POTENTIOMETRIC ELECTRODES FOR ENVIRONMENTAL MONITORING OF ORGANOPHOSPHORUS PESTICIDES

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ABSTRACT Potentiometric electrodes based on the detection of choline esterase inhibition by analytes have been developed. The detection of choline esterase activity is based on the novel principle of molecular transduction. Immobilized peroxidase, acting as the molecular transducer, catalyzes the electroreduction of hydrogen peroxide by direct (mediatorless) electron transfer. The development of the following sensing elements are involved: screening different carbon-based electrode materials and modification of the electrode surface and testing of different types of enzyme immobilization. A number of different carbon-based materials were screened and tested: carbon fiber, felt and matt, graphite materials, and some highly dispersed carbon materials. A butyryl-choline sensitive, tri-enzyme electrode has been developed employing Teflonized carbon black as an electrode material of choice. The immobilization procedure is based on physical adsorption of peroxidase and co-immobilization of choline oxidase and choline esterase by the glutaraldehyde binding technique. Incubation of the electrode in a solution containing organophosphorus pesticide (trichlorfon) for 10 min results in a notable decrease of electrode activity. This allows for the determination of trichlorfon in nanomolar concentration with a detection limit of 1.3 ppb.

KEYWORDS: organophosphorus pesticide, determination, biosensor

INTRODUCTION

Organophosphorus compounds (OPCs) are significant environmental and food chain pollutants due to their intensive use as pesticides in agriculture. Other important sources of such pollutants are manufacturing sites, spills during their transportation, and inappropriate use and storage. Chemicals of this group are also the basis for several different chemical weapons (Sarin, VX, etc.) and a potential source of serious environmental problems due to deliberate use, accidents, or improper disposal. Pollutants of this type are found to be present in many sampled soils, streams, ground, and waste waters. One of the most important preventive measures in this case is to rapidly determine the source of the pollutant and magnitude of the threat using on-site measurements. Analysis of low levels of organophosphorus pesticides in foods is another important task.

Generally chromatographic techniques are the most commonly used methods for determination of OPCs. These techniques allow selective and quantitative determination. However, they have a number of disadvantages: (i) the currently available equipment is complex and expensive, which prohibits use for rapid analyses under field conditions; (ii) the pre-treatment and assay procedures are lengthy, hence fast analyses are impossible; (iii) the techniques are expensive and can only be performed by highly trained technicians. Environmental issues require far more sensitive, selective, and quantitative methods, capable of low-level OPCs

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detection under field conditions in streams, ground, and waste waters, soils, and plants as well as in food [1].

The most common approach to enzymatic determination of OPCs is based on their inhibition of the activity of choline esterase enzymes. Choline esterase enzymes catalyze the reaction or hydrolysis of a particular choline ester (butyryl choline, acetyl choline, etc.) to the corresponding carboxylic acid with the release of choline. The presence of low concentrations of inhibitors—especially OPCs—strongly and specifically affects enzyme activity. Therefore, by measuring enzyme activity in the presence and absence of inhibitors, the concentration of the inhibitor compounds can be assayed.

Different types of pesticide biosensors based on inhibition of the activity of choline esterase enzymes are described in the literature. Inhibitor assay based on pH shift detection has been described [2-6]. Another group of OPC biosensors are based on amperometric detection of the product of the choline esterase reaction [7-14].

A popular approach for OPC detection is to base the design of enzyme biosensors on the coupling of choline esterase enzyme with a choline electrode. Different kinds of transducers have been used for the development of this type of biosensor. A hydrogen peroxide electrode was employed as a basic transducer [15-20]. Oxygen amperometric sensors have also been used as basic transducers for choline electrode construction [21]. Low-molecular weight redox mediators [22, 23] and redox polymer [24, 25] have been used in the construction of choline electrodes for hydrogen peroxide detection. Horseradish peroxidase has been used as a catalyst for hydrogen peroxide reduction with the enzymatic oxidation of a redox mediator. The use of redox mediators facilitate electron exchange but lead to system complications.

The approach based on potentiometric mediatorless electrocatalytic detection of hydrogen peroxide has been described for the development of glucose [26] and lactate [27] sensors. The present work describes development of an OPCs biosensor based on bioelectrocatalytic (mediatorless) transduction. Immobilized peroxidase, acting as the molecular transducer, catalyzes the electroreduction of hydrogen peroxide by direct (mediatorless) electron transfer [28]. The sensing element consists of a carbon-based electrode containing an assembly of co-immobilized enzymes: choline esterase, choline oxidase, and peroxidase. The biosensors based in this approach demonstrate the advantages of this practical application based on the following:

- simplicity of assay procedures (due to potentiometric assay principle)—hence easy to use in field conditions by relatively untrained personnel;
- small size of the sensing elements (electrodes) and portable sensor arrangements (due to electrochemical measurement principle);
- possibility for multiassay—the simultaneous quantitative determination of several analytes by an array of potentiometric sensors; and
- low price, disposable sensing elements (due to molecular transducing principle).

EXPERIMENTAL

Reagents and materials

Horseradish peroxidase, choline oxidase, butyryl choline esterase, trichlorfon ([2,2,2,-thrichloro-1-hydroxyethyl] phosphonic acid), Woodward's Reagent K (N-ethyl-5-phenylisoxazolium-3'-sulfonate), and

glutaraldehyde were products of Sigma Chem. Co. (St. Louis, MO). WDF graphite felt, WCA graphite cloth fiber, and VCK carbon cloth fiber were gifts from National Electrical Carbon Corp. (North Olmsted, OH). Carbon rod (1.3 mm) was a gift from DFI Pultruded Composites, Inc. (Erlanger, KY). Carbon rod (0.74 mm) was a product of NEPTCO Inc. (Pawtucket, RI). Carbon Mat was a gift from Aerospace Composite Products (San Leandro, CA). Graphite was a product of Schunk Graphite Technology (Menomonee Falls, WI). Teflonized acetylene carbon black 35% w/w PTFE (TCB-35) and 5% w/w (TCB-5) were products of Central Laboratory of Electrochemical Power Sources, Bulgalian Academy of Science (Sofia, Bulgaria). Ultra Low Temperature Isotropic (ULTI) carbon micro-dispersed powder was a gift from Carbomedics Inc. (Austin, TX). Branched polyethyleneimine (Serva, Germany), successively modified with cetylbromide and ethylbromide, was kindly supplied by Dr. A.K. Gladilin of Moscow State University (Moscow, Russia).

Electrode preparation

Electrodes based on carbon materials (felt, mat, cloth, rod, and highly dispersed carbon).

Carbon materials were fixed by heat shrinkable tubing on the top of a carbon rod. The carbon rod was used to provide an electrical connection with the measuring device. Carbon rods (1.3 mm and 0.74 mm diameter) were also used as electrodes.

Electrode based on carbon/polymer composite.

A carbon black/polymer composite electrode was made according to a modification of the method described elsewhere [26, 27]. A suspension of 15 mg of carbon black in 0.25 ml of polymer (polyethylenimine successively modified with cetylbromide and ethylbromibe) in benzene (10 mg/ml) was spread on the surface of a carbon rod (0.74 mm) and dried. The electrode was then soaked for 4 hours in 12.5% solution of glutaraldehyde in 0.1 M sodium phosphate buffer solution with a pH of 7.4 and containing 0.15 M of sodium chloride (PBS) at 37ºC.

Enzyme immobilization

1. The carbon or carbon/polymer composite electrode was soaked overnight at 4ºC in peroxidase solution (1-3 mg/ml) in PBS. The electrode was then soaked for 2 hours in 12.5% solution of glutaraldehyde in PBS at room temperature. The electrode then was immersed into choline oxidase solution in PBS (1-3 mg/ml) and stored overnight at 4ºC. This resulted in the preparation of a bi-enzyme electrode. For the preparation of a tri-enzyme electrode, the procedure of treatment by glutaraldehyde was repeated and the electrode was then stored overnight at 4ºC in butyryl choline esterase solution in PBS (1-3 mg/ml).

2. The carbon electrode was initially soaked for 2 hours in an aqueous solution of Woodward's Reagent K (20 mg/ml), pH 4 (adjusted by sulfuric acid), at room temperature. Further steps for immobilization were the same as described above.

Measurements of electrode activity

Measurements were performed in a cell containing 1 ml of PBS. The reaction was started by the addition of an aliquot amount of substrate (choline or butyryl choline) into the cell. Potential changes were measured by means of a high impedance voltmeter. As a reference, an Ag/AgCl electrode was used. The potentiometric output (electrode response) was obtained as the rate of

potential change (mV/min). The electrode response for a particular substrate concentration characterizes the electrode activity.

Inhibitor assay

The initial activity of the tri-enzyme electrode (for 1 mM concentration of butyryl choline in the cell) was measured according to the procedure described above. Then the electrode was incubated in a solution containing the inhibitor (trichlorfon) and measurement of the electrode activity was repeated. Inhibition for each particular concentration of inhibitor was expressed as a ratio of electrode activity after incubation with the inhibitor to the initial activity of the electrode.

RESULTS AND DISCUSSION

Bi-enzyme choline-sensitive electrode

The bi-enzyme electrode consists of a layer of immobilized peroxidase and a layer of choline oxidase co-immobilized upon the peroxidase layer. The addition of choline to the reaction media results in an enzymatic oxidation of choline:

$$\text{choline} + O_2 \xrightarrow{\text{choline oxidase}} \text{betaine} + H_2O_2. \quad (1)$$

This leads to a formation of hydrogen peroxide. Peroxidase catalyses the electroreduction of hydrogen peroxide by a mediatorless mechanism:

$$H_2O_2 + 2e + 2H^+ \xrightarrow{\text{peroxidase}} 2H_2O. \quad (2)$$

Thus, the peroxidase-modified electrode serves as a sensing element for peroxidase detection. The appearance of the latter at the peroxidase-modified electrode surface results in a sharp increase in the electrode potential due to the catalytic removal of overvoltage. The rate of potential increase is proportional to the rate of hydrogen peroxide formation (Equation 2) and, hence, this potential shift rate is proportional to the choline concentration (Equation 1) in a certain concentration range.

Immobilization of the enzymes on the electrode surface is a critical step in biosensor development. Two factors mainly determine the performance of immobilization: the nature of the carbon surface and the capacity of immobilization. According to the developed technique, only the first enzyme layer (peroxidase layer) is attached directly to the electrode surface. Other enzymes (choline oxidase and butyryl choline esterase) are immobilized upon the peroxidase layer by glutaraldehyde binding. Therefore, the critical step of bi- and tri-enzyme electrode development is the immobilization of the peroxidase layer. Several immobilization approaches were tested and a number of the electrode materials were screened.

The simplest way for immobilization is a physical sorption. In some cases, sorption can be improved when the carbon surface is additionally oxidized. This also was experimentally tested with most of the investigated carbon materials. For surface oxidation, electrodes were polarized anodically at a potential of +2 V (vs Ag/AgCI) for 15 minutes. Then the electrodes were polarized cathodically at a potential of -1 V (vs Ag/AgCl) for 5 minutes.

Chemical immobilization, based on covalent binding of the amino groups of peroxidase with the carboxyl groups of the carbon surface, was also tested. Woodward's Reagent K was used as a binding agent. Chemical immobilization was conducted for both an unoxidized and an oxidized carbon surface.

In all cases, choline oxidase was immobilized upon a peroxidase layer according to the standard glutaraldehyde binding technique described above. The method of evaluation of electrode performance was the method of the calibration curves. All electrodes were comparatively tested in order to obtain the dependence of the electrode response on the concentration of choline. The criteria for electrode sensitivity was the slope of the initial part of the calibration curve. The results of comparative study are presented in Table 1.

The data presented in the Table 1 show that pre-oxidation of the carbon surface in most cases results in a decrease of electrode sensitivity towards choline. That is probably due to the damage of the peroxidase molecule by surface oxidized groups. The chemical immobilization by Woodward's Reagent K leads to an improvement of electrode sensitivity only in a limited number of cases.

According to the data obtained, the electrode material based on TCB-S was selected, and the immobilization procedure based on physical adsorption (for peroxidase) and glutaraldehyde binding (for choline oxidase) was developed for a bi-enzyme electrode. The bi-enzyme electrode was used for the further development of the

TABLE 1. THE COMPARATIVE STUDY OF BI-ENZYME ELECTRODES FOR SELECTION OF ELECTRODE MATERIAL.

Material	Pre-treatment	Slope of the calibration curve (mV/min/mM)
VCK Carbon Fiber	None	93.2
	Oxidized	6.8
	Woodward's Reagant K	0
WDF Carbon Felt	None	23
	Oxidized	0
	Woodward's Reagant K	23
Carbon Matt	None	25.7
	Oxidized	0
	Woodward's Reagant K	0
Graphite	None	242
	Oxidized	82
	Woodward's Reagant K	0
WCA Graphite Fiber	None	86
	Oxidized	11
	Woodward's Reagant K	0
TCB-35	None	133
	Oxidized	22
	Woodward's Reagant K	170
	Oxidized + Woodward's Regant K	41
TCB-5	None	622
	Oxidized	178
ULTI Carbon	None	47
Carbon-Polymer Composite	Glutaraldehyde immobilization	230
Carbon Rod	None	37

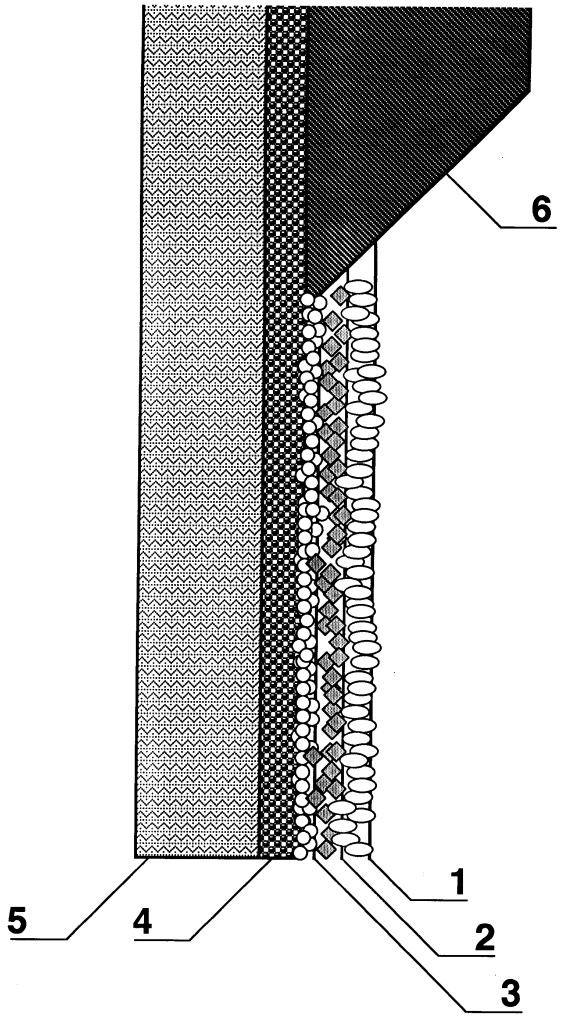

FIGURE 1. CROSSECTIONAL SCHEMATIC OF THE TRI-ENZYME ELECTRODE. 1 = BUTYRYL CHOLINE ESTERASE LAYER; 2 = CHOLINE OXIDASE LAYER; 3 = PEROXIDASE LAYER; 4 = PRESS PRINTED CARBON LAYER; 5 = PAPER SUPPORT; 6 = CARBON ROD POTENTIAL COLLECTOR.

tri-enzyme (butyryl choline esterase/choline oxidase peroxidase) electrode.

Tri-enzyme butyryl choline sensitive electrode

The tri-enzyme butyryl choline-sensitive electrode contains one additional enzyme layer of butyryl choline esterase co-immobilized upon the layer of choline oxidase of bi-enzyme electrode. Butyryl choline esterase catalyses the hydrolysis of butyryl choline leading to the formation of choline:

$$\text{butyryl choline} + H_2O \xrightarrow{\text{choline esterase}} \text{butyric acid} + \text{choline}. \quad (3)$$

The formation of choline results in the detection of butyryl choline by coupling with Equations 1 and 2.

The selection of electrode material and the development of the immobilization procedure for the bi-enzyme electrode for choline was the prerequisite for the development of the tri-enzyme electrode for OPC assay. One of the important advantages of the selected TCB-5 material is its highly dispersed structure. This allows the electrodes to be manufactured by press-printing technique. The schematic of the tri-enzyme electrode is presented in Figure 1. A paper strip cut from the press-printed material was used for electrode preparation.

Figure 2 demonstrates the dependence of electrode activity (as % of initial electrode activity) on the time of storage at 4°C for bi-enzyme (curve 1) and tri-enzyme (curve 2) electrodes. The tri-enzyme electrode demonstrates a much higher stability than the bi-enzyme electrode. The tri-enzyme electrode remains active for at least one month. The preparation of the tri-enzyme electrode involves the additional stage of incubation in glutaraldehyde solution. This probably results in improvement of the stability of the electrode due to cross-linking of choline oxidase molecules.

Figure 3 shows the tri-enzyme electrode response to choline (curve 1) and butyryl choline (curve 2). It can be seen that the response of such an electrode with respect to

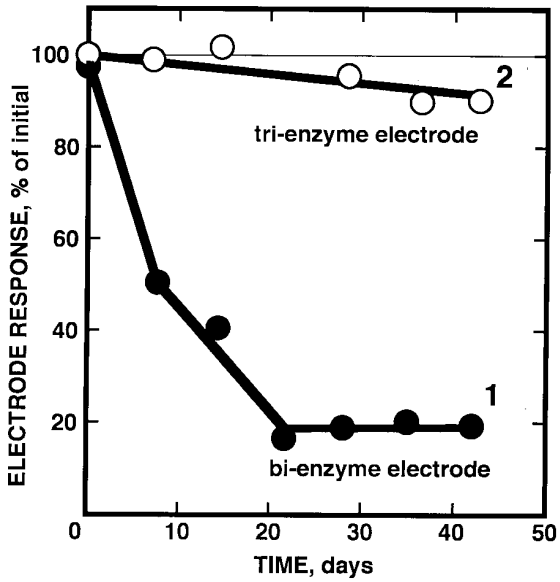

FIGURE 2. THE DEPENDENCE OF ELECTRODE ACTIVITY (AS % OF INITIAL ELECTRODE ACTIVITY) ON THE TIME OF STORAGE AT 4ºC FOR BI-ENZYME (CURVE 1) AND TRI-ENZYME (CURVE 2) ELECTRODES. CONDITIONS: 0.5 mM CONCENTRATION OF CHOLINE (FOR BI-ENZYME ELECTRODE) AND THE SAME CONCENTRATION OF BUTYRYL CHOLINE (FOR TRI-ENZYME ELECTRODE) WAS USED.

butyryl choline is lower (the slope of the calibration curve, curve 2) in comparison with its activity with respect to choline (the initial slope of the calibration curve, curve 1). From this it can be concluded that the rate-limiting reaction of the processes taking place on the electrode is the hydrolysis of butyryl choline catalyzed by butyryl choline esterase (Equation 1). It can be assumed that the enzyme butyryl choline esterase immobilized on the electrode is not present in excess. Analysis of organophosphorus compounds through inhibition of butyryl choline esterase activity is hampered by an excess of enzyme activity. Hence the sensitivity of inhibitor assay with such systems is increased when the rate of butyryl choline hydrolysis is the limiting process.

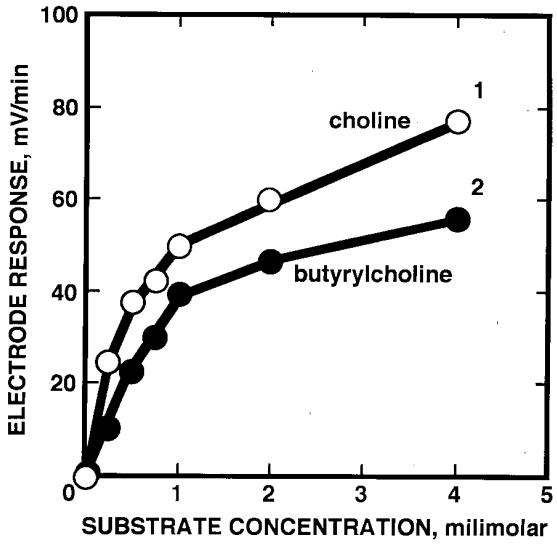

FIGURE 3. TRI-ENZYME ELECTRODE RESPONSE TO CHOLINE (CURVE 1) AND BUTYRYL CHOLINE (CURVE 2).

Inhibitor assay

Inhibitor assay for the determination of organophosphorus pesticides was demonstrated using trichlorfon as a model analyte. Figure 4 shows the effect of concentration of trichlorfon on the tri-enzyme electrode response to butyryl choline. The technique permits fast determination of trichlorfon with detection limit as low as 5 nM (1.3 ppb). The electrode was incubated in the inhibitor-containing solution for 10 min. The duration of measurements of electrode activity before and after incubation with inhibitor did not exceed 5 min. each. Therefore, the overall assay time was about 20 min. Further improvement in the reproducibility of electrode performances within one manufacturing set of electrodes will avoid the need for measurement of initial activity of each electrode. Only one electrode of the set will need to be tested to obtain initial activity of the electrode set. This could further decrease the assay time.

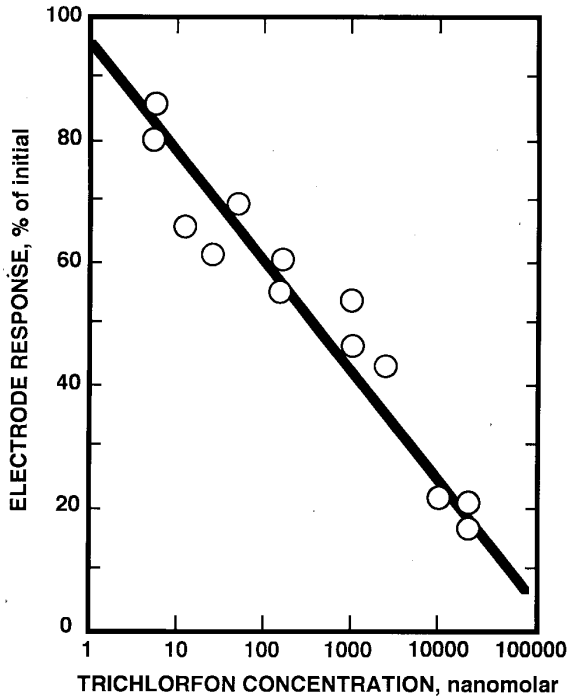

FIGURE 4. TRICHLORFON DETERMINATION CALIBRATION CURVE. THE EFFECT OF CONCENTRATION OF TRICHLORFON ON THE TRI-ENZYME ELECTRODE RESPONSE TO BUTYRYL CHOLINE. CONDITIONS: TEMPERATURE OF INCUBATION 39ºC; pH OF INHIBITOR-CONTAINING MEDIA 9.1; CONCENTRATION OF BUTYRYL CHOLINE FOR MEASUREMENT OF ELECTRODE ACTIVITY 1 mM.

CONCLUSIONS

This work demonstrates the potential for application of potentiometric enzyme electrodes based on mediatorless enzyme electrocatalysis for fast and sensitive assay of organophosphorus pesticides. The approach described herein permits future development of simple, inexpensive, and highly miniaturized measuring equipment for environmental control of organophosphorus pesticides.

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