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An acetylcholinesterase biosensor based on a conducting polymer using multiwalled carbon nanotubes for amperometric detection of organophosphorous pesticides

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ABSTRACT

A novel amperometric biosensor based on a conducting polymer using multi walled carbon nanotube modified electrode was developed for detection of organophosphorus pesticides. Acetylcholinesterase (AChE) was successfully immobilized by covalent linkage on the modified graphite electrode. Carbon nanotubes were functionalized by electrochemical treatment. A conducting polymer; poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine) (poly(SNS-NH_2)) was synthesized via electropolymerization to examine its matrix properties for biomolecule immobilization. This strategy enhanced electron transfer rate at a lower potential (+100 mV vs. Ag reference) and catalyzed electrochemical oxidation of acetylthiocholine effectively. Scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), contact angle measurements and electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) techniques were used to monitor changes in surface morphologies and electrochemical characterizations. The proposed biosensor design offered a fast response time (6 s), a wide linear range (0.05 mM and 8.00 mM) and a low detection limit (0.09 mM) with a high sensitivity (24.16 μA mM^{-1} cm^{-2}) for acetylthiocholine. The inhibition responses of paraoxon, parathion and chlorfenvinphos on the enzymatic activity of AChE were detected. The fabricated biosensor was tested for the detection of pesticides in fortified tap water samples. The results were found to be in good agreement with the ones determined by HPLC/DAD technique.

1. Introduction

Acetylcholinesterase (AChE) is a crucial enzyme present in the central nervous system of living organisms. In the active site of AChE, a serine residue catalyzes the hydrolysis of neurotransmitter acetylcholine and terminates the impulse transmission at cholinergic synapses [1]. Organophosphorus pesticides (OPs), known as cholinesterase inhibitors, are widely used in agriculture, medicine, industry and chemical warfare. OPs exhibit high toxicity and their presence in the environment can be fatal for human health as they inhibit the catalytic activity of AChE irreversibly by forming a stable complex in the active site of AChE [2]. During the inhibition mechanism, the serine residue is blocked. The enzyme is essential for the functioning of central nervous system. The resulting high production of acetylcholine interferes with brain response since acetylcholine level depends on availability of active AChE [3,4]. For this reason, the need of monitoring devices for detection of OPs in the environment is vital and subjected to keen interest for several decades.

Global attention is given in developing analytical systems to monitor OPs in the environmental surveillance and protection [5,6]. The common methods for pesticide detection are based on chromatographic separation such as gas and liquid chromatography. Despite of their sensitivity and reliability, they are

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time consuming, require expensive equipment, high-qualified personnel and are not adapted for in situ detection [7]. Meanwhile, the development of enzyme-based electrochemical biosensors appears as a promising alternative to the classical methods owing to their simple measurement procedure, short response time, sufficient sensitivity and selectivity. Therefore, biosensors based on the inhibition of acetylcholinesterase enzyme are attractive for the detection of acetylcholine or organophosphorous pesticides. Various types of amperometric AChE-based biosensors have been described [8–11]. The inhibition of enzyme activity is monitored by measuring the oxidation current of acetylthiocholine upon a certain applied potential. The reaction mechanism is as follows [12]:

$$\text{Acetylthiocholine chloride} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine (red)} + \text{acetic acid} + Cl^-$$

$$2\,\text{Thiocholine (red)} \xrightarrow{\text{anodic oxidation}} \text{thiocholine (ox)} + 2e^- + 2H^+$$

Contrary to the advantages of AChE biosensors for detection of OPs, they suffer from a major drawback; loss of enzyme activity. In order to keep fragile enzyme bioactivity during the electrochemical measurements, the adopted immobilization method should be strong enough to maintain mechanical stability of the biosensor and sufficiently soft to arrange optimal conformation of the enzyme [13]. Several immobilization strategies and materials are developed such as cross linking [14], covalent binding [15], electrostatic adsorption [16] and physical adsorption [17].

For the fabrication of the biosensors conducting polymers (CPs) attracted considerable attention due to their high conductivity, ease of preparation, good environmental stability [18]. Moreover, they allow tuning the structural and electronic properties to use them as an immobilization platform in biosensors. CPs are used to produce sensitive microenvironment for biomolecules. Hence, conducting polymers are preferred as immobilization matrices, offering significant advantages owing to their good conductivities and mechanical properties and good adhesion to the transducer [19].

CNTs are fascinating materials for sensing applications due to several properties like small dimensions, functional surface, good conductivity, excellent biocompatibility, modifiable side walls and high reactivity [20,21]. In addition to enhanced electrochemical reactivity, CNT-modified electrodes are widely used for the immobilization of important biomolecules [22]. Therefore, π–π electronic and hydrophobic interactions allow them to interact with some aromatic compounds [23]. To take advantage of such superior properties in electrochemical sensing applications, the CNT should be properly functionalized. With their unique electron transfer property and desirable shapes for surface design, CNTs are valuable candidates for surface modifications especially in the case of electrochemical processes.

The combination of carbon nanotubes with conducting polymers has attracted much attention due to their biomolecule anchorage tools. Synergistic effects lead to a significant enhancement in the electronic and mechanical properties of each single component. Also, the electrical wiring effect of MWCNT incorporated with the polymer film diminishes the diffusion problems [24]. In the nanoscale interface of CNTs and conducting polymer, the electron transfer can be easily achieved due to the high affinity of both groups.

Herein a conducting polymer; poly(4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine), poly(SNS-NH_2), was used as the immobilization matrix for acetylcholinesterase. Pendant amino groups in the structure of the polymer serve as a host matrix for immobilization of AChE via covalent immobilization. Functionalization of MWCNT (f-MWCNT) was achieved via electrochemical etching method [25–27] to possess free carboxylic acid moieties. By this way, both pendant amino groups of poly(SNS-NH_2) and free carboxylic acid groups of f-MWCNT were linked with AChE through covalent binding using a two step carbodiimide coupling method simultaneously. This method involves the formation of amide bonds between modified transducer with poly(SNS-NH_2) and f-MWCNT and the enzyme molecules. In order to increase lifetime stability of the enzyme electrode, covalent immobilization procedure was chosen since there exists a strong and efficient bonding between the enzyme molecules and the support. The aim of this work is to develop sensitive amperometric biosensor based on AChE for indirect measurements of OPs on the basis of their inhibitory effect on AChE activity. After investigation of the experimental conditions related to the performance of the fabricated biosensor, paraoxon, parathion and chlorfenvinphos in tap water samples were analyzed with the proposed sensor. The results were compared with those determined by high pressure liquid chromatography with diode array detector (HPLC/DAD) as a reference method with solid phase extraction (SPE) technique in order to validate the accuracy of the biosensor. To the best of our knowledge, there are no reports presenting such a single biosensor detecting paraoxon, parathion and chlorfenvinphos.

2. Materials and methods

2.1. Materials

Acetylcholinesterase (AChE, EC 3.1.1.7, 518 U mg^{-1} from *Electrophorus electricus* (electric eel)), acetylthiocholine chloride, paraoxon, parathion, chlorfenvinphos, multi walled carbon nanotube, N-hydroxysuccinimide (NHS) and pyridine 2-aldoxime methochloride (2-PAM) and chemicals used for the synthesis of the monomer and electropolymerization were purchased from Sigma–Aldrich Co. LCC. (St. Louis, USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was purchased from Fluka (Buchs, Switzerland). Acetonitrile (ACN), sodium hydroxide were purchased from Merck (Darmstadt, Germany) and tetrahydrofuran (THF) from Acros (Geel, Belgium). All chemicals were analytical grade.

2.2. Instrumentation

All amperometric measurements were performed with the potentiostat CompactStat (Ivium Technologies B.V., Eindhoven, Netherlands) in a three-electrode cell configuration consisting of a graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode. A platinum wire as the counter electrode and a silver wire as the pseudo reference electrode (vs. Ag/AgCl (0.05 V)) were used. Amperometric measurements were performed in a three-electrode system. In amperometric analyzes, the data were given as the average of three measurements and standard deviations were recorded as ±SD. All measurements were performed at ambient conditions (25 °C). For the investigation of surface characteristics, scanning electron microscopy (SEM) (JEOL JSM-6400 model, Japan) and X-ray photoelectron spectroscopy (XPS) (PHI 5000 Versa Probe (FULVACPHI, Inc., Japan/USA) with monochromatized Al Kα radiation (1486.6 eV) 10 as an X-ray anode at 24.9 W were used. Contact angle measurements of a drop of water (2.0 μL) on the polymer surfaces were carried out using the sessile drop method with a CAM 100 KSV (KSV, Finland). Recording the drop profile with a CCD camera allowed monitoring the changes in contact angle. All reported data were given as the average of three measurements ±SD. FTIR spectra were recorded on a Varian 1000 FTIR spectrometer. Electrochemical Impedance Spectroscopy (EIS) was performed with a GAMRY Reference 600 (GAMRY Instruments Inc., Pennsylvania, USA). HPLC (Agilent 1100 Series, Waldbronn, Germany) equipped with a diode array detector (DAD) and a LiChrospher 100 RP-18