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

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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

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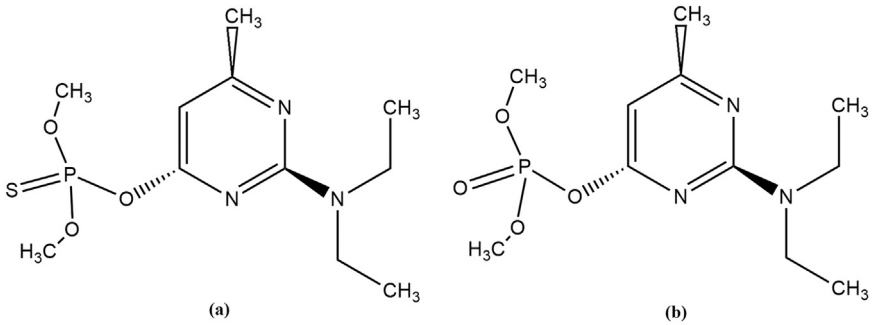

Fig. 1. Chemical structure of: (a) Pirimiphos-methyl and (b) Pirimiphos-methyl oxon.

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).