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Lowering the detection limit of the acetylcholinesterase biosensor using a nanoporous carbon matrix

Sofia Sotiropoulou, Nikos A. Chaniotakis*

Laboratory of Analytical Chemistry, Department of Chemistry, University of Crete, Knossou Avenue, 71409 Iraklion Crete, Greece

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Abstract

Nanostructured carbon matrix has been used for the immobilization and stabilization of the enzyme *E.el.* AChE. The use of this activated carbon matrix is shown to provide both, significant enzyme stabilization, as well as the means for lowering the detection limit of the biosensor. The enzyme is immobilized by adsorption into the nanostructured conductive carbon, which also acts as the working electrode. The proposed biosensor showed very good stability under continuous operation conditions (L_{50} > 60 days), allowing its further use in inhibition mode. Using this biosensor, the monitoring of the organophosphorus pesticide dichlorvos at picomolar levels (1000 times lower than other systems reported so far) was achieved. The linear range of detection in flow injection system was six orders of magnitude (10^{-12} to 10^{-6} M). It is suggested that the ability of activated carbon to selectively concentrate the pesticide, as well as the enzyme hyperactivity within the nanopores is the reason for the decrease in the detection limit of the biosensor.

Keywords: Selective concentration; Biosensor; Picomolar; Dichlorvos

1. Introduction

The direct detection of pesticides is an area of intense scientific and industrial research. Insecticides and especially organophosphorus compounds (OPs), are being used extensively in agricultural processes, such as crop, animal protection, and storage of products, as well as in stables and homes for pest control. The use of these hazardous materials in living environments mandates the close monitoring of their levels for safety considerations. Due to their high acute toxicity [1], the safety levels set by the national and international regulatory agencies are quite low. For example, EPA has set the maximum concentration of the organophosphorus pesticide Dichlorvos in drinking water at 5×10^{-10} M. The low levels of concentration of these compounds in drinking water, foodstuffs, and air cannot be easily monitored directly using standard analytical instrumentation. Sophisticated techniques are required to detect pesticides at ppb levels [2,3] which however are not suitable for direct monitoring of OPs in real samples.

Enzyme-based biosensors have emerged the last few years as one of the most promising technologies for direct monitoring of pesticides. Among the numerous biosensor-related reports dealing with this subject in literature [4–6] the systems that seem to offer the highest sensitivity are biosensors based on the inhibition of cholinesterases [7,8]. Despite their high sensitivity though, these systems lack the detection limits required for the direct monitoring of pesticides, since the lowest detection limit reported so far for this class of biosensors does not exceed the level of 10^{-9} M for OP detection [9–11]. There have been very few instances in literature in which an improvement in the detection limit of the biosensors is reported. Low detection limits have been achieved by the use of diffusion barriers [12], or nanosensor arrays [13,14]. In general, it is believed that detection limit improvements can be achieved only with the use of highly sensitive genetically engineered enzyme [4,15].

* Corresponding author. Tel.: +30 2810 393 618; fax: +30 2810 393 601.
E-mail address: nchan@chemistry.uoc.gr (N.A. Chaniotakis).

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While this is a subject of intense research activity in our laboratory, we have also considered a new method for improving the detection limit of the biosensors by means of a novel biosensor technology.

This work describes our efforts in designing an amperometric biosensor system for the detection of organophosphorus pesticides based on the idea of pesticide selective concentration into activated nanoporous carbon nanostructures, which is also used as the enzyme immobilization matrix. The adsorption of organic compounds, such as pesticides, onto activated carbon surfaces is a well-known phenomenon, but it has not been previously used as the means for detection limit improvement. The carbon matrix is also used as the sensor transducer, since its resistance is very low, while it maintains a high surface area since the sensor design protocol does not require the use of any organic matrices that would passivate the electrode surface. Additionally, the nanostructure of the carbon aids in the stabilization of the enzymes, providing biosensors with higher operational and storage stabilities.

2. Experimental

2.1. Materials

Electric eel acetylcholinesterase (*E.el.* AChE, EC 3.1.1.7, type VI-S, specific activity 301 U/mg), Bovine serum albumin and acetylthiocholine chloride (ATCh-Cl) and iodide (ATCh-I) were purchased from SIGMA. Dichlorvos was purchased from Riedel-de Haën. The substrate acetylthiocholine chloride was diluted in 0.9% NaCl at a final concentration of 0.1 M and was kept at −20 °C in aliquots. Stock solutions (10^{-3} M) of dichlorvos were prepared in acetonitrile and were stored at 4 °C for approximately 1 month. Porous carbon was obtained ready for use from Electrochemical Analytical Systems Iraklion Crete. The density of the carbon is 1.16 ± 0.03 g/cm^3 with porous volume of 0.336 ± 0.003 cm^3/g. The pores in the carbon are in the range of nanosize (50–500 nm) and microsize (1–10 μm) [16]. The porous carbon is obtained in the form of rods and is cut at pellets of appropriate size (diameter 5.6 mm, height 1 mm). The porous carbon pellets are then cleaned by sonication for 10 min in a water bath and then for another 10 min in an ethanol bath. After drying at 150 °C for 30 min the porous carbon pellets are ready for use. The porous carbon pellets are placed into a laboratory-built biosensor holder described previously [17] for testing. In all experiments nanopure water (∼18 MΩ, EASYpure model D7033, Barnstead) was used. All other reagents used were of analytical grade.

2.2. Experimental setup

The electrochemical system was based on amperometric measurements. The electrochemical cell consisted of the biosensor element (carbon pellet with the adsorbed *E.el.* AChE) placed in the biosensor holder, a silver/silver chloride double junction reference electrode and a stainless steel counter electrode. The cell was incorporated in a flow injection system that consisted of a wall-jet flow cell with a dead volume of 22 μL, a low pressure 6-Port injection valve (model V 540, UPCHURCH Scientific) with a loop volume of 182 μL, while the solvent was delivered using a syringe pump (Model 362, ORION Research Inc.) at a flow rate of 0.5 mL/min. The Metrohm 641-VA Detector was used as the potentiostat, and the signal was recorded via a personal computer equipped with a 16-bit A/D converter and controlled with software written in basic. Temperature control at 25.0 ± 0.1 °C was achieved using a circulating bath (Model 362, PolyScience).

2.3. *E.el.* AChE immobilization and activity

The enzyme was immobilized in the porous carbon rod by adsorption according to a previously reported procedure [18]. In short, the enzyme solution consisted of *E.el.* AChE, diluted in phosphate buffer (25 mM phosphate, 0.1 M KCl buffer of pH = 7.0) and stabilized with 0.1 mg/ml bovine serum albumine (BSA). The flow solution contains 25 mM phosphate buffer, 0.1 M KCl and the pH is adjusted to 7.0. The activity of the free enzyme was evaluated photometrically at 412 nm according to the Ellman method [19]. The amount of immobilized enzyme inside the carbon matrix was calculated indirectly by the decrease in absorbance at 412 nm after the adsorption step, using again the Ellman method.

3. Results and discussion

Theoretical studies on the response of inhibitor biosensors [20] have shown that dichlorvos acts as an irreversible inhibitor of the enzyme, and thus detection is based on the decrease of the enzymatic activity towards the substrate acetyl(thio)choline before and after the inhibition stage. More specifically, the detection scheme is based on the following three stages. Initially, acetylthiocholine is enzymatically hydrolyzed to thiocholine, which in turn is oxidized at low potentials at the carbon transducer producing the initial biosensor response. At the second step, inhibition takes place and then at the third step the final response to acetylthiocholine is recorded following the same procedure as in the first step. The % inhibition can then be correlated to the log[dichlorvos] as described elsewhere [21].

3.1. Optimal operating potential

Fig. 1 shows the hydrodynamic voltammogram of the *E.el.* AChE biosensor over the potential range 0.1–0.5 V. The sharp increase in biosensor's sensitivity observed at +400 mV is expected, since the anodic oxidation of thiocholine starts at