Talanta 155 (2016) 258–264

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Biosensor based on electrospun blended chitosan-poly (vinyl alcohol) nanofibrous enzymatically sensitized membranes for pirimiphos-methyl detection in olive oil

A.Y. El-Moghazy [a], E.A. Soliman [a], H.Z. Ibrahim [b], J.-L. Marty [c], G. Istamboulie [c], T. Noguer [c,*]

[a] Polymeric Materials Research Department, Advanced Technology and New Materials Research Institute, City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab City 21934, Alexandria, Egypt
[b] Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Alexandria 832, Egypt
[c] BAE: Biocapteurs-Analyses-Environnment, Universite de Perpignan Via Domitia, 52 Avenue Paul Alduy, Perpignan Cedex 66860, France

ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form
5 April 2016
Accepted 7 April 2016
Available online 22 April 2016

Keywords:
Biosensor
Nanofibers
Electrospinning
Genetically-Modified Acetylcholinesterase
Organophosphorus Pesticides
Olive Oil

ABSTRACT

An ultra-sensitive electrochemical biosensor was successfully developed for rapid detection of pirimiphos-methyl in olive oil, based of genetically-engineered acetylcholinesterase (AChE) immobilization into electrospun chitosan/poly (vinyl alcohol) blend nanofibers. Due to their unique properties such as spatial structure, high porosity, and large surface area, the use of nanofibers allowed improving the biosensor response by two folds. The developed biosensor showed a good performance for detecting pirimiphos-methyl, with a limit of detection of 0.2 nM, a concentration much lower than the maximum residue limit allowed set by international regulations (164 nM). The biosensor was used for the detection of pirimiphos-methyl in olive oil samples after a simple liquid-liquid extraction, and the recovery rates were close to 100%.

1. Introduction

As a consequence of the increase of world population, the intensification of modern agriculture has been accompanied by a dramatic increase of the use of pesticides. Among these pesticides, organophosphates (OPs) and carbamates are widely used as insecticides due to their high activity and relatively low persistence. Pirimiphos-methyl (O-[2-(diethylamino)-6-methyl-4-pyrimidinyl] O,O-dimethylphosphorothioate) is a contact and fumigant organophosphorus pesticide, which is applied on a wide range of plants such as vegetables, olives, ornamentals, bulb flowers, sugar cane, vines, citrus and other fruit for controlling whiteflies, thrips, mealybugs, aphids, and mites (Fig. 1). This pesticide is also used against a broad-spectrum of pests in grains storage silos, animal houses, industrial buildings [1]. As others organophosphorus compounds, pirimiphos-methyl acts by phosphorylating acetylcholinesterase (AChE) enzyme in tissues, causing accumulation of acetylcholine at synapses and neuro-muscular junctions, which can finally lead to death [2].

In the last decade, an increased attention has been focused on pesticides residues in food. Whilst the great majority of food analyses are performed by chromatographic methods (GC–MS and HPLC) [3,4], the resulting public concern created a demand for the development of reliable, simple and low-cost methods for “in field” detection. In the recent years biosensor technology has been proved a smart alternative to these traditional analytical tools, providing many advantages such as accuracy, simplicity, rapidity, specificity, sensitivity, relatively economic equipment, and user-friendly operation. Electrochemical biosensors based on the inhibition of acetylcholinesterases (AChE) have been intensively studied in the aim of detecting carbamate and organophosphorus insecticides [5]. The sensitivity of AChE-based biosensors can be increased using two different strategies. The first approach rests upon the use of genetically modified enzymes, which have opened new horizons for increasing biosensor selectivity and sensitivity to pesticides [5,6]. The second strategy is based on developing conducting polymers and nanotechnology for allowing a subsequent improvement of signal intensity and selectivity, where, a special attention has been paid to nanomaterials, especially in form of nanoparticles or nanotubes, as they allow enhancing the specific surface area, thus promoting the electron transfer from the biomolecule to the transducer [7–9]. In this context, polymeric nanofibers have been tried as another nano-structured form of polymeric immobilization matrices to enhance the electrochemical

* Corresponding author.

http://dx.doi.org/10.1016/j.talanta.2016.04.018
0039-9140/

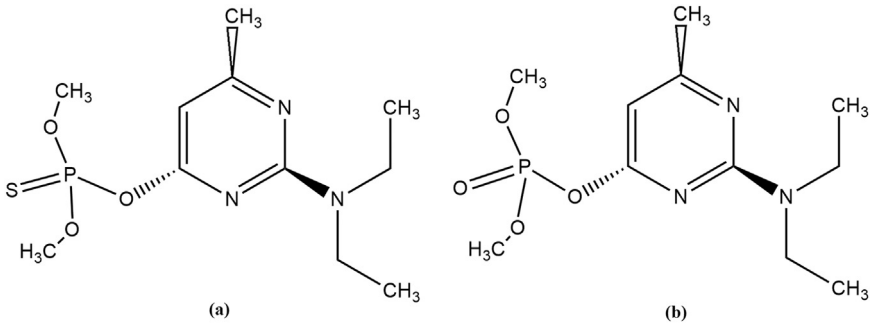

Fig. 1. Chemical structure of: (a) Pirimiphos-methyl and (b) Pirimiphos-methyl oxon.

performance of biosensors [10]. Electrospinning has been shown an efficient technique for nanofibers formation, with many unique characteristics including high surface area to volume ratios and tunable porosity [11]. Electrospun nanofibers have been applied in many fields such as medical applications, filtration, textiles and sensors [12–14]. In sensor applications, gas, optical and colorimetric sensors have been successfully developed by employment of nano-fibers [15–17], however, to best of our knowledge a few of studies on the construction of biosensor based on electro-spun polymeric nano-fibers. Employment of these electro-spun polymeric nano-fibers as supports for biological molecules provides higher loading and enhancing in the catalytic activity by reducing the diffusion resistance of the substrate [18–20]. This impelled us to employ electrospun nanofibrous membranes based on non-toxic, biodegradable and cost-effective blended polymers of chitosan (CS) and poly (vinyl alcohol) (PVA) as a support to immobilize genetically modified acetylcholinesterase, where the bioactive nanofibrous CS-PVA membrane is directly applied onto surface of screen-printed electrodes. The constructed biosensor prototype has been tested for detecting pirimiphos-methyl in olive oil.

2. Material and methods

2.1. Reagents, enzymes and solutions

Acetylcholinesterase (EC 3.1.1.7) from electric eel (EE) (Type V-S, 1000 U/mg), chitosan (low molar weight), and other chemicals were purchased from Sigma Chemical Co. (Germany). Poly (vinyl alcohol) (MW =72000) was purchased from Merck Co. (Germany). Acetylcholinesterase from *Drosophila melanogaster* Wild type enzyme (B131) and genetically-modified enzyme (B394) were produced by the Centre de Recherche de Biochimie Macromoléculaire (CRBM) (Montpellier, France). Graphite (Electrodag 423SS) and silver/silver chloride (Electrodag 418SS) inks were obtained from Acheson (Plymouth, UK). Cobalt phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). Poly (vinyl) chloride (PVC) sheets (200 mm x 100 mm x 0.5 mm) (SKK, Denzlingen, Germany) were used as support for the screen-printed electrodes. A glycerophtalic paint (Astral, France) was used as insulating layer. All solutions were prepared daily with distilled water and stored at 4 °C.

2.2. Determination of enzyme activity

The studies of AChEs activity were carried out with a Shimadzu UV-1800 spectrophotometer. Enzyme kinetics were measured using Ellman's method [21], which is based on the reaction between the reaction product thiocholine and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), leading to a yellow compound (5-thio-2-nitrobenzoate) absorbing at 412 nm ($\varepsilon = 13{,}600\ M^{-1}cm^{-1}$). 1 enzymatic unit (U) was defined as the amount of enzyme hydrolysing 1.0 µmole of acetylthiocholine per min.

2.3. Determination of enzymes inhibition constant (k_i)

Organophosphates induce an irreversible inhibition of acetylcholinesterase by phosphorylation of a serine residue in the active site [22]. In this work, the inhibition constant k_i for the three types of AChE was determined at 30 °C as described by Villatte et al. [23]. AChE was incubated for 0, 10, 30, 50, 70 and 90 s with different concentrations of pesticides, and the enzyme activity was determined spectrophotometrically as described before. The graphs obtained by plotting log of residual activity versus incubation time for each inhibitor showed a linear representation. The apparent reaction rate k_{obs} (min^{-1}) were obtained by measuring the slope of this straight line. Plotting $1/k_{obs}$ versus 1/[I] allowed calculating the inhibition constant k_i, which corresponds to the reciprocal value of the obtained slope.

2.4. Biosensor construction

2.4.1. Fabrication of screen-printed electrodes (SPEs)

Screen-printed electrodes (SPEs) were fabricated by a semi-automatic DEK248 printing machine in a three-electrode configuration [6,7]. The working electrode incorporating cobalt phtalocyanine as mediator consisted of a 4 mm-diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight track.

2.4.2. Nanofibrous CS-PVA membrane electrospinning

To prepare electrospun CS-PVA blend nanofibrous membrane, PVA aqueous solution (8% w/w) was prepared by dissolving the definite amount of PVA in water with stirring for 3 h at 75 °C. Chitosan (3% w/w) was prepared by dissolving the definite amount of chitosan in acetic acid (90% v/v) with stirring for 3 h at room temperature. A homogenous PVA-CS blend was achieved by mixing of both of PVA and CS solutions with a mass ratio of 60/40. The obtained solution was loaded into a 5 mL syringe fitted with a 0.8 mm needle (internal diameter), which was connected to a high voltage power supply. Electrospinning process was carried out using a ESP200D electrospinning machine (Nanonc, Germany), at 18 kV, respecting a 15 cm distance between the needle tip and the electrode surface. The resulting CS-PVA blend nanofibrous membranes formed directly onto the surface of SPEs under these conditions were structurally characterized by Fourier transform infrared spectral analysis using a Shimadzu FT-IR 8400 S (Shimadzu, Japan). Scanning electron microscopy characterization was performed using a Joel JSM 6360LA SEM (JEOL, Japan).

2.4.3. Activation of fibrous polymeric membrane and immobilization of AChE

The electrospun CS-PVA blend nanofibrous membranes collected on surface of the SPEs were activated with glutaraldehyde 1% for 2 h. After completion of this activation process, nanofibers-activated SPEs were rinsed with distilled water to remove unreacted glutaraldehyde. Activated electrospun nanofibrous membranes deposited onto SPEs were then incubated with AChE (1mU in buffer) for 6 h at 4 °C. The resulting sensor (AChE/ CS-PVA NFM/ SPE) was rinsed with buffer until no soluble enzyme is detectable in washing buffer. Relative activity (A_r %) was considered as a basic indicator for efficiency of the immobilization process and it is calculated as follows:

$$A_r\% = [a/b] \times 100$$

where, a=activity of immobilized enzyme, b=activity of total amount of free enzyme (1 mU).

SPE based on blended CS-PVA casted membranes (AChE/CS-PVA CM/SPE) were prepared in parallel and their performances were compared to those of AChE/CS-PVA NFM/SPE (Fig. 2).

2.5. Amperometric measurements

The reactions involved in the developed AChE biosensor have already been described in previous works [6,7], they are summarized in Fig. 3.

Thiocholine, the product of the enzymatic hydrolysis of the acetylthiocholine, reacts with the oxidized form of cobalt phtalocyanine (CoPc), an electronic mediator incorporated in the electrode core. The reduced form of CoPc is then reoxized at 100 mV vs Ag/AgCl reference electrode, using a 641 VA potentiostat (Metrohm, Switzerland) connected to a BD40 flatbed recorder (Kipp & Zonen, The Netherlands). The flow of generated electrons reflects the rate of acetylthiocholine hydrolysis, which is reduced while increasing enzyme phosphorylation. For performing amperometric measurements, the SPE was immersed vertically in a thermostated cell (30 °C) containing 5 mL of phosphate buffer pH 7, under magnetic stirring. After current stabilization, acetylthiocholine chloride (ATChCl) was added at final concentration 1 mM, allowing determination of the initial intensity A_0. Inhibition measurements were carried out in the same conditions, except that a 10-min incubation was performed in presence of a known amount of insecticide before acetylthiocholine injection. Electrodes and cell were thoroughly washed with distilled water between each measurement. The inhibition percentage was related with the insecticide concentration; it was calculated according to the following formula:

$$I(\%) = \left[(A_0 - A)/A_0 \right] X100$$

where A and A_0 are respectively the current intensity after and before the inhibition.

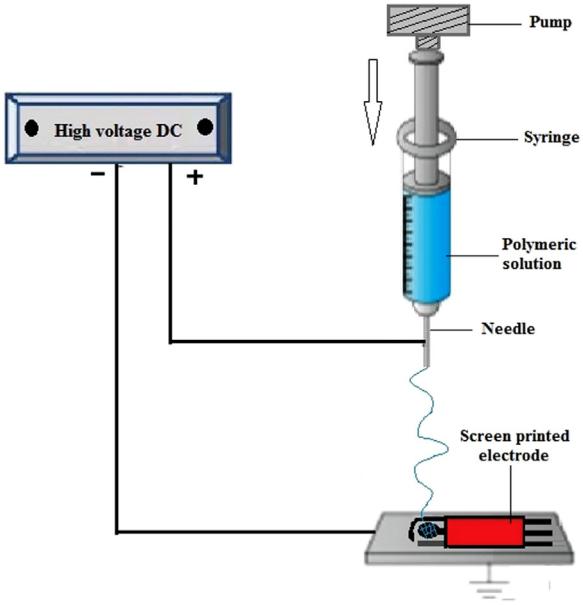

Fig. 2. Schematic representation of the electrospinning process.

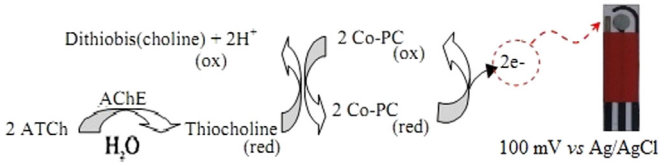

Fig. 3. Enzymatic and electrochemical reactions involved in the AChE-based biosensor.

2.6. Pesticide extraction from olive oil

The application of the developed biosensor on olive oil real samples was performed with a commercially available bio extra virgin olive oil purchased in a local supermarket, which was spiked with a known concentration of pesticides. Prior to analysis, the pesticide was extracted by a simple liquid-liquid extraction procedure as described by Ben Oujji et al. [24] as follows: 500 μL of the olive oil was added to 400 μL of acetonitrile and 100 μL of dichloromethane, the mixture was centrifuged at 13,400 rpm for 90 s, the resulting supernatant was then collected and used as pesticide mother solution. Like other thiophosphoryl compounds, pirimiphos-methyl is more active and toxic in its phosphate "oxon" form, which is generally obtained in vivo by metabolization process. For this reason, this study focuses on the detection of the oxidized product of pirimiphos-methyl, called pirimiphos-methyl-oxon. The oxidation step was carried out using N-bromosuccinimide (NBS) as already described in literature [25]. It was shown that a treatment with 10^{-6} M NBS was sufficient for the total oxidation of pirimiphos-methyl solution. The effect of NBS on the biosensor was tested to ensure that this oxidizing agent does not affect AChE, no effect of NBS on the enzyme was observed under the conditions used.

3. Results and discussion

3.1. Determination of inhibition constant (k_i)

The inhibition constant k_i is an indicator of the pesticide affinity for the enzyme. This parameter is useful for comparing the potency of inhibitors and assessing the sensitivity of different enzymes. Most of the biosensors already described in literature are based on the classical acetylcholinesterase from electric eel (*Electrophorus electricus*), while some other studies involve human or horse acetyl- and butyrylcholinesterases, but the resulting sensors showed low sensitivities to organophosphorus insecticides [26]. In this work, the sensitivity of electric eel AChE to pirimiphos-methyl oxon was compared to drosophila wild type and mutant acetylcholinesterases. As shown in Table 1, AChE B394 mutant enzyme displayed the best sensitivity to pirimiphos-methyl-oxon, its inhibition constant k_i is 28 and 358-fold higher than those of wild type drosophila and electric eel enzymes, respectively. Based on these results, the mutant drosophila AChE was used for developing

Table 1
Inhibition constants calculated for pirimiphos-methyl oxon using the three acetylcholinesterases.

Acetylcholinesterase	k_i (μmol^{-1} min^{-1})
Electric eel (wild type)	0.90
Drosophila (wild type)	11.4
Drosophila (B394 mutant type)	322

the biosensor for pirimiphos-methyl.

3.2. Characterization of electrospun PVA/chitosan nanofibers and cast membranes based on CS-PVA blend

3.2.1. FT-IR spectroscopic analysis

FT-IR spectral analysis was used to assess the structure of blended CS-PVA membranes. Moreover, spectra of CS-PVA blend were recorded after their activation and immobilizing AChE to study structural changes owing to these processes. The resulting spectra are presented in Fig. 4.

The recorded FT-IR spectrum of PVA (Fig. 4(a)) presented the dominant –CH (as/sym) stretching vibration of the polymer backbone in the spectral range 2916 – 2844 cm^{-1}. The adsorption bands of OH free groups (un-reacted non-bonded groups) in PVA appeared at 3734 cm^{-1}, while the adsorption peak owing to stretching vibration of OH groups involved in intra- and inter-molecular hydrogen bonding between PVA chains were observed at 3389 cm^{-1} because of their high hydrophilicity. A red shift and broadening of adsorption band owing to –OH stretching which appeared in a range of 3500–3600 cm^{-1} can be attributed to the inter-molecular hydrogen bonding between the polymer chains through the terminal hydroxyl groups. The shoulder nearby 2600 cm^{-1} is ascribed to combination of the adsorption bands belonging to stretching vibration of C=O and out–of–plane deformation of –OH. A broad adsorption bands due to C–H stretching appeared at 2924, 1324 and 843 cm^{-1} and bands due to – CH_2 bending appeared at 1429 cm^{-1}, the adsorption bands appeared at 1678 and 1560 cm^{-1}due to stretching C=O involved in the residual acetate groups remained from non-hydrolyzed polyvinyl acetate. Moreover, high intensive absorption band appeared at 1097 cm^{-1} is attributed to the crystallization or entanglement of PVA chains. FT-IR spectrum of chitosan (Fig. 4(b)) showed main characteristic bands which appeared at 3479 and 2891 cm^{-1} due to the axial stretching of –OH and C–H groups respectively, which appears superimposed to the N–H stretching band; N–H angular deformation at 1598 cm^{-1}; $-CH_3$ symmetrical angular deformation at 1359 cm^{-1} and C–N amino groups axial deformation, besides the two characteristic polysaccharide bands, 1026 and 1126 cm^{-1} due to the C–O stretching of β (1-4) glycosidic bonds. However, intensity of the adsorption bands appeared in both of CS and PVA spectra in a range of 3300–3500 cm^{-1} which are the characteristic of the N–H and –OH stretching vibration decreased significantly and overlapped in a characteristic peak at 3309 cm^{-1} in CS-PVA spectrum (Fig. 4(c)) that can be attributed to the intra-molecular hydrogen bonding of OH with NH_2 groups of both polymers. Also, the FT-IR spectra of CS-PVA blend showed that the N–H bending vibrations at 1599 cm^{-1} and C=O stretching bands appeared at 1678 and 1560 cm^{-1} shifted to 1587 cm^{-1} and 1682 cm^{-1} which suggested occurring interactions between $-NH_2$ and C=O groups. On the other side, a slight shift and increase of intensity of adsorption band due to C–N stretching that appeared at 1678 cm^{-1} in spectrum of CS-PVA blend after its activation with glutaraldehyde (Fig. 4(d)) prove occurring reaction of $-NH_2$ groups of chitosan with C=O groups of glutaraldehyde. Another evidence of amide bond formation is decrease intensity of amine group of chitosan at 3309 cm^{-1}. Fig. 4(e) is for enzyme immobilized on CS-PVA nanofiber, the evidence of enzyme immobilization can be clear in increasing peak intensity of amide group at 1668 due to bonds formed between NH_2 for enzyme and the free C=O of glutaraldehyde to form C=N bond, also presence of peak at 1074 is for secondary amines or amide of peptide for enzyme.

3.2.2. Morphological features

The morphological and micro-structural features of electrospun nanofibrous or casted membranes based on blended CS-PVA on screen-printed electrodes were explored using scanning electron microscopy (SEM). Micrographs (X5000) of these polymeric membranes deposited onto SPEs by electrospinning or casting techniques (Fig. 5) showed that the nanofibrous membrane produced using electrospinning really exhibited homogenous fibrous lattice structure with smooth surfaces and uniform diameters around 100 nm (monolayer or thin membrane) (Fig. 5(a)). No noticeable changes were shown in the unique three-dimensional structure of this nanofibrous lattice after its activation using

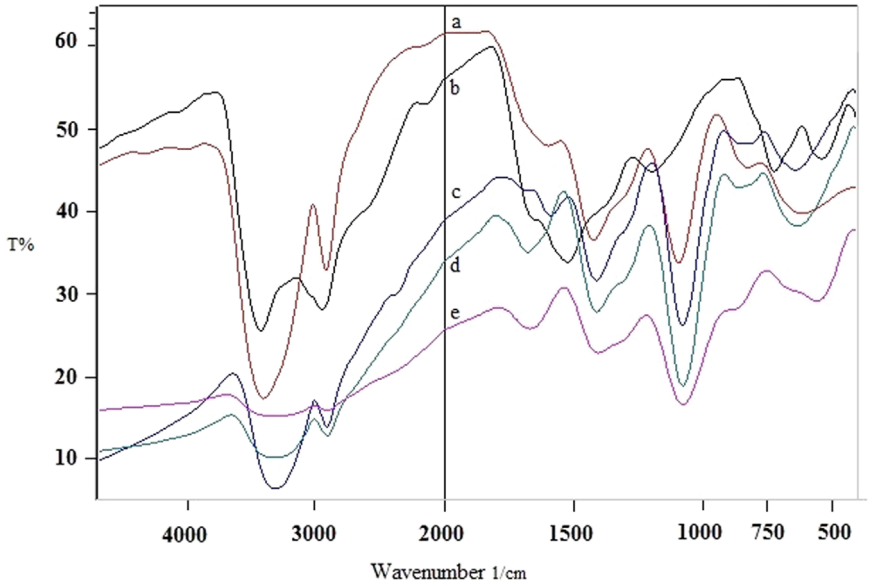

Fig. 4. FTIR Spectra for: (a) PVA, (b) CS, (c) CS-PVA, (d) GA/CS-PVA and (e) AChE/GA/CS-PVA.

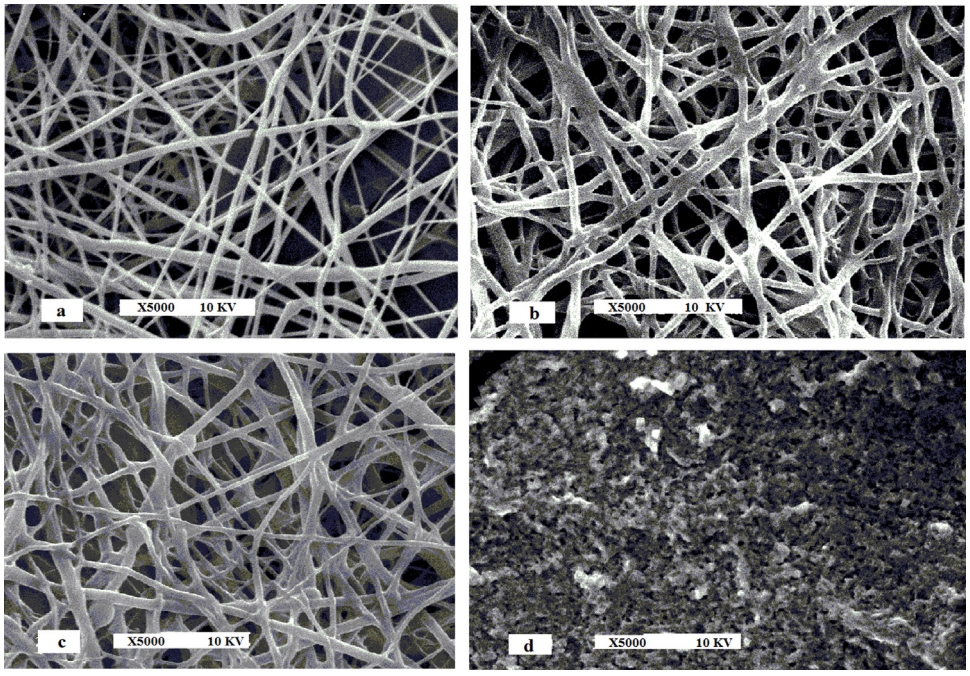

Fig. 5. SEM Micrographs (X5000) of electrospun CS-PVA NFM (a), activated electrospun CS-PVA NFM(b), AChE/electrospun CS -PVA NFM (c), AChE/CS-PVA CM (d).

glutaraldehyde, however this treatment slightly affected the diameter uniformity and alignment of CS-PVA nanofibers (Fig. 5(b)). Immobilized AChE onto the electrospun nanofibrous membrane appeared in form of nodes on this fibrous lattice (Fig. 5(c)). On the other side, CS-PVA blend membranes produced using casting technique loaded with AChE emerged massive structure (multi-layered and thick membrane) with crimpy surface (Fig. 5(d)).

3.3. Optimization of preparation process of AChE/CS-PVA NFM/SPE

Impacts of glutaraldehyde concentration, activation and immobilization time were studied to optimize the preparation processes that determine the activity of bioactive nanofibrous polymeric membranes and consequently performance of AChE/CS-PVA NFM based biosensor.

3.3.1. Activation

Glutaraldehyde concentration can be considered a critical factor determining the catalytic activity of the resulting sensitized membrane. Nanofibrous polymeric membranes were activated by using glutaraldehyde at different concentrations in range of 0.25–2% for constant time (2 h). The activity of immobilized AChE increased linearly while increasing glutaraldehyde concentration and a maximum activity was observed using 1% glutaraldehyde. Increasing glutaraldehyde concentration induces an optimum functionalization of chitosan, and therefore a higher immobilization yield. However, a loss of activity was observed with increasing the concentration over this value, probably due to multi-point binding between the enzyme molecules and polymeric nano-fibrous support or “protein-protein” interactions and hinder availability of their active sites [27] (see Fig. S1, Supplementary data).

The coupling process was optimized by varying the incubation time of glutaraldehyde 1% from 0.25 up to 8 h. It was shown that the enzyme relative activity increased with extending the activation time up to 2 h, due to an increased amount of AChE immobilized onto the blended nano-fibers. However, bioactive membrane activity noticeably decreased by using treatment times longer than 2 h; this could be attributed to the formation of an excess of immobilization sites, resulting in enzyme rigidification and/or to an increase of steric hindrance limiting accessibility to the active sites for substrate [27] (see Fig. S1, Supplementary data).

3.3.2. Immobilization

The impact of immobilization time on the activity of enzymatically-sensitized electrospun nanofibrous membranes was investigated by performing the immobilization process at 4 °C for different times ranged from 0.5 to 14 h. As shown from Fig. 6(c), increasing the incubation time of activated nanofibers with AChE up to 6 h resulted in increasing the activity of immobilized enzyme close to 90%. Notwithstanding, using longer contact time did not result in higher enzyme activity, indicating that immobilization sites of nanofibers were saturated [28].

The final protocol for designing AChE/CS-PVA NFM-based biosensors was thus based on a 2 h activation with glutaraldehyde 1% (v/v), followed by 6 h incubation with the enzyme.

3.4. Amperometric response to acetylthiocholine

The response of the developed AChE/ CS-PVA NFM/SPE biosensor to acetylthiocholine (ATCh) was studied and compared to the response of casted electrodes (AChE/ CS-PVA CM/SPE). The responses of electrospun electrodes non-modified by AChE (CS-PVA NFM/SPE) were also evaluated (see Fig. S2, Supplementary Data). It was observed that electrospun electrodes lead to current responses more two times higher than casted electrodes. These results are in good agreement with previous works comparing immobilized enzyme on nanofibers and casted membranes [29–31]. This behavior can be attributed to increase quantity of AChE molecules immobilized onto polymeric nanofibers with good conformational stability which are frequently a fundamental prerequisite for functional activity, whereas, the unique structure of nanofibers, which offer a large surface area and facilitates the access of substrate to the enzyme, thus enhancing the biosensor response. On the opposite, the dense structure of chitosan/PVA casted membrane showed higher diffusion resistance to the substrate, leading to lower biosensor responses.

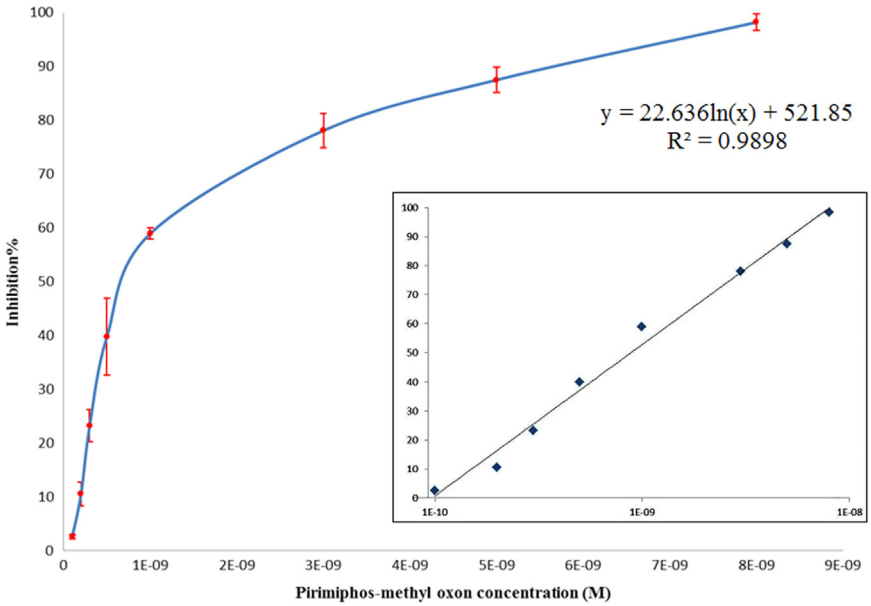

Fig. 6. Calibration curves obtained for the amperometric detection of pirimiphos-methyl oxon using the (AChE/CS-PVA NFM/SPE) biosensor. (window: semi-log plot).

Table 2
Analysis of olive oil samples spiked with pirimiphos-methyl. Found concentrations and recovery rates for 3 series of samples.

Series of spiked olive oil samples	Concentration of pirimiphos-methyl in spiked samples (M)	Found concentration (M)	Recovery rate (%)
1	3×10^{-8}	$2.95 \pm 0.064 \times 10^{-8}$	98.3 ± 2
2	1.5×10^{-7}	$1.53 \pm 0.032 \times 10^{-7}$	102 ± 2
3	5×10^{-7}	$4.8 \pm 0.084 \times 10^{-7}$	96 ± 1.6

3.5. Inhibition assays and determination of biosensor analytical performance

Under the optimal conditions, inhibition assays were carried out using pirimiphos-methyl oxon concentrations ranging from 1×10^{-10} to 8×10^{-9} M. The inhibition ratio was determined for each concentration after a 10 min incubation of the biosensor in the pesticide solution. As can be seen in Fig. 6, a logarithmic response curve was obtained in the range of concentrations tested ($r^2 = 0.9898$, $n = 8$). The biosensor detection limit, calculated as the concentration inducing a 10% inhibition, was calculated to be 0.2 nM, corresponding 6×10^{-5} ppm. Such performances are compatible with international regulations, establishing the maximum residue limit (MRL) for pirimiphos-methyl in olive at 5×10^{-2} ppm [32]. The developed biosensor was much more sensitive than another biosensor based on a bienzymatic system, which allowed detecting pirimiphos-methyl oxon with a LOD of 0.12 μM [33].

Concerning operational stability, the biosensor showed an excellent stability and reproducibility during 10 consecutive measurements, showing a mean electrodes response of 285 ± 5 nA ($n = 10$). When stored in sealed plastic boxes at 4 °C, the biosensor activity decreased by less than 10% after 42 days of storage.

3.6. Analysis of real samples

The developed AChE/ CS-PVA NFM/SPE biosensor was applied for analysis of olive oil after a simple liquid-liquid extraction. The samples were first analysed before spiking confirming the absence of residual insecticides in oil, no inhibition being observed in that case. Oil samples were then spiked with pirimiphos-methyl at concentrations of 3×10^{-8} M, 1.5×10^{-7} M and 5×10^{-7} M. These measurements were carried out in triplicate. Table 2 shows the results obtained for each series of samples after extraction and biosensor analysis. It was shown a very good performance of both extraction and analysis steps, with recovery rates close to 100% (Table 2).

4. Conclusion

Due to their unique properties such as spatial structure, high porosity, and large surface area, the use of electrospun chitosan/polyvinyl alcohol (PVA) nanofibers allowed considerable improvement of AChE-based biosensor performances. Moreover, the biosensor development based on mutant acetylcholinesterase (B394) enhanced the sensitivity of the biosensor. The developed biosensor showed very good analytical performance in terms of reproducibility, operational, and storage stability. The detection limit for pirimiphos-methyl was 0.2 nM, a concentration much lower than the maximum residue limit allowed (MRL) set by international regulations (164 nM). Furthermore, the developed biosensor was successfully used for the detection of pirimiphos-methyl in olive oil samples after a simple liquid-liquid extraction, without any laborious pre-treatment. Theses results show the remarkable potential of biosensors based on electrospun nanofibers for food and environmental monitoring.

Acknowledgements

This work was supported by the French Government through a fellowship granted by the French Embassy in Egypt (Institut Français d'Egypte) (Grant no. 855444G).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.04.018.

References

[1] W.H. Organization, WHO specifications and evaluations pirimiphos-methyl (O-2-diethylamino-6-methylpyrimidin-4-yl-O, O-dimethyl phosphorothioate), 2006.

[2] D.M. Quinn, Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states, Chem. Rev. 87 (1987) 955–979.

[3] G.R. van der Hoff, P. van Zoonen, Trace analysis of pesticides by gas chromatography, J. Chromatogr. A. 843 (1999) 301–322.

[4] H. Mitobe, T. Ibaraki, A. Tanabe, K. Kawata, A. Yasuhara, High performance liquid chromatographic determination of pesticides in soluble phase and suspended phase in river water, Toxicol. Environ. Chem. 81 (2001) 97–110.

[5] P.R.B. De Oliveira Marques, G.S. Nunes, T.C.R. Dos Santos, S. Andreescu, J. L. Marty, Comparative investigation between acetylcholinesterase obtained from commercial sources and genetically modified Drosophila melanogaster: application in amperometric biosensors for methamidophos pesticide detection, Biosens. Bioelectron. 20 (2004) 824–831.

[6] G. Istamboulié, S. Andreescu, J.-L. Marty, T. Noguer, Highly sensitive detection of organophosphorus insecticides using magnetic microbeads and genetically-engineered acetylcholinesterase, Biosens. Bioelectron. 23 (2007) 506–512.

[7] A.Y. El-Moghazy, E.A. Soliman, H.Z. Ibrahim, T. Noguer, J. Marty, G. Istamboulie, Ultra-sensitive biosensor based on genetically engineered acetylcholinesterase immobilized in poly (vinyl alcohol)/Fe–Ni alloy nanocomposite for phosmet detection in olive oil, Food Chem. 203 (2016) 73–78.

[8] H. Wu, J. Wang, X. Kang, C. Wang, D. Wang, J. Liu, et al., Glucose biosensor based on immobilization of glucose oxidase in platinum nanoparticles/graphene/chitosan nanocomposite film, Talanta 80 (2009) 403–406.

[9] T.M.B.F. Oliveira, M. Fátima Barroso, S. Morais, P. de Lima-Neto, A.N. Correia, M. B.P.P. Oliveira, et al., Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification, Talanta 106 (2013) 137143.

[10] J. Fu, H. Qiao, D. Li, L. Luo, K. Chen, Q. Wei, Laccase biosensor based on electrospun copper/carbon composite nanofibers for catechol detection, Sensors 14 (2014) 3543–3556.

[11] N. Bhardwaj, S.C. Kundu, Electrospinning: a fascinating fiber fabrication technique, Biotechnol. Adv. 28 (2010) 325–347.

[12] J. Lannutti, D. Reneker, T. Ma, D. Tomasko, D. Farson, Electrospinning for tissue engineering scaffolds, Mater. Sci. Eng. C 27 (2007) 504–509.

[13] D. Liang, B.S. Hsiao, B. Chu, Functional electrospun nanofibrous scaffolds for biomedical applications, Adv. Drug Deliv. Rev. 59 (2007) 1392–1412.

[14] Z.-M. Huang, Y.-Z. Zhang, M. Kotaki, S. Ramakrishna, A review on polymer nanofibers by electrospinning and their applications in nanocomposites, Compos. Sci. Technol. 63 (2003) 2223–2253.

[15] R. Luoh, H.T. Hahn, Electrospun nanocomposite fiber mats as gas sensors, Compos. Sci. Technol. 66 (2006) 2436–2441.

[16] X. Wang, C. Drew, S.-H. Lee, K.J. Senecal, J. Kumar, L.A. Samuelson, Electrospun nanofibrous membranes for highly sensitive optical sensors, Nano Lett. 2 (2002) 1273–1275.

[17] J. Yoon, S.K. Chae, J.-M. Kim, Colorimetric sensors for volatile organic compounds (VOCs) based on conjugated polymer-embedded electrospun fibers, J. Am. Chem. Soc. 129 (2007) 3038–3039.

[18] P. Ye, Z.-K. Xu, Z.-G. Wang, J. Wu, H.-T. Deng, P. Seta, Comparison of hydrolytic activities in aqueous and organic media for lipases immobilized on poly(acrylonitrile-co-maleic acid) ultrafiltration hollow fiber membrane, J. Mol. Catal. B Enzym. 32 (2005) 115–121.

[19] P. Ye, Z.-K. Xu, J. Wu, C. Innocent, P. Seta, Nanofibrous poly(acrylonitrile-co-maleic acid) membranes functionalized with gelatin and chitosan for lipase immobilization, Biomaterials 27 (2006) 4169–4176.

[20] Z.-M. Liu, Z.-K. Xu, J.-Q. Wang, Q. Yang, J. Wu, P. Seta, Surface modification of microporous polypropylene membranes by the grafting of poly(γ-stearyl-L-glutamate), Eur. Polym. J. 39 (2003) 2291–2299.

[21] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.

[22] W.N. Aldridge, Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl p-nitrophenyl thiophosphate (E 605) and analogues, Biochem. J. 46 (1950) 451–460.

[23] F. Villatte, V. Marcel, S. Estrada-Mondaca, Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides, Biosens. Bioelectron. 13 (1998) 157–164.

[24] N. Ben Oujji, I. Bakas, G. Istamboulié, I. Ait-ichou, E. Ait-addi, R. Rouillon, T. Noguer, Sol-gel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors, Food Control. 30 (2013) 657–661.

[25] M.B. Kralj, P. Trebše, M. Franko, Oxidation as a pre-step in determination of organophosphorus compounds by the AChE-TLS bioassay, Acta Chim. Slov. 53 (2006) 43–51.

[26] A. Galezowska, T. Sikora, G. Istamboulie, T. Noguer, M. Trojanowicz, I. Polec, G. S. Nunes, J.-L. Marty, Application of genetically engineered acetylcholinesterases in screen-printed amperometric biosensor for detection of organophosphorus insecticides, Sens. Mater. 20 (2008) 299–308.

[27] H. Chen, Q. Zhang, Y. Dang, G. Shu, The effect of glutaraldehyde cross-linking on the enzyme activity of immobilized β-galactosidase on chitosan bead, Adv. J. Food Sci. Technol. 5 (2013) 932–935.

[28] L.E. Shi, Y. Yi, Z.X. Tang, W.Y. Xiong, J.F. Mei, G.Q. Ying, Nuclease P1 immobilized on DEAE cellulose, Braz. J. Chem. Eng. 27 (2010) 31–39.

[29] J. Xie, Y. Lo Hsieh, Ultra-high surface fibrous membranes from electrospinning of natural proteins: casein and lipase enzyme, J. Mater. Sci. 38 (2003) 2125–2133.

[30] A.C. Patel, S. Li, J.-M. Yuan, Y. Wei, In situ encapsulation of horseradish peroxidase in electrospun porous silica fibers for potential biosensor applications, Nano Lett. 6 (2006).

[31] K.H. Lee, C.S. Ki, D.H. Baek, G.D. Kang, D.-W. Ihm, Y.H. Park, Application of electrospun silk fibroin nanofibers as an immobilization support of enzyme, Fibers Polym. 6 (2005) 181–185.

[32] Reasoned opinion of EFSA: Risk assessment for pirimiphos-methyl residues resulting from cross-contamination. EFSA J., vol. 9, 2011 2436, 48 pp.

[33] M. Del Carlo, A. Pepe, M. Mascini, M. De Gregorio, A. Visconti, D. Compagnone, Determining pirimiphos-methyl in durum wheat samples using an acetylcholinesterase inhibition assay, Anal. Bioanal. Chem. 381 (2005) 1367–1372.