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Electrochemical biosensing of carbofuran based on acetylcholinesterase immobilized onto iron oxide–chitosan nanocomposite

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ABSTRACT

We report a highly sensitive square wave voltammetric biosensor for the determination of carbofuran using an acetylcholinesterase (AChE) enzyme immobilized iron oxide–chitosan nanocomposite film modified glassy carbon electrode (AChE/Fe_3O_4–CH/GCE). The Fe_3O_4–CH nanocomposite was prepared by a simple solution mixing process and its formation was confirmed by FT-IR spectroscopy. The effective enzyme immobilization onto the nanocomposite matrix was confirmed by electrochemical impedance, scanning electron and atomic force microscopy studies. Various experimental parameters such as effect of scan rate, inhibition time and substrate concentration were optimized. The nanocomposite-based biosensor could detect carbofuran as low as 3.6×10^{-9} M. The reproducibility of the AChE/Fe_3O_4–CH/GCE was ascertained by performing intra-assay and inter-assay experiments using cyclic voltammetry. The practical application of the biosensor was ascertained by the determination of carbofuran from cabbage samples and by comparing the results with those obtained by the standard high-performance liquid chromatography (HPLC) method.

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a carbamate compound which is used as an insecticide in agriculture [1]. It is one of the most toxic carbamate pesticides. In agriculture, it is applied to alfalfa, corn, peanuts, peppers, strawberries, tobacco, bananas, sorghum, potato, cabbage, cottonwood trees, sugarcane and rice. The lethal dosage of carbofuran in mammals is found to be 5–50 mg/kg [2]. Numerous analytical methods such as colorimetry [3], capillary electrophoresis [4], mass spectrometry [5], gas chromatography [6], high performance liquid chromatography [7] and a rapid magnetic particle-based ELISA technique [8] have been employed for the detection of carbofuran.

The development of electrochemical biosensors for the detection of various pesticides has been an active research area in the past decade [9,10]. Biosensors offer great advantages over conventional analytical techniques, including high specificity for real-time analysis in complex mixtures, high sensitivity, simple operation without the need for extensive sample pretreatment and low cost. A majority of electrochemical pesticide biosensors is based on inhibition of the enzyme *acetyl cholinesterase* (AChE) [11,12]. The main approach used to measure this inhibition is based on the amperometric/voltammetric detection of thiocholine, which is the enzymatic reaction product of acetylthiocholine by its oxidation at a constant potential at the electrode.

The most important step in the development of an enzyme sensor is the immobilization of the enzyme onto the electrode surface. This process is governed by various interactions between the enzyme and the electrode material and strongly affects the performance of the biosensor in terms of sensitivity, stability, response time, and reproducibility. A judicious choice of the enzyme support material can significantly enhance the operational performance of the biosensor. In this context, the use of a nanostructured chemically modified electrode (NCME) in an electrochemical biosensor can be expected to offer certain definite advantages. Compared to bulk electrodes, the presence of nanoparticles on the electrode surface increases the electroactive surface area and enables fast electron-transfer kinetics [13]. The increase in electroactive surface area allows for lower detection limits and higher sensitivity to analytes [14]. In particular, nanoparticles can enhance the enzyme loading at the electrode surface [15]. The high surface-to-volume ratio creates more binding sites on the electrode surface for easier contact with enzyme molecules. The NCME maximizes the utilization of the bioactive sites of the enzyme and acts as electron-transfer pathway.

There have been very few reports on the use of NCMEs for the enzymatic detection of carbofuran. Carbofuran was detected at an AChE enzyme electrode stabilized by an electrodeposited

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gold nanoparticle layer with a detection limit of 3.4×10^{-8} M using chronoamperometry [16]. An amperometric biosensor based on immobilization of AChE on gold nanoparticles and silk fibroin modified platinum electrode was reported [17]. Dounin et al. [18] developed a disposable electrochemical printed chip for the trace level detection of carbofuran. The nanostructured chip enabled highly sensitive oxidation of the thiocholine product even in the absence of surface modification. Using differential pulse voltammetry, the limit of detection for carbofuran was found to be 1.8×10^{-8} M. A layer-by-layer self-assembled AChE/poly(amido amine)-Au/CNTs multilayer electrode was employed for the detection of carbofuran by differential pulse voltammetry in the concentration range from 4.8×10^{-9} M to 0.9×10^{-7} M with a detection limit of 4.0×10^{-9} M [19]. There have been some interesting reports on carbofuran immunosensors based on NCMEs as well [20–23].

Recently, magnetic-chitosan nanocomposites have received great attention for the removal of heavy metal ions from water [24,25], biosensors [26,27], drug delivery and other biomedical applications [28,29] and also as dye adsorbent [30,31]. In this study, we developed an AChE enzyme biosensor for carbofuran based on Fe_3O_4–CH nanocomposite formed by a simple solution mixing process. Fe_3O_4 nanoparticles can be a good choice for an electrode matrix in enzyme biosensors because of its good biocompatibility, low toxicity, high electron transfer capability and high adsorption ability [32]. CH, the *N*-deacetylated derivative of chitin, possesses several useful properties such as excellent film-forming ability, good adhesion, biocompatibility and high mechanical strength. The amino groups of CH provide a hydrophilic environment compatible with the biomolecules [33]. Therefore, CH can be a good choice as a carrier material for enzyme immobilization [34]. In the present study we demonstrate that the distinct physico-chemical properties of Fe_3O_4 and CH can help in the effective immobilization of the AChE enzyme onto the nanocomposite. Especially the presence of CH prevents not only the aggregation of the magnetic nanoparticles but also the loss of the enzyme molecules by providing a biocompatible microenvironment to maintain the enzyme activity. The Fe_3O_4–CH nanocomposite shows a good electrocatalytic ability to oxidize thiocholine and accordingly this leads to the development of a highly sensitive and reliable biosensor for real sample analysis.

2. Experimental

2.1. Instruments

Electrochemical measurements were performed on an electrochemical work station (CH Instruments, USA, Model 680). A one compartment cell with provision for three electrodes comprising glassy carbon electrode (GCE, 0.07 cm^2) as the working electrode, Ag/AgCl as the reference and a large platinum foil as the counter electrode was used. Surface morphological studies were carried out using a scanning electron microscope (SEM) (Hitachi 3000H). The surface topography of the samples was obtained using an atomic force microscope (AFM) (Shimadzu 9500). The IR data were obtained using a FT-IR spectrometer (Shimadzu 8400S). High performance liquid chromatography (HPLC) measurements were made using a Shimadzu LC-6AD instrument.

2.2. Reagents

Acetylthiocholine chloride (ATCl), AChE from *Electrophorus electricus* (electric eel), bovine serum albumin (BSA), carbofuran, nano iron oxide (<50 nm), chitosan hydrogen chloride (CH), acetic acid and sodium acetate were purchased from Sigma–Aldrich and used as received. A stock solution of carbofuran (1.0×10^{-3} M) was prepared by dissolving the appropriate quantity of the pesticide in 10 mL of acetonitrile. A measured volume of the stock solution was diluted with 0.1 M phosphate buffer (pH 7.4) to prepare the pesticide solution of desired concentration. A stock solution of AChE was prepared by dissolving 500 U in 1 mL of PBS and to this 1 mg of bovine serum albumin (BSA) was added for stability and stored in a refrigerator [35]. The stock solution was diluted appropriately to obtain the required concentration (0.1 U) of the enzyme. All electrochemical experiments were carried out in 0.1 M phosphate buffer solution PBS (pH 7.4). All aqueous solutions were prepared using double distilled water. Prior to each experiment, all the solutions were deoxygenated by passing pre-purified N_2 gas for 15 min.

2.3. Preparation of Fe_3O_4–CH nanocomposite and immobilization of AChE

A CH (1%) solution was prepared by dissolving CH (100 mg) in 100 mL of 5.0×10^{-2} M acetate buffer (pH 4.0) solution. About 1 mg of commercial Fe_3O_4 nanoparticles was dispersed in the CH solution and sonicated for 30 min to form Fe_3O_4–CH. About 5 μL of Fe_3O_4–CH solution was drop coated onto a GCE and dried at room temperature for about 2 h. This Fe_3O_4–CH nanocomposite film was carefully rinsed with deionized water to remove any unbound particles and the modified electrode is denoted as Fe_3O_4–CH/GCE. About 5 μL of 0.1 U of the enzyme solution was drop coated onto Fe_3O_4–CH/GCE and dried for 1 h. Finally, the electrode was rinsed with PBS and stored at 4 °C when not in use. The AChE enzyme immobilized electrode is denoted as AChE/Fe_3O_4–CH/GCE.

2.4. Measurement procedure

The principle of an electrochemical biosensor based on AChE is shown in Scheme 1. In the absence of the pesticide analyte, the substrate ATCl is converted by hydrolysis to thiocholine chloride and acetic acid. Thiocholine is electroactive and can be oxidized to dithiocholine at an appropriate applied voltage. In the presence of the pesticide (inhibitor), the conversion of ATCl is decreased. The anodic oxidation current of thiocholine is inversely proportional to the concentration of pesticide.

The inhibition (%) is estimated using the formula shown in Eq. (1) [36].

$$\text{Inhibition}\ (\%) = \frac{i_{\text{p, control}} - i_{\text{p, exp}}}{i_{\text{p, control}}} \times 100 \quad (1)$$

where $i_{p,control}$ is the oxidation peak current of ATCl at the enzyme modified electrode in the absence of pesticide and $i_{p,exp}$ is the peak current of ATCl at the enzyme modified electrode after inhibition of the enzyme by pesticide.

3. Results and discussion

3.1. Characterization of Fe_3O_4–CH nanocomposite

FTIR spectroscopy was used to identify the functional groups present in Fe_3O_4–CH. The FT-IR spectrum of CH (Fig. 1A) shows a broad band at 3396 cm^{-1} which can be assigned to the stretching vibration mode of OH and NH_2 groups. The bands in the region 2850–3000 cm^{-1} are attributed to the CH_3 and CH_2 groups due to CH antisymmetric and symmetric modes. The band at 1637 cm^{-1} is due to the C—O stretching along with N—H deformation mode in the amide group. Another band at 1415 cm^{-1} is due to C—N axial deformation. The band at 1058 cm^{-1} corresponds to CH—OH group. Fig. 1B shows the FT-IR spectrum of Fe_3O_4 with a vibrational band at 560 cm^{-1} which could be assigned to the stretching vibration mode and the torsional vibration mode of Fe—O bonds in the tetrahedral