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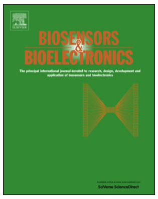

Amperometric biosensing of organophosphate and organocarbamate pesticides utilizing polypyrrole entrapped acetylcholinesterase electrode

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ABSTRACT

The work presented here describes a novel, easy and low-cost method of fabrication of a highly sensitive acetylcholinesterase biosensor and its application to detect organophosphate and organocarbamate pesticides. Acetylcholinesterase was electro-immobilized into a thick conducting layer of polypyrrole. Porcine skin gelatin and gluteraldehyde mixture was used for stabilizing the system. Acetylthiocholine chloride was used as the substrate. Polypyrrole catalyzed the electrochemical oxidation of thiocholine and promoted the electron transfer, thus lowering the oxidation potential and increasing the detection sensitivity. Electro oxidation of thiocholine in polypyrrole matrix occurred at 0.1 V under low potential scan rate. The thiocholine sensitivity of the electrode was found to be 143 mA/M. The sensor was applied to detect the sample organophosphate pesticide ethylparaoxon and organocarbamate pesticide carbofuran. The detection limit for paraoxon was found to be 1.1 ppb and that for carbofuran is 0.12 ppb. The sensor showed good intra and inter state precision with relative standard deviation (RSD) 0.742% and 6.56% respectively. Both dry and wet storage stability were studied. The sensor stored at 0 °C in dry condition had a good storage stability retaining 70% of its original activity for 4 months. During wet storage, the activity decrease followed the same trend, however, the operational stability at the end of the storage period was found to be less compared to the dry storage case. The developed biosensor is as a promising new tool for analysis of cholinesterase inhibitors.

1. Introduction

Organophosphates (OPs) and organocarbamates (OCs) have been widely used as pesticides in modern agriculture due to their low persistence and high insecticidal activity (Amine et al., 2006; Carlsson et al., 2000; Rekha et al., 2000). Although they degrade faster than the organochloride, they have greater acute toxicity, posing risk to people who may be exposed to large amounts (Jeyaratnam, 1990). That is why rapid determination and reliable quantification of OPs and OCs have become of great importance. Numerous analysis methods including gas chromatography (Asensio-Ramos et al., 2010), liquid chromatography (Cappiello et al., 2002), ultraviolet spectroscopy (Ganzera et al., 2006), gas-mass spectroscopy (Wong et al., 2007), fluorimetry (Dams et al., 2002) and surface plasmon resonance (SPR) (Chand and Gupta, 2007) have been developed which can estimate the OPs and OCs in contaminated samples with high sensitivity, reliability and precision. Despite their advantage, these methods require expensive instrumentation, highly trained personnel, and long work up time, making them unsuitable for rapid analysis of field samples. Hence, simple, sensitive, selective, and field deployable tools are still highly desired for monitoring of OP and OC contamination. Enzyme based electrochemical biosensors have emerged in the past few years as the most promising alternative to detect pesticides (de Albuquerque, Ferreira, 2007; Marinov et al., 2010; Pohanka et al., 2011; Pohanka et al., 2012; Roepcke, et al., 2010; Skladal, 1996; Xavier et al., 2000). Among them, amperometric acetylcholinesterase (AChE) biosensors based on the inhibition of AChE have shown satisfactory results for pesticides analysis (Crew et al., 2011; Du et al., 2010a,2010b; Raghu et al., 2012; Sun and Wang, 2010; Tuoro et al., 2011) in which the enzyme activity is employed as an indicator of quantitative measurement of insecticides.

Effective immobilization of enzyme to solid electrode surface still remains as a great challenge for the fabrication of biosensor. A key consideration for immobilizing enzyme is how to retain its bioactivity. The usual immobilization methods include direct physical adsorption on a solid support (Sotiropoulou et al., (2005)), cross linking (Kandimalla and Ju, 2006), encapsulation into a hydro gel (Anitha et al., 2004; Yadavalli et al., 2004), covalent binding

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Abbreviations: Geltn, gelatin; Glut, gluteraldehyde

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(Du et al., 2007; Lin et al., 2004), entrapment in different substrate materials (Gong and Lin, 2003; Jeanty et al., 2001), self-assembly into multilayer film (Liu and Lin, 2006), immobilization on controlled-pore glass (Lee et al., 2002), and immobilization on magnetic particles (Kindervater et al., 1990). The conducting polymers have attracted much attention due to their interesting electrical and electrochemical properties (Cosnier, 1999; Mu et al., 1997) and also due to their potential application in miniaturized electronic devices. Polypyrrole (PPy), a key member of the organic conducting polymers, has been widely used as the enzyme-hosting matrix in electrochemical biosensors due to its advantages of permitting a facile electronic charge flow through the polymer matrix, easy preparation, high conductivity and good stability (Ramanavičius et al., 2006). In these PPy based biosensors the enzyme was either electro entrapped inside the PPy matrix or attached to the surface of PPy film in pure or composite form. These include electro entrapment of glucose oxidase (GOX) (Fortier et al., 1990; Njagi and Andreescu, 2007; Umana and Waller, 1986) for glucose determination, electro entrapment of tyrosenase for determination of phenolic compounds (Narlı et al., 2006), surface attachment of acetylcholinesterase (AChE) on PPy and polyaniline (PANI) composite polymer film doped with multi-walled carbon nanotube (Du et al., 2010a,2010b) and on Au nanoparticles–polypyrrole nano wire composite film (Gong et al., 2009) for determination of OP and OC pesticides. Though the surface immobilized PPy-AChE-nanomaterial sensors can give high sensitivity, their durability is poor due to bio-fouling and washing out of the enzyme. Moreover the fabrication processes are very much cumbersome and expensive due to involvement of multiple chemical steps and costly chemicals such as the nanoparticles. Electro entrapment procedures are easy, single step procedure and due to the encapsulation of the enzyme by the macromolecular matrix, the enzyme is better protected from washing out during analytic application of the sensor and hence expected to be more durable compared to the surface immobilized AChE sensors. To our knowledge so far no work has been reported that has documented the use of electro entrapped AChE for pesticide sensing.

Aim of the present work was to test the feasibility of using PPy electro entrapped AChE as a biosensor for OP and OC pesticides without using nanoparticles, and hence to optimize the fabrication process and operational conditions for high sensitivity, reusability and long time stability. An optimum fabrication process has been developed in which AChE electro entrapped inside PPy using a low (0.02 M) concentration of KCl. The enzyme was stabilized inside the conducting polymer matrix by cross linking with gluteraldehyde and porcine skin gelatin. The performance of the sensor was tested with sample organophosphate pesticide ethyl paraoxon and organocarbamate carbofuran. This approach may help in imparting selectivity to the enzyme biosensors through control of the film porosity. Also, since polypyrrole film is not easily degraded by some of the organic solvents, therefore, such an approach may pave the way for real sample analysis in organic phase.

2. Experimental

2.1. Materials

Acetylcholinesterase, Type VI-S (500 U/mg) EEL, acetylthiocholine chloride (ATChCl) (99%), Bovine Serum Albumin (BSA) (96%), were procured from Sigma Chemicals Co., USA. Pyrrole (98%), gelatin powder (Type A, from porcine skin), 5,5′-dithiobis (2-nitro benzoic acid) (DTNB) (99%), carbofuran (98%), paraoxon-ethyl (90%), gluteraldehyde (50 wt% in H_2O) were all procured from Sigma-Aldrich, USA. KH_2PO_4 and K_2HPO_4 were of analytical reagent grade and procured from E. Merck, Germany. Doubled distilled water was used throughout the experiments.

2.2. Instrumentation

PAR 273-A potentiostate/galvanostate was used both for film deposition and electrochemical measurements. Platinum working electrode used was from CH Instrument, USA. UV–vis spectrophotometer used was UV-2550, Shimadzu, Japan.

2.3. Preparation of the sensor

The sensor probe was prepared by immobilizing AChE at the tip of a platinum (Pt) electrode (2 mm) with PPy as the support matrix. The Pt electrode was cleaned by polishing with fine alumina powder followed by sonicating for 15 min. For electro deposition 2 mL of 0.5 M pyrrole solution in phosphate buffer (pH 7.2) containing 0.02 M KCl and 5 μL (100 U mL^{-1}) of the enzyme were mixed together in a three electrodes cell setup comprised of platinum working electrode, platinum coil auxiliary electrode and Ag/AgCl saturated with 3 M NaCl as the reference electrode. Electrolysis was carried out at 0.8 V for 30 min. Before starting the electrolysis, the solution mixture was sonicated for 5 min. A thick film of PPy-AChE was developed at the tip. The film was then washed with phosphate buffer saline (PBS) (pH 7.2) and dried at room temperature for 30 min. Then, holding the electrode vertical, with its film containing end upwards, 5 μL of 5% gelatin solution (prepared by warming gelatin water mixture up to 60 °C followed by cooling down to room temperature) was added to it and then kept at room temperature for 1 h. Then gluteraldehyde (35% in water) was added in two steps. At first, 5 μL gluteraldehyde was added to the electrode tip with a micro syringe, keeping the electrode vertical. The electrode was then kept at room temperature in the same vertical position until the film appeared dry (for approximately 1 h). Then the electrode tip was again treated with gluteraldehyde by immersing it in the stock solution (35%) for 1 min. The electrode was then kept at room temperature for 4 h followed by overnight storage at 0 °C. Finally it was transferred to a –20 °C freezer where it was stored for 5 days before use.

2.4. Measurement procedure

Quantitative estimation of sensor response was done mostly through the chronoamperometric (CA) method, but cyclic voltammetry was also applied in few cases. The cell setup comprised of three electrodes, the sensor probe as working, Pt coil as auxiliary and Ag/AgCl saturated with 3 M NaCl as reference electrodes in PBS (pH 7.2) electrolyte. Initial voltage $E_0=0.0$ V was applied for 60 s, final potential $E=0.70$ V for 400 s and ATChCl was added at 300 s, i.e., after sufficient current stabilization. After taking out from -20 °C freezer, prior to its application, the sensor was soaked in PBS for 30 min and then potentiostated at 0.8 V for 15 min. The experiments were performed at 32 °C with constant magnetic stirring. Triplicate measurements were made in all experiments, with four cycles of potential sweep for the CVs, unless stated otherwise.

Time dependence of the inhibitory action of the pesticides was studied by evaluating the percent residual activity (A_r%) with time and the concentration dependence of the same was studied by evaluating the relative inhibition percentage (I%) with concentrations, using respectively Eqs.(1) and (2).

$$A_r\% = \frac{I_2}{I_1} \times 100\% \quad (1)$$