of $120 \text{ mA M}^{-1} \text{ cm}^{-2}$ [24]. The enzyme acetylcholinesterase was also immobilized on a modified glassy carbon electrode with a dispersion of porous-reduced graphene oxide and chitosan. The enzymatic activity was monitored using acetylthiocholine as substrate at an applied potential of 750 mV, obtaining a linear range between 0.72 and 1.76 mM. This biosensor was then employed to detect carbaryl, which is a well-known inhibitor of acetylcholinesterase, with a detection limit of $0.5 \mu\text{g L}^{-1}$ and an incubation time of 12 min [25].

Recently, Vicentini et al. used ultrasound-assisted carbon black (CB) dispersion with chitosan to modify glassy carbon electrodes by drop casting, revealing an excellent sensitivity and a faster heterogeneous electron transfer rate of the CB modified electrode, compared with bare glassy-carbon and edge-plane pyrolytic graphite electrodes [26]. Thus they evidenced the advantages of using CB as a modifying agent, confirming the results obtained by our group. In fact, we highlighted the improved electrochemical performances of screen-printed electrodes (SPEs) modified with CB N220 as a carbonaceous nanomaterial, fabricating CB-SPE by drop

casting bare SPE with stable dispersions of CB in acetonitrile or in a mixture of dimethylformamide-water. CB-SPE encompasses the advantages of SPEs like disposability, miniaturization, easiness of modification, and suitability to be mass-produced, along with the advantages of CB, such as cost-effectiveness, and good electrochemical performances of raw material as well [9, 27–33].

In this work, a one step procedure for an easy, sustainable, and cost-effective biosensor production is proposed for the first time, adding only some μL of a dispersion of acetylcholinesterase (AChE), chitosan, and CB onto the working electrode surface. In fact, in the literature chitosan is mainly employed for enzyme immobilization using a multi-step procedure [34–36], whereas in this work chitosan was exploited to obtain a stable dispersion of CB (nanomaterial chosen for its valuable electrocatalytic properties) and AChE (the enzyme irreversibly inhibited by organophosphorus pesticides).

Materials and methods

Apparatus and reagents

Cyclic voltammetry and chronoamperometry were performed using a PalmSens portable instrument (Utrecht, The Netherlands). Carbon black (commercial CB N220) having diameters of nanoparticles comprised between 17.95 and 32.5 nm [9] was obtained from Cabot Corporation (Ravenna, Italy). Acetylcholinesterase (AChE from *electric eel*) was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Screen-printed electrodes

Screen-printed electrodes were produced in our laboratory with a 245 DEK (Weymouth, UK) screen-printing machine according to the procedure reported in our previous papers [30–33].

CB and chitosan dispersion

A 0.5% w/w chitosan stock solution was prepared by dissolving chitosan flakes in a hot (80–90 °C) aqueous solution of 0.05 M HCl. The solution was cooled at room temperature, and the pH was adjusted to 5.0 using concentrated NaOH. The chitosan solutions were stored in a refrigerator (4 °C) when not in use. All chitosan solutions were colorless. Different CB dispersions in chitosan solutions were prepared at a concentration of 1, 3, 5, and 10 mg/mL, by adding 1, 3, 5, and 10 mg of CB in 1 mL of chitosan solution (0.5% w/w), followed by stirring for 30 min. To select the optimal chitosan amount, different concentrations of chitosan solution (0.50, 0.25, 0.10, and 0.05% w/w) were used to disperse CB. The best

dispersion was obtained using 3 mg/mL of CB and 0.05% w/w of chitosan.

SPE modified with chitosan and CB dispersion

The modified SPEs were prepared by one step drop casting. 0.5 μ L of dispersion of chitosan and CB (CS/CB dispersion) were cast onto the working electrode surface and left to dry at room temperature. CS/CB-SPEs were stored at RT.

Thiocholine determination

Since thiocholine is not commercially available, the thiocholine was enzymatically produced by AChE using acetylthiocholine as substrate, according to the procedure reported in our previous papers [37, 38]. The concentration of thiocholine was measured spectrophotometrically using Ellman's method [39].

Biosensor based on AChE/CS/CB-SPE

A suitable volume of enzyme was added to 1 mL of 3 mg/mL of CB dispersed in chitosan 0.05% w/w to obtain 1.5 U/mL of enzyme as final concentration. The biosensor was prepared by a drop casting technique, placing 0.5 μ L of AChE/CS/CB dispersion onto the working electrode surface. The AChE/CS/CB-SPEs were stored at 4 °C under vacuum and after 1 week 85% of the initial enzymatic activity was measured.

Acetylthiocholine determination

Acetylthiocholine analyses were carried out using an amperometric “drop” procedure in phosphate buffer solution (0.05 M + KCl 0.1 M, pH 7.4) with an applied potential of +300 mV versus Ag/AgCl. In detail, a drop (50 μ L) of buffer containing different amounts of acetylthiocholine was placed onto the AChE biosensor in such a way that the working, counter, and reference electrode areas were covered. After applying the potential, the signal was continuously recorded and the current was detected soon after a steady state was reached.

Paraoxon determination

The inhibitory effect of paraoxon on AChE biosensor was evaluated by determining the current decrease attributable to the oxidation of thiocholine produced by this enzyme. The experiment was performed recording the response toward the substrate as described above. Thereafter, the AChE biosensor was incubated in the paraoxon solution for a fixed period of time (incubation time), then rinsed more times with distilled water. After

that, the response toward the substrate was again measured, and the degree of inhibition was calculated using the following equation (Eq. 1):

$$I\% = \left[(I_0 - I_i) / I_0 \right] \times 100 \quad (1)$$

where I_0 and I_i represent the response of biosensor before and after the paraoxon exposure, respectively.

Sample collection and measurement

Drinking water was collected from a water bottle purchased from a local market. The sample was diluted 1:2 with phosphate buffer 0.1 M + KCl 0.2 M, pH = 7.4, before the electrochemical analyses. In order to evaluate the accuracy of the biosensor, the sample was fortified with 0.5 μ g L⁻¹ of paraoxon and diluted 1:2 v/v with phosphate buffer 0.1 M + KCl 0.2 M.

Results and discussion

Optimization of chitosan and CB dispersion

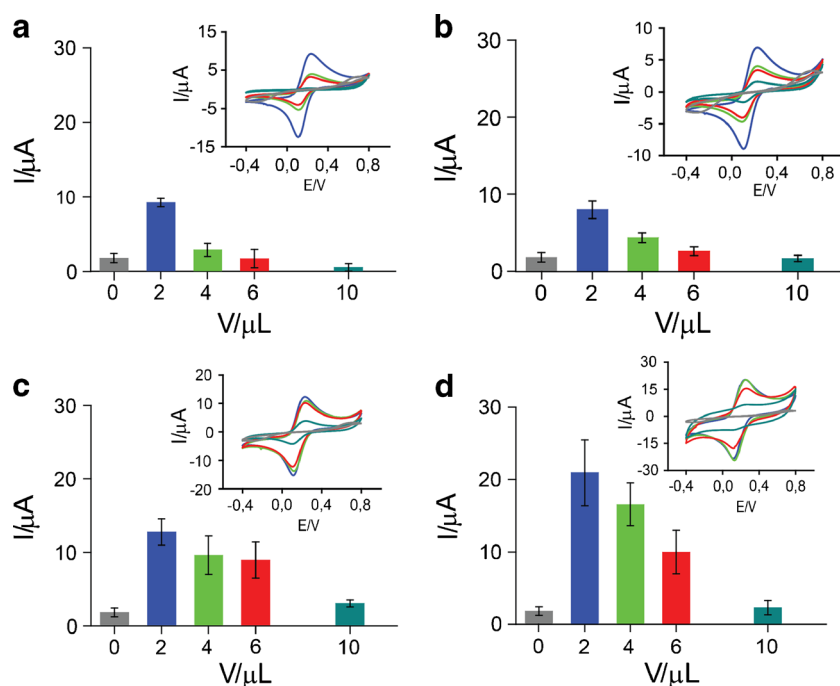
In our previous studies, several solvents for the preparation of effective dispersions of CB were investigated, and the mixture of water and dimethylformamide 1:1 v/v was chosen to achieve an ultrasound-assisted stable dispersion suitable for SPE modification by drop casting [27, 30, 32].

Despite the good results obtained, the organic solvents and the sonication are not compatible with many enzymes. In this work, chitosan was selected to promote the disagglomeration of CB with the advantage to obtain a sonication-free and bio-compatible dispersion. To optimize the amount of CB, SPEs were modified with different volumes (2, 4, 6, and 10 μ L, by a successive deposition of 2 μ L aliquots) of different dispersions (1, 3, 5, and 10 mg/mL) of CB in chitosan 0.5% w/w (CS/CB-SPEs).

In Fig. 1, cyclic voltammograms of ferricyanide and the relative anodic peak intensity using bare and SPEs modified with different volumes of the same CB dispersion are shown. As depicted in Fig. 1, well resolved ferro/ferricyanide peaks were observed using CS/CB-SPEs, demonstrating the retention of the electrochemical properties of CB in aqueous chitosan solution. An increase of peak intensity was attained using the same volume of CS/CB dispersion at higher CB concentration placed on the working electrode surface. However, a worsening of electrochemical behavior was observed with the increase of the chitosan amount (higher volume placed on the working electrode surface), because of the insulating properties of chitosan.

CS/CB-SPE prepared using 2 μ L of 3 mg/mL of CB dispersion was selected as a compromise between a good

Fig. 1 Study of amount of CS/CB cast onto working electrode surface. Anodic peak current detected by cyclic voltammeter recorded in ferricyanide 1 mM, phosphate buffer 0.05 M, and KCl 0.1 M as electrolyte, pH 7 scan rate 100 mV/s using CS/CB-SPE modified with different volumes of CS/CB dispersion at concentration of (a) 1 mg/mL; (b) 3 mg/mL; (c) 5 mg/mL; (d) 10 mg/mL. Inset: the relative CVs obtained



electrochemical behavior in terms of peak intensity and peak to peak separation, stability of CS/CB-dispersion, and time of CS/CB-SPE preparation. It is worthy of note that in the presence of chitosan, a stable dispersion with a higher content of CB (3 mg/mL) was obtained (data not shown), compared with CB dispersion in dimethylformamide-water mixture, where 1 mg/mL is the maximum content of CB for a stable dispersion and where three depositions of 2 μL of 1 mg/mL of CB dispersion were required [27].

The chitosan concentration was also optimized and the data reported in Fig. 2 clearly evidenced an enhancement of the probe electrochemical performances with the decrease of the chitosan concentration. The best results in terms of peak intensity ($50 \pm 1 \mu\text{A}$) (Fig. 2a) and peak to peak separation ($0.080 \pm 0.001 \text{ V}$) (Fig. 2b) were obtained using a chitosan concentration of 0.05% w/w. These experimental evidences could be ascribed to the thickness of the CS/CB layer on the

working electrode surface; low concentrations of chitosan could entail a layer with a thinner thickness, allowing better substrate diffusion; 0.05% w/w of chitosan was selected as a compromise between good electrochemical performance and stability of the dispersion.

Electrochemical characterization of the CS/CB-SPE

The SPE fabricated by drop casting 2 μL of CB (3 mg/mL) dispersion in chitosan solution at 0.05% w/w was then electrochemically characterized. A linear relationship between the current of the anodic and cathodic peaks versus the square root of the scan rate was observed varying the scan rate from 10 to 200 mV/s in the presence of potassium ferricyanide 1 mM, indicating a semi-infinite linear diffusion-controlled reaction (data not shown). The heterogeneous rate constant (k_0) using the Nicholson method [40] was $1.85 \times 10^{-2} \text{ cm/s}$, in agreement with our previous works using dimethylformamide-water mixture ($2.0 \times 10^{-2} \text{ cm/s}$ [27]) or acetonitrile ($1.59 \times 10^{-2} \text{ cm/s}$ [9]) as solvents, demonstrating the robustness of the SPE modification procedure with CB.

In addition, the electroactive area was calculated by using Randles-Sevcik equation [41], obtaining a value of $23.2 \pm 0.4 \text{ mm}^2$. This value is higher than that previously revealed using SPE modified with CB dispersion in dimethylformamide-water mixture ($9.3 \pm 1.2 \text{ mm}^2$) [42], suggesting that the presence of chitosan is able to produce highly dispersed CB.

Then CS/CB-SPE was used for the measurement of caffeic acid, hydroquinone, thiocholine, ferricyanide, and the results were compared with the SPE modified with only chitosan as well as with the bare SPE. In the case of ferricyanide, caffeic

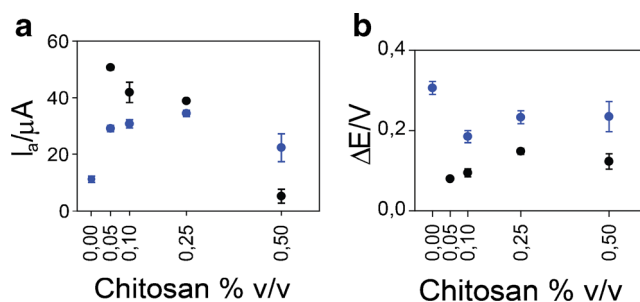


Fig. 2 Optimization of chitosan concentration. Anodic peak current (a) and peak to peak separation (b) detected by cyclic voltammeter recorded in ferricyanide 1 mM, phosphate buffer 0.05 M, and KCl 0.1 M as electrolyte, pH 7; scan rate 100 mV/s using CS/CB-SPE (black dot) or CS-SPE (blue dot)

acid, and hydroquinone detection, decreases of peak to peak separation and increases of the current intensity were observed using CS/CB-SPE compared with CS-SPE and bare SPE [see [Electronic Supplementary Material](#) (ESM), Fig. S1]. In the case of thiocholine measurements, a relevant shift of the anodic potential was observed for the CS/CB-SPE (Fig. 3a). The relevant shift of the anodic potential makes possible the detection of thiocholine at a lower applied potential. Taking into account that thiocholine is the enzymatic product of acetylcholinesterase (AChE), modified SPE was used as a platform to develop an AChE biosensor.

Development of an AChE biosensor

Choice of applied potential

To demonstrate the biocompatibility of our platform, the AChE-biosensor was first challenged by using the cyclic voltammetry technique. The AChE biosensor was put in contact with a solution of acetylthiocholine 1 mM in phosphate buffer for 15 min and after that the cyclic voltammogram was recorded observing an excellent response of the thiocholine enzymatically produced by the immobilized AChE. To remark on the contribution of CB, the same procedure was performed by using AChE/CS-SPEs and, as depicted in Fig. 3b, no peaks were detected. It is interesting to note the retention of the enzymatic activity as well as the improved electrochemical performances of CB that allow thiocholine detection at a low applied potential even in the presence of the enzyme. In order to achieve better biosensor sensitivity, the amperometric mode was chosen as electrochemical technique and the applied potential was selected. Taking into account the data obtained using the cyclic voltammetry technique (that showed a significant increase of anodic current at an applied potential of 0.3 V) as well as the advantage of using low applied potentials [43], we selected and tested applied potentials of 0.2, 0.3, and 0.4 V versus Ag/AgCl. As depicted in Fig. 3c, working at an applied potential of 0.3 V, we obtained better reproducibility; thus this potential was selected for further studies. The result achieved is better than the ones reported using a glassy carbon electrode modified with a dispersion of porous-reduced graphene oxide and chitosan, which required 0.75 V as applied potential [25], a glassy carbon electrode modified with platinum nanoparticles-carboxylic graphene-nafion, which required 0.5 V applied potential [44], and a glassy carbon electrode modified with a gold nanoparticle-polypyrrole-reduced graphene oxide, which required 0.68 V applied potential [45].

Optimization of enzymatic units

This biosensor was assembled to detect organophosphorus pesticides, which are irreversible inhibitors of AChE. In the case of irreversible inhibition, the optimization of the

immobilized enzymatic units is a crucial step to achieve a low detection limit. The reaction of enzymes with inhibitors follows stoichiometric laws, and this means that to detect low concentrations of inhibitor, low enzymatic units should be used. We tested the response towards the substrate at an applied potential of 0.3 V versus Ag/AgCl, using the biosensor fabricated with different amounts of AChE immobilized on the working electrode surface (varying from 0.005 to 0.2 U), as reported in Fig. 3d. We selected 0.01 U of AChE as the best compromise in terms of the substrate response (around 200 nA) and the repeatability attained (RDS % intra-electrode equal to 3.2%).

Optimization of pH

The pH could affect the response of biosensor because the acetylcholinesterase enzymatic activity is pH-dependent, and the best response using immobilized AChE is usually comprised between pH = 6 and pH = 8 [46]. We investigated three pH values in this range (i.e., pH = 6, 7.4, and 8 (Fig. 3e)). We observed an increment of the response with the pH increasing, however, at pH = 8, a lack of reproducibility was revealed. This behavior could be ascribed to the pH effect on chitosan because at this pH, the positively charged amino groups are deprotonated, and this decreases the electrostatic interactions between the chitosan and the enzyme [47]. The value of pH = 7.4 was thus selected as a compromise between sensitivity and reproducibility.

Acetylthiocholine measurement

Once optimized, the biosensor was then tested towards acetylthiocholine (Fig. 3f). The enzymatic substrate was analyzed in the range between 1×10^{-5} M and 5×10^{-3} M, observing a Michaelis-Menten behavior with a $K_{\text{Mapp}} = 0.69 \pm 0.07$ mM, which, as expected, is higher than the one obtained using the enzyme free in solution [38]. The detection limit of acetylthiocholine was found 3×10^{-6} M (calculated as a ratio signal to noise equal 3). The acetylthiocholine concentration that gave V_{max} was 1.8 mM, and 2 mM was chosen for the organophosphorus pesticide determination to have a high rate of enzymatic activity, while avoiding the inhibition due to a high concentration of substrate [48]. To evaluate the stability of the biosensor, in terms of adhesion of the AChE/CS/CB layer onto the working electrode surface, successive measurements ($n = 5$) of acetylthiocholine after a period of 20 min in stirring conditions were taken. A RSD % lower than 8% was obtained, demonstrating the good stability of the biosensor in working conditions.

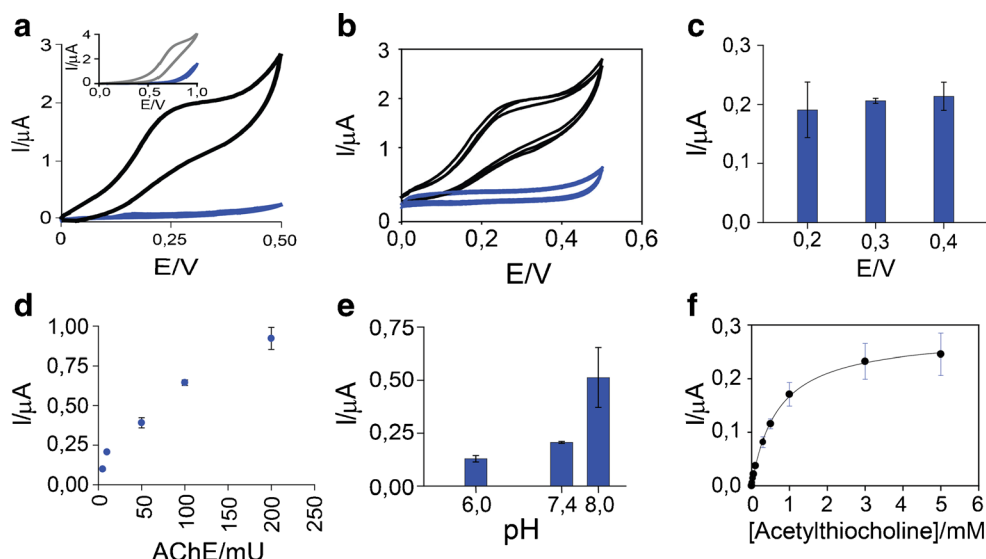


Fig. 3 (a) CVs of thiocholine 1 mM in phosphate buffer 0.05 M and KCl 0.1 M as electrolyte, pH 7 and scan rate 100 mV/s using CS/CB-SPE (black line) and CS-SPE (blue line). Inset: CVs with a wider potential range using CS-SPE (blue line) and bare SPE (grey line). (b) CVs of using biosensor based on SPE modified with chitosan and CB (AChE/CS/CB-SPE, black line) and SPE modified with chitosan (AChE/CS-SPE, blue line) in presence of acetylthiocholine 1 mM; scans recorded in phosphate buffer 0.05 M and KCl 0.1 M as electrolyte, pH 7.4; scan rate 50 mV/s. The measurements were taken in triplicate. (c) Choice of applied potential. AChE/CS/CB-SPE using acetylthiocholine 1 mM. Applied potential: +300 mV versus Ag/AgCl, phosphate buffer 0.05 M

+ KCl 0.1 M, pH 7.4. The measurements were taken in triplicate. (d) Choice of enzymatic units. AChE/CS/CB-SPE using acetylthiocholine 1 mM. Applied potential: +300 mV versus Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. The measurements were taken in triplicate. (e) Choice of pH. AChE/CS/CB-SPE using acetylthiocholine 1 mM. Applied potential: +300 mV versus Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M. The measurements were taken in triplicate. (f) Calibration plot of acetylthiocholine. Applied potential: +300 mV versus Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. The measurements were taken in triplicate

Characterization of the (bio)sensor

Electrochemical impedance spectroscopy was employed to evaluate the changes on the working electrode surface by chitosan, CB, and AChE. The value of electron transfer resistance (R_{ct}) using ferro/ferricyanide as redox probe was estimated by the evaluation of the electron transfer between the solution and the electrode, and R_{ct} was calculated according to the diameter of the semicircle present at the high frequency region [49]. Electrochemical impedance spectroscopy was carried out using bare SPE, SPE modified with CB (CB-SPE), chitosan (CS-SPE), CB and chitosan (CS/CB-SPE), and CB, chitosan, AChE (AChE/CS/CB-SPE) at an open circuit potential (OPC). Fitting of spectra was performed using the Randles equivalent circuit [50] shown in Fig. 4 (inset), which comprises the electrolyte resistance, R_s , (around 140 Ω) in series with a parallel combination of R_{ct} (interfacial charge transfer resistance), Z_w (diffusion of the analytes in solution and corresponding to Warburg impedance straight-line of the curves), and CPE (constant phase element). Figure 4 shows the Nyquist plots for bare SPE, CB-SPE, CS-SPE, CS/CB-SPE, and AChE/CS/CB-SPE. The R_{ct} value for bare SPE was much higher ($82250 \pm 210 \Omega$) than the one found using the modified SPE (R_{ct} CB-SPE = $54 \pm 3 \Omega$; R_{ct} CS-SPE = $1429 \pm 23 \Omega$; R_{ct} CS/CB-SPE = $46 \pm 1 \Omega$), confirming the improvement of the electron transfer using

chitosan and/or CB. In the case of CS-SPE, the decrease of R_{ct} was probably ascribed to the charge effect, since the ferri-cyanide is negative and chitosan is positive; a different explanation is mandatory in the case of CB. In fact, as demonstrated by some previous papers [27, 51], the CB confers to the electrode surface a low R_{ct} , improving the electrochemical performances, probably due to its good conductivity, high specific surface area, and high number of defect sites. In the case of the AChE/CS/CB-SPE, the R_{ct} value is equal to $77 \pm 10 \Omega$, and

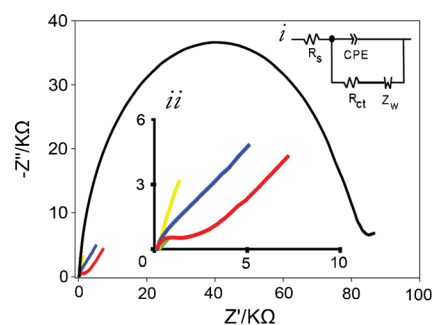
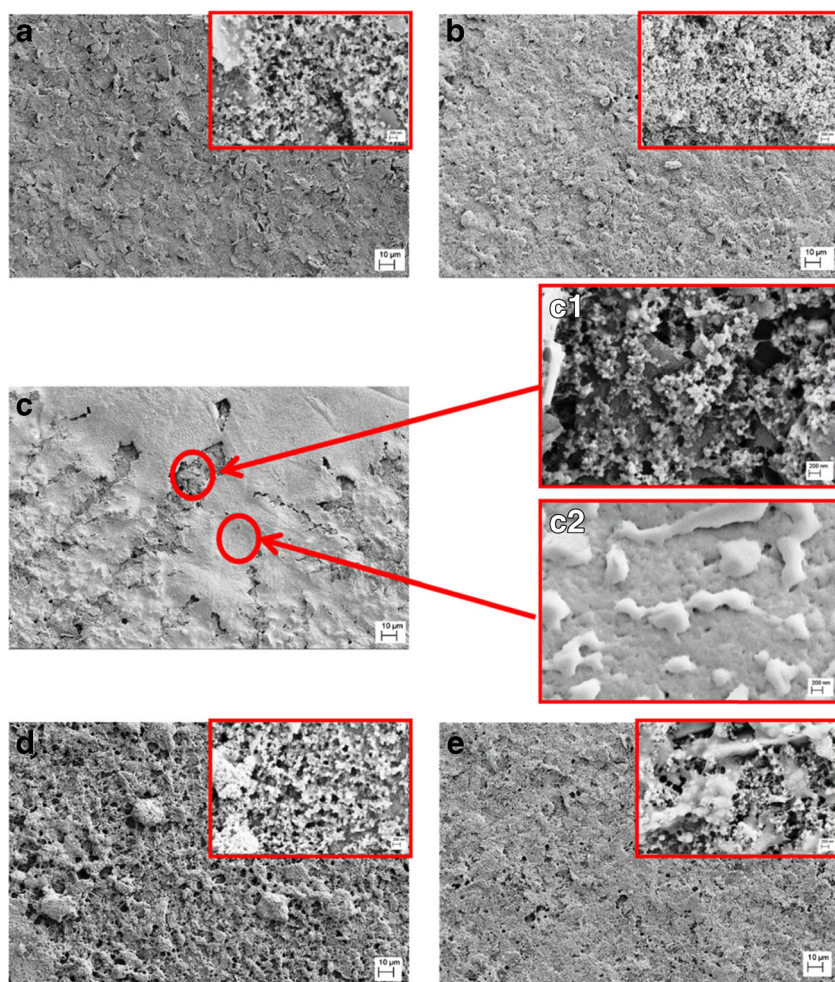


Fig. 4 Complex plane impedance plots at an open circuit potential for bare-SPE (black line), CB-SPE (blue line), CS-SPE (red line), CS/CB-SPE (yellow line), and AChE/CS/CB-SPE (green line), using a 5 mM ferri-cyanide and 5 mM ferrocyanide solution in KCl 0.1 M. Inset (i): Randles circuit (ii) highlight of the complex plane impedance plot at the high frequency for CB-SPE (blue line), CS-SPE (red line), CS/CB-SPE (yellow line), and AChE/CS/CB-SPE (green line)

Fig. 5 SEM micrographs of (a) bare SPE, (b) CB-SPE, (c) CS-SPE (c1 and c2 higher magnification of selected areas), (d) CS/CB-SPE, (e) AChE/CS/CB-SPE



this value is rather different from the case of the enzymatic membrane deposited on CB-SPE ($R_{ct} = 2842 \pm 89 \Omega$) [51]. These results suggest the absence of the enzymatic layer that does not hamper the electron transfer of the electrochemical probe (ferro/ferricyanide). In order to confirm these results, the (bio)sensors were morphologically characterized.

In Fig. 5, SEM micrographs of all the prepared (bio)sensors are compared. In the case of CB-SPE, it is possible to evidence a continuous, uniform, and homogeneous layer of CB nanoparticles (Fig. 5b), with the presence of numerous cauliflower-like CB aggregates (Fig. 5b, inset) which completely cover the underneath ink. In fact, the bare SPE is characterized by an inert polymeric binder and randomly orientated micrometric graphite flakes (Fig. 5a), which cannot be clearly observed for CB-SPE because of the uniform CB nanoparticles deposition on its surface (Fig. 5b).

In the case of CS-SPE (Fig. 5c), it is possible to observe a thick, cracked polymeric layer, characterized by a rough surface, as evidenced in Fig. 5c2, completely covering the underneath ink which can be revealed in correspondence of the several cracks, exposing the below surface (Fig. 5c1).

It is worthy to highlight that the chitosan layer was not detected on the CS/CB-SPE surface (Fig. 3d), confirming the chitosan efficacy to completely envelop the CB nanoparticles, acting as surfactant. Moreover, the interaction between the CB nanoparticles and the chitosan promoted the formation of a rougher and more porous CB layer with respect to the continuous and compact one of CB-SPE sample (Fig. 5b), originating a network characterized by the presence of numerous and diffuse voids (Fig. 5d). In fact, the CS/CB-SPE was composed of CB particles and cauliflower-like structures that were linked together by the excess of chitosan with respect to CB (Fig. 5d), as evident from the higher magnification shown in the inset of Fig. 5d.

Finally, regarding the AChE/CS/CB-SPE, significant morphologic differences can be evidenced (Fig. 5e), probably due to the different features of the starting drop, composed of chitosan, CB, and enzyme with resultant different solvent evaporation modality and rate after drop casting on SPE. Apparently less cauliflower like structures can be detected, being completely embedded within an organic net, as emphasized in the inset of

Fig. 5e. Thus, it is possible to hypothesize an interaction between the enzyme, the polymer, and the carbon nanomaterial, and to confirm the absence of an enzymatic membrane, in agreement with the electrochemical impedance spectroscopy data.

Paraoxon pesticide detection

The analytical features of biosensors for paraoxon detection were assessed, taking paraoxon as the organophosphorus pesticide model. The calibration curve was obtained using 20 min as incubation time. Since paraoxon is able to irreversibly inhibit AChE, the sensitivity of the measurement increases with the increment of the incubation time, and usually a compromise between analysis time and sensitivity is selected. Taking into account that 10–20 min is a reasonable incubation time [48], 20 min time was selected. We observed a linear range up to $0.5 \mu\text{g L}^{-1}$ described by the following equation $y = 126x + 3$, $R^2 = 0.958$ (Fig. 6). The detection limit, calculated as 10% of inhibition [48], was found equal to $0.05 \mu\text{g L}^{-1}$. The detection limit is lower than the one achieved using butyrylcholinesterase immobilized on SPE modified with CB and cobalt phthalocyanine ($5 \mu\text{g L}^{-1}$) by cross-linking [33] or using acetylcholinesterase–choline oxidase immobilized on a gold–platinum bimetallic nanoparticles modified glassy carbon electrode (around $40 \mu\text{g L}^{-1}$) [52], suggesting that the improved performances can be due to the novel enzyme immobilization supported by the combined use of chitosan and CB.

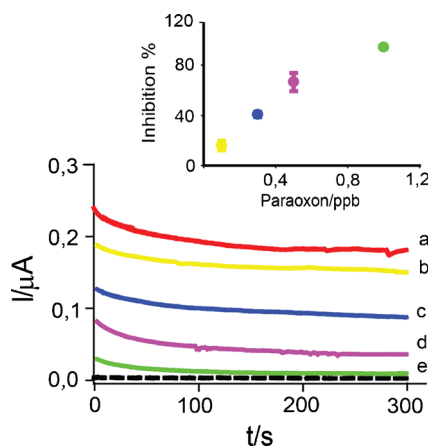


Fig. 6 Chronoamperograms in presence of 2 mM acetylthiocholine without (a) and with exposure of biosensor to (b) 0.1, (c) 0.3, (d) 0.5, and (e) $1 \mu\text{g L}^{-1}$ of paraoxon. Measurements were taken in phosphate buffer 0.05 M and KCl 0.1 M as electrolyte, pH 7.4, 20 min of incubation time, applied potential of +300 mV versus Ag/AgCl. Inset: the calibration curve. The black dashed line is the response of biosensor in buffer solution

The inter-electrode reproducibility, obtained measuring the paraoxon solution at a concentration of $0.5 \mu\text{g L}^{-1}$ using different biosensors ($n = 5$), was found equal to 10%. It is not possible to quantify the intra-electrode reproducibility because of the irreversible character of inhibition. For pesticide measurements using the same biosensor, a reactivation step with compounds like Pyridine 2-aldoxime methiodide (2-PAM) [53] and obidoxime [54] is required, and usually it should be carried out immediately after the inhibition to avoid the phenomenon called “aging” of the chemical bond between the inhibitor and the enzyme [55]. Herein, we propose disposable biosensors to avoid the reactivation procedure, with the advantage of an easier and faster measurement.

This biosensor was then applied for the detection of paraoxon in drinking waters, since the legal limit for the total amount of pesticides is $0.5 \mu\text{g L}^{-1}$ [56]. In order to avoid electrochemical and enzymatic interferences, the “medium exchange method,” proposed by us in a previous work [51], was adopted. Briefly, this method consists in three steps: in the first step, the enzymatic activity is measured in buffer solution in the presence of only the enzymatic substrate; in the second step, the biosensor is placed in contact with the sample contaminated with pesticide for a selected time (the incubation time), followed by an extensive rinsing of the biosensor with distilled water; in the last step, the enzymatic residual activity is finally determined in a new buffer aliquot in the presence of only the enzymatic substrate. In this way, it is possible to avoid electrochemical interferences such as phenolic compounds, ascorbic acid, etc., since the enzymatic activity is quantified in phosphate buffer. Indeed, washing the biosensor with distilled water after the inhibition step, only irreversible inhibitors (e.g., organophosphates) able to link the enzyme by covalent bond can be detected, while the other types of inhibitors that could be present in waste water samples such as F^- , Cd^{2+} , Cu^{2+} (reversible inhibitors) are avoided being washed away in the second step [51]. This biosensor is thus capable of detecting the family of organophosphorus and carbamic pesticides because they are able to irreversibly inhibit AChE, while in the case of a mixture of these compounds, only an “anti-cholinesterase activity index” can be calculated [57]. As underpinned in our recent review on enzyme inhibition-based biosensors [48], this type of biosensor can be considered as a “Family Doctor” and further analyses have to be performed by a “Specialist Doctor”, such as a high performance liquid chromatography to separate and quantify each organophosphorus and carbamic pesticide present in contaminated samples.

The sample analyzed using the “medium exchange method” showed the absence of inhibition; thus to evaluate the accuracy of the biosensor, the drinking water sample was

Table 1 Comparison among some biosensors for paraoxon detection based on cholinesterase inhibition

Biosensor	Technique	Incubation time	LOD	Linear range	Ref.
BChE-BSA-Nafion/GA/CoPc-CB/SPE ^a	Amperometry (0.05 V versus Ag/AgCl)	20 min	18 nM (5 ppb)	36–110 nM (10–30 ppb)	[33]
BChE-BSA-Nafion/GA/CBSPE ^b	Amperometry (0.3 V versus Ag/AgCl)	20 min	5 ppb	5–30 ppb	[51]
cGO-NTA-Ni-*AChE/GCE ^c	Amperometry (0.2 V versus Ag/AgCl)	10 min	0.65 nM (0.18 ppb)	0.65 nM–10 μ M (0.18 ppb–2.75 ppm)	[58]
GA/Gel/PPy- AChE/Pt ^d	Chronoamperometry (0.7 V vs. Ag/AgCl)	60 min	1.1 ppb	12.5–150 ppb	[59]
AChE/GA/Cyst(AuSPE ^e)	Amperometry (0.4 V versus Ag/AgCl)	15 min	2 ppb	2–40 ppb	[60]
AChE/CB-CS/SPE ^f	Chronoamperometry (0.3 V versus Ag/AgCl)	20 min	0.05 ppb	0.1–0.5 ppb	This work

^a Butyrylcholinesterase-Bovine Serum Albumin-Nafion/Glutaraldehyde/Cobalt phthalocyanine-Carbon Black /Screen-Printed Graphite Electrode

^b Butyrylcholinesterase-Bovine Serum Albumin-Nafion/Glutaraldehyde/Carbon Black/Screen-Printed Graphite Electrode

^c Carboxyl Graphene Oxide-Na₂Na-Bis(carboxymethyl)-L-lysine hydrate-Nichel-His-tagged Acetylcholinesterase /Glassy Carbon Electrode

^d Glutaraldehyde/Gelatin/Polypyrrole-Acetylcholinesterase/Platinum Electrode

^e Acetylcholinesterase /Glutaraldehyde/Cysteamine/Screen-Printed Gold Electrode

^f Butyrylcholinesterase/Chitosan-Carbon Black/Screen-Printed Graphite Electrode

spiked with 0.5 μ g L⁻¹ obtaining a satisfactory recovery value of 97 $\pm$ 15% (n = 3).

Conclusions

In this work, the suitability of chitosan and carbon black for the acetylcholinesterase enzyme immobilization was exploited and evaluated by the construction and assembling of an acetylcholinesterase-based biosensor. The presence of chitosan entails the production of a sustainable dispersion, avoiding the use of organic solvents and of ultrasound-assisted sonication. Thus, using these mild conditions, it is possible to add the enzyme directly in the dispersion, and modify the screen-printed electrode in one step by a drop casting technique. Of note, even in presence of chitosan and enzyme, carbon black is still able to detect thiocholine at a low potential without fouling. The enzyme immobilization performed in the favorable environment and the excellent electrochemical features of the carbon black allow the construction and assembling of a biosensor with low detection limit (Table 1) suitable for pesticide monitoring in drinking waters.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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