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Biosensor analysis of blood esterases for organophosphorus compounds exposure assessment: Approaches to simultaneous determination of several esterases

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ABSTRACT

This paper reviews our previously published data and presents new results on biosensor assay of blood esterases. Tyrosinase and choline oxidase biosensors based on nanostructured polyelectrolyte films were developed for these purposes. Experiments were performed on the quantitative determination of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CaE), and neuropathy target esterase (NTE) in samples of whole blood of rats, mice, and humans. Good agreement was found between biosensor and spectrophotometric assays for AChE, BChE, and CaE. No direct comparison could be made for NTE because its activity cannot be measured spectrophotometrically in whole blood. A new method of simultaneous quantitative determination of AChE and BChE in test mixtures is also described. This method represents a bifunctional biosensor for the simultaneous analysis of choline and phenol based on integration of individual sensors. Algorithms for calculation of separate concentrations of AChE and BChE in the mixture were developed. The mean error of calculated component concentrations was ~6% for binary test mixtures. The present work provides a foundation for building multiplexed systems for the simultaneous determination of multiple esterases with applications to biomonitoring for exposures to organophosphorus compounds.

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1. Introduction

Defending against organophosphorus compounds (OPs) of known structure as well as those inadvertently produced by destruction of chemical weapons, natural disasters, or terrorist attacks requires rapid, sensitive, and specific detection of them and their biological effects by developing biomarkers and biosensors of exposure. A notorious demonstration of the availability, rapidity, and lethality of these agents was the release of sarin into the Japanese commuter train system in Tokyo, in which blood cholinesterase (ChE) activity was used as a tool for confirming exposure to the agent [1].

OPs phosphylate primary targets, e.g., acetylcholinesterase (AChE; acute toxicity) and neuropathy target esterase (NTE; delayed neuropathy, OPIDN); as well as secondary targets, e.g., butyrylcholinesterase (BChE) and carboxylesterase (CaE), which act as stoichiometric scavengers of OPs. We denote the set of activities of these esterases as well as that of paraoxonase-1 (PON-1), one of the A-esterases that can act as a catalytic scavenger of some OPs by hydrolyzing and thereby detoxifying them, as the "esterase status" of an organism—a determinant of individual sensitivity to OPs and a complex biomarker of exposure [2,3].

Currently, individual activities of blood esterases are determined mainly by using spectrophotometric techniques with corresponding substrates and specific inhibitors: this is a time-consuming process, suffering from high interference from blood hemoglobin and limited sensitivity. The application of modern electrochemical biosensors is a good alternative to routinely used methods, mainly due to high sensitivity achieved in recent years for analytes, such as phenol and choline [4,5].

The layer-by-layer (LBL) technique has demonstrated its promise as a versatile way to form organized multilayer thin films with predictable physical and chemical properties [6,7]. Based on the consecutive adsorption of positively and negatively charged polyelectrolytes on a solid substrate, the technique provides control of surface properties and enables the insertion of enzymes or other functional elements into the assembly [8,9]. Recently, we used LBL technology to create new amperometric biosensors for phenol and choline by integrating tyrosinase and choline oxidase into self-assembled polyelectrolyte layers [10,11].

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This paper reviews our previously published data [12,13] and presents new results on biosensor assay of blood esterases. Here we demonstrate the applicability of the previously developed choline oxidase and tyrosinase biosensors for the analysis of individual activities of AChE, BChE, CaE, and NTE in whole blood using esters of choline and phenol as esterase substrates. To validate the new method, biosensor measurements were compared with standard spectrophotometric methods. In addition, we produced a bifunctional biosensor system for the simultaneous analysis of choline and phenol, optimized its detection, and developed an algorithm for quantification of binary test mixtures (AChE + BChE).

2. Materials and methods

2.1. Materials

Tyrosinase from mushroom (EC 1.14.18.1), activity 2870 U/mg for L-tyrosine; AChE from bovine erythrocytes (EC 3.1.1.7), activity 0.31 U/mg; BChE from horse serum (EC 3.1.1.8), activity 264 U/mg; 20% (w/w) aqueous solution of poly(dimethyldiallylammonium chloride) (PDMDAACl, MW 400–500 kDa); eserine hemisulfate; paraoxon; phenol; choline; acetylcholine chloride; butyrylcholine chloride; phenyl acetate; butyrylthiocholine iodide; and graphite rods (d = 3 mm) were from Sigma. Ethopropazine hydrochloride; 1-naphthyl acetate; acetylthiocholine iodide; tetraisopropylpyrophosphoramide (iso-OMPA); and bis-*p*-nitrophenyl phosphate were from Sigma–Aldrich. Choline oxidase from Alcaligenes species (EC 1.1.3.17), activity 13.5 U/mg for choline; and glutaraldehyde (50% (w/v) aqueous solution) were from Fluka. Phenyl valerate and mipafox were from Oryza. All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using deionized Milli-Q water.

2.2. Blood sampling

White outbred male mice and Wistar male rats were used in the experiments. The animals were sacrificed by decapitation under CO_2-induced anesthesia. Trunk blood from each animal was collected immediately in glass vials containing citrate (3.8% (w/v) sodium citrate, 0.2 ml citrate/ml blood). Blood from 4 anonymous human (female) donors stabilized with citrate (0.2 ml citrate/ml blood) was used in the experiments. Blood samples were aliquoted, frozen in liquid nitrogen, and stored at −70 °C before use.

2.3. Preparation of blood for analysis

Frozen blood samples were thawed in an ice-water bath. Whole blood hemolysates (1:100, v:v) were prepared by adding 1 volume of blood to 99 volumes of ice-cold diluting buffer (see below for buffer for each esterase). Aliquoted hemolysates were frozen immediately in liquid nitrogen to facilitate complete hemolysis and kept at −20 °C until analysis.

2.4. Preparation of tyrosinase/choline oxidase-based biosensors

Polished graphite rods used as is (for tyrosinase entrapping) or electrochemically modified by MnO_2 (for choline oxidase entrapping) were first immersed in PDMDAACl solution for 10 min, rinsed with deionized water, and dipped into enzyme solution for 10 min followed by rinsing with deionized water. Cross-linking by glutaraldehyde was carried out in aqueous solution of 1% (w/v) glutaraldehyde for 60 min followed by rinsing thoroughly with deionized water. Sensors were stored under air at +4 °C and used for analysis during the next several days. PDMDAACl was used as a 0.5% (w/v) solution in 0.05 M sodium phosphate (pH 7.0). Tyrosinase and choline oxidase were prepared at concentrations of 0.1 mM in 0.05 M sodium phosphate (pH 7.0) and at 0.1 mM in 50 mM Hepes with 30 mM KCl (pH 7.5), respectively.

2.5. Electrodeposition of MnO_2 onto graphite rods

Graphite rods were modified by an H_2O_2-sensitive layer of MnO_2 at a constant potential of +600 mV *vs* Ag/AgCl reference electrode in 0.1 M NH_4Cl (pH 9.5) containing 1 mg/ml $MnCl_2$. After 15 min of deposition, the electrodes were washed with deionized water, incubated at 60 °C for 1 h, and stored at room temperature for at least 3 months until use.

2.6. AChE/BChE assay

AChE/BChE activity was determined in blood hemolysates (prepared in 0.1 M sodium phosphate pH 7.5) with 1 mM of acetylthiocholine/butyrylthiocholine and 0.4 mM DTNB for the spectrophotometric method of Ellman [14] or with 1 mM of acetylcholine/3 mM of butyrylcholine for the biosensor method. AChE assay was carried out in the presence of ethopropasine (0.02 mM, 10 min preincubation), for elimination of BChE activity in whole blood [15,16].

2.7. CaE assay

Spectrophotometric and biosensor determinations of CaE activity in blood hemolysates (prepared in 0.1 M sodium phosphate, pH 8.0) were carried out with 1 mM of phenyl acetate. 1-Naphthyl acetate (1 mM) was also used as a substrate in spectrophotometric CaE assays. To discriminate CaE activity 10 min preincubation with inhibitors of PON-1/arylesterase (2 mM EDTA) and ChEs (40 μM eserine) was used [17,18].

2.8. NTE assay

NTE activity was assayed according to the differential inhibition method of Johnson [19] with an electrochemical endpoint as described previously [20,21]. Briefly, blood hemolysate samples were incubated at 37 °C with 50 μM paraoxon (sample B) or 50 μM paraoxon plus 250 μM mipafox for 20 min (sample C), followed by incubation with phenyl valerate (final apparent concentration 0.54 mM) for the next 40 min at 37 °C. The reaction was stopped by addition of 50 μl of 1% (w/v) aqueous SDS. Phenol product was assayed amperometrically after 20-fold sample dilution in 50 mM sodium phosphate with 100 mM NaCl (pH 7.0). NTE activity was calculated as the difference in phenol production between samples B and C.

2.9. Spectrophotometric esterase activity determinations

All spectrophotometric esterase determinations were carried out using a Gilford-250 spectrophotometer in 3 ml 1-cm pathlength cuvettes at 25 °C by recording the increase in absorbance due to accumulation of the specified chromophore. The amount of 1:100 (v:v) blood hemolysate was 0.8–1.0 ml in the 3 ml incubation mixture, representing a final blood dilution of 1:300–1:375 (v:v). All data were automatically corrected for spontaneous hydrolysis of each substrate measured at the same time in the reference cuvette. For AChE and BChE assays the wavelength was changed to 436 nm (ε_{436} = 10,600 M^{-1} cm^{-1}) [15] to reduce interference from the high absorbance of hemoglobin at 412 nm. In the CaE assay, when phenyl acetate was used as the substrate, appearance of phenol product was measured at 270 nm (ε_{270} = 1310 M^{-1} min^{-1}); when 1-naphthyl acetate was used as substrate, appearance of 1-

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