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Dual amperometric–potentiometric biosensor detection system for monitoring organophosphorus neurotoxins

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Abstract

A dual-transducer flow-injection biosensor detection system for monitoring organophosphorus (OP) neurotoxins is described. Such simultaneous use of different physical transducers in connection to the same (organophosphorous hydrolase (OPH)) enzyme enhances the information content and provides discrimination between various subclasses of OP compounds. While the potentiometric biosensor responds favorably to all OP compounds, reflecting the pH changes associated with the OPH activity, the amperometric device displays well-defined signals only towards OP substrates (pesticides) liberating the oxidizable *p*-nitrophenol product. The potentiometric detection has been accomplished with a silicon-based pH-sensitive electrolyte-insulator-semiconductor (EIS) transducer, operated in the constant-capacitance (ConCap) mode. Both transducers are prepared by a thin-film fabrication technology, and respond rapidly and independently to sudden changes in the level of the corresponding OP compound, with no apparent cross reactivity. Relevant experimental variables were evaluated and optimized. Such development holds great promise for field screening of OP neurotoxins in connection to various defense and environmental scenarios. The multiple-transduction concept could be extended for increasing the information content of other 'class-enzyme' biosensor systems.

Keywords: Biosensor; Organophosphorous hydrolase; Enzyme electrode; Pesticides; Nerve agents

1. Introduction

Organophosphorus (OP) compounds are among the most toxic substances and are thus commonly used as pesticides and chemical warfare agents (CWA). Early detection of OP neurotoxins is important in the defense against terrorist activity, for protecting our water resources and food supplies, and for monitoring detoxification processes. Accordingly, there is a considerable interest in the development of highly reliable devices for sensitive, selective, and rapid detection of

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OP compounds. Biosensors are well-suited for meeting the challenges of on-site (decentralized) analyses. Enzymatic devices, based on the inhibition of acetylcholine esterase (AChE), have been widely used for the detection of OP compounds [1–3]. However, such AChE biosensors are not selective (due to their response to a wide range of toxic inhibitors), and are indirect and slow (owing to prolonged incubation and regeneration periods). A preferred avenue is to employ enzymes capable of selective recognition of OP compounds. In particular, fast, reversible, and direct measurements of such substances are possible utilizing the biocatalytic action of organophosphorous hydrolase (OPH) [4]. Organophosphorus hydrolase is an organophosphotriester-hydrolyzing enzyme able to hydrolyze a wide range of OP neurotoxins [5]. Several types of OPH-based biosensors—based on different signal transduction mechanisms—have been introduced, including potentiometric [6,7], amperometric [8,9] or optical [10,11] devices.

This article describes a dual-transducer flow-injection biosensing protocol for simultaneous amperometric and potentiometric measurements of OP compounds. Such use of different physical transducers in connection to OPH greatly enhances the information content, allows distinguishing the presence of OP pesticides (e.g. paraoxon, parathion) in sample mixtures containing other OP neurotoxins, and can minimize false alarms (through proper cross checking). Such capability reflects the fact that potentiometric pH-electrode transducers detect a wide range of OP compounds liberating protons upon OPH hydrolysis [6,7], while amperometric transducers monitor OP pesticides producing *p*-nitrophenol [8,9]. The potentiometric detection has been accomplished with a silicon-based pH-sensitive electrolyte-insulator-semiconductor (EIS) transducer, operated as a capacitor in the constant-capacitance (ConCap) mode [12]. Such EIS transduction, derived from the physical field effect, was recently employed for enzymatic (penicillinase-based) detection of penicillin [12]. While the concept of dual-transduction biosensing is illustrated here in connection to OPH, it can be applied for discriminating among substrates of other ‘class’ enzymes. The new protocol also opens the door for the creation of cross-reactive biosensor array systems based on the integration of additional transducers and/or OPH variants, along with advanced signal processing. The attractive performance characteristics of the new dual-transducer biosensing system are reported in the following sections.

2. Experimental

2.1. Apparatus

The flow-injection system (Fig. 1C) consisted of a carrier reservoir, an injector (with a 500 or 1000 μl loop), interconnecting PTFE tubing, an Alitea FIAlab pump (Sweden), and the electrochemical detectors. Two closely spaced plexiglas flow cells were used for accommodating the potentiometric and amperometric biosensors. The detectors were connected via a 10 cm long teflon tubing. A waste container was connected to the outlet of the second detector. The preparation of the sensor chips and the enzyme immobilization protocols are described in the next sections.

Amperometric experiments were carried out with the BAS CV-27 voltammetric analyzer (BAS, W. Lafayette), in connection with a BAS X–Y recorder. The disk-shaped gold working electrode (1.9 mm diameter), the Ag/AgCl ink reference, and the gold counter electrode were integrated on a silicon chip (Fig. 1B). The chip was tightly inserted into a special groove in the detector body, allowing exposure of the electrodes to the flow channel.

Potentiometric measurements were performed with an impedance analyzer (“EIS: sense”, constructed at the Jülich Research Center). The set-up of the silicon-based potentiometric field-effect sensor is shown in Fig. 1A. The EIS sensor consisted of a *p*-doped silicon substrate, a 30 nm SiO_2 insulating layer and the pH-sensitive tantalum pentoxide layer (8 mm diameter). An O-ring was placed on the surface of the potentiometric EIS sensor for defining the contact area of the transducer and preventing leaks. An internal ohmic rear contact to the aluminum layer was achieved with a gold-coated screw.

2.2. Reagents

Organophosphorus hydrolase (OPH) (specific activity 427 U/ml, 2.7 mg protein/ml; activity measured using paraoxon as substrate) was produced according to a previously described methods [8]. Paraoxon,

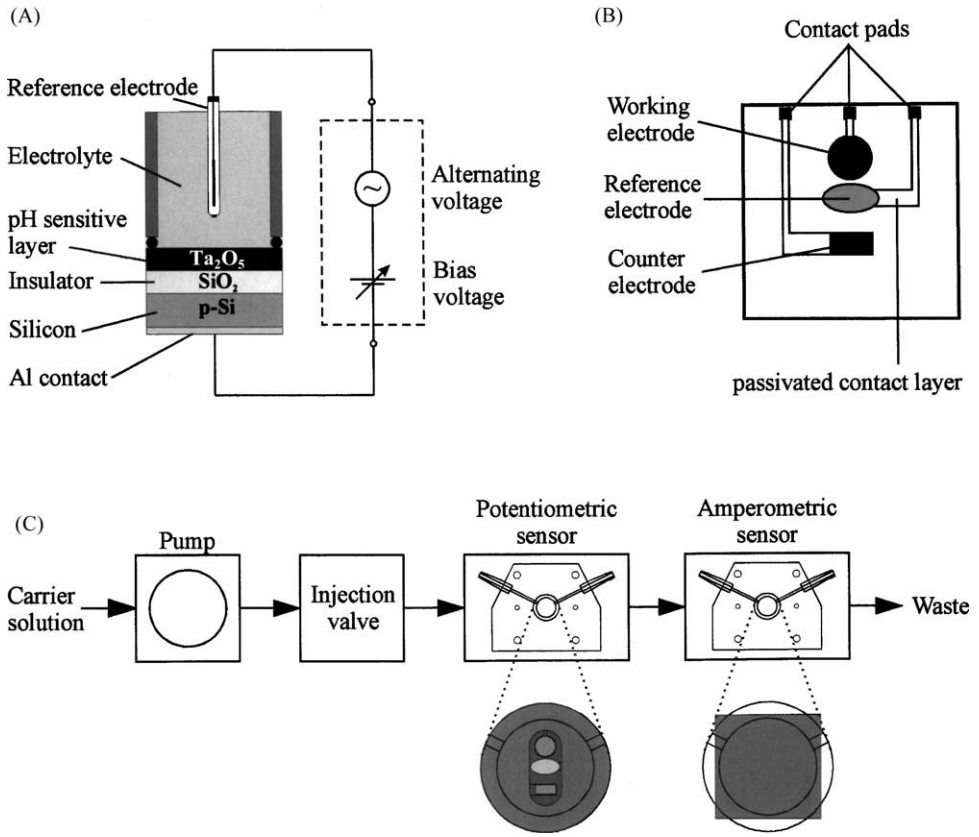

Fig. 1. (A) Schematic diagram of the potentiometric EIS sensor system. (B) Design of the thin-film amperometric gold electrode. (C) Schematic diagram of the dual-biosensor flow-injection system (see text for details).

cystamine and glutaraldehyde (70% aqueous solution) were obtained from Sigma (St. Louis, MO), while parathion, diazinon and dichlorvos were from Chemservice (West Chester, PA). *p*-Nitrophenol, sodium hypochlorite and potassium hydroxide were purchased from Aldrich. Sulfuric acid, hydrochloric acid (36%) and tetraethoxysilane were purchased from VWR (West Chester, PA), Spectrum (Garden, CA) and Fluka (Milwaukee, WI), respectively. Most experiments employed a phosphate buffer (0.5 mM, pH 9.0)/10 mM KCl medium as the flow-injection carrier solution. All solutions were prepared using deionized water.

2.3. Electrode fabrication

2.3.1. Amperometric detection

The gold-electrode structures were prepared by conventional thin-film fabrication techniques. A *p*-doped silicon with a resistivity of >1000 Ωcm was used as substrate material. Silicon dioxide (400 nm) served as insulating layer on top of the silicon. The metallic gold electrodes were embedded and photolithographically patterned into the insulating layer by means of lift-off process. The thickness of the microstructured electrodes was about 280 nm; the electrodes consisted of a three layer system (30 nm Ti, 50 nm Pt, 200 nm Au). Finally, the entire wafer (with the exception of the gold electrodes) was passivated by coverage with a 1 μm thick polyimide layer. The resulting gold working electrode had an area of 3.14 mm^2. Further details of the fabrication procedure were reported elsewhere [13].

The gold electrode was cleaned prior to the OPH immobilization by applying a potential of 0.5 V for 10 s in an aqueous solution of 0.5 M sulfuric acid/0.2 M hydrochloric acid/0.3 M sodium hypochlorite. The

electrode was then treated electrochemically by scanning the potential between −0.5 and +0.85 V in a 0.5 M potassium-hydroxide solution for 20 cycles at a rate of 100 mV/s.

The enzyme was immobilized by immersing the electrode in a 10 mM aqueous solution of cystamine for 12 h. Then, the electrode was rinsed with distilled water and was dried with nitrogen. It was subsequently dipped in a phosphate buffer (50 mM, pH 9.0) solution containing 2.5% glutaraldehyde solution for 1 h. The electrode was then washed with water and dried with nitrogen. This was followed by casting 5 μl of the OPH solution on the gold electrode and allowing it to dry for 4 h. The OPH-modified gold electrode was stored at 4 °C when not in use.

2.3.2. Potentiometric detection

A cleaned *p*-doped silicon wafer was used as substrate. The top of the silicon was oxidized to a 30 nm thick silicon dioxide layer. Subsequently, a 50 nm thick pH-sensitive tantalum pentoxide layer was deposited by the means of a pulsed laser deposition. Finally, electron beam evaporation was used to deposit the aluminum rear side contact [14]. A 20 μl tetraethoxysilane solution droplet was placed on the transducer surface, was allowed to dry, and was rinsed with water. Subsequently, 20 μl of a mixture containing the OPH enzyme solution and 2.5% glutaraldehyde (in a phosphate buffer; 50 mM, pH 9), in a 2:1 ratio, was cast on the sensor surface. The electrode was then rinsed with water, was allowed to dry for at least 4 h, and stored at +4 °C.

2.4. Procedure

2.4.1. Amperometric measurements

Experiments were performed by applying the desired potential (usually +0.75 V), stabilizing the background current in the presence of the flowing carrier solution (0.5 mM phosphate buffer, pH 9.0) and injecting the sample solution.

2.4.2. Potentiometric measurements

Electrolyte-insulator-semiconductor measurements were performed using the ConCap mode, with a capacitance of 22.0 nF (in connection to a total impedance of 15 kΩ). The sample injections were initiated after a 30 min stabilization of the bias voltage drift in the presence of the flowing carrier solution. Details of such EIS operation were described elsewhere [12].

3. Results and discussion

The dual-transducer OPH biosensor system enhances the information content and enables differentiation between OP pesticides (e.g. paraoxon, parathion) and other OP compounds. Fig. 2 displays typical amperometric (A) and potentiometric (B) peaks recorded simultaneously with the combined OPH biosensor system for injections of micromolar solutions of paraoxon (a); dichlorvos (b); parathion (c); and diazinon (d). The silicon-based potentiometric biosensor responds favorably to all four OP compounds, reflecting the pH changes associated with the OPH activity. In contrast, the amperometric device displays well-defined signals for paraoxon and parathion (associated with the oxidation of the liberated nitrophenol), and no response to dichlorvos and diazinon. Both transducers respond rapidly and independently to the corresponding OP compound, with a nearly instantaneous rise in the signal, a slower decay, and peak widths (at half-height) of around 60 s.

The realization of an effective OP monitoring system requires a systematic optimization of parameters

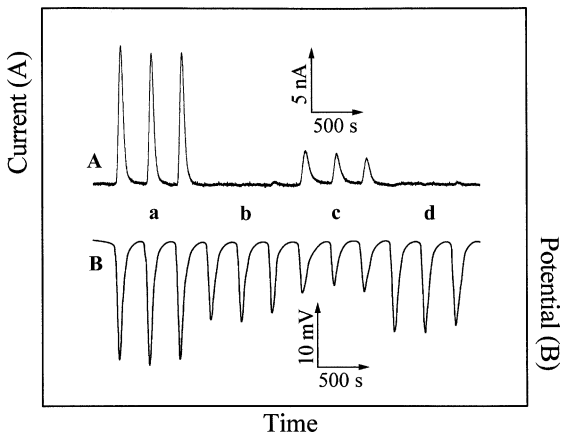

Fig. 2. Simultaneous amperometric (A) and potentiometric (B) measurements of (a) 50 μM paraoxon; (b) 100 μM dichlorvos; (c) 200 μM parathion; (d) 200 μM diazinon. Carrier solution, phosphate buffer (0.5 mM, pH 9.0)/10 mM KCl; flow rate, 1.8 ml min^{-1}; injection loop, 1000 μl; operating potential (A), +0.75 V; constant capacitance (B), 22 nF.

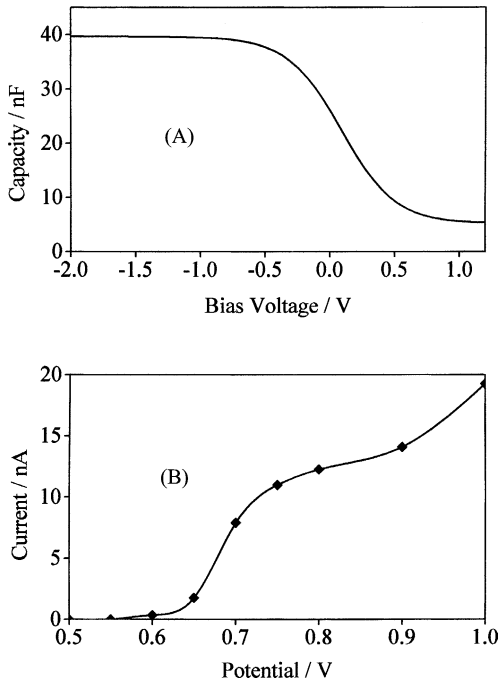

Fig. 3. (A) Capacitance–voltage curve of the potentiometric EIS sensor. Carrier solution, phosphate buffer (0.5 mM, pH 9.0)/10 mM KCl; flow rate, 1.8 ml min^{-1}. (B) Hydrodynamic voltammogram for 5 μM *p*-nitrophenol at the bare gold-electrode detector. Carrier solution, phosphate buffer (50 mM, pH 7.0)/10 μM KCl; flow rate, 1.3 ml/min; injection loop, 500 μl; operating potential, +0.75 V.

relevant to both transducers. Fig. 3 shows the influence of the capacitance (A) and applied potential (B) upon the corresponding potentiometric and amperometric signals. A sigmoidal C/V profile, expected for EIS transducers [12], is observed. The corresponding electrical behavior of this EIS sensor can be probed by its small-signal capacitance. The linear range of the capacitance lies between 30 and 10 nF. Such C/V curve shifts along the voltage axis upon changing the concentration of the OP substrate (i.e. with different degrees of proton adsorption at the Ta_2O_5 layer). Setting the capacitance at a fixed value (around 60% of its maximum), i.e. at ca. 22 nF, thus offers an optimal response (maximal change of the bias voltage). The nitrophenol current response (of the amperometric transducer) increases slowly with the applied potential between +0.55 and +0.65 V, rises sharply up to +0.75 V, and starts to level off thereafter (B). All subsequent biosensing work em-

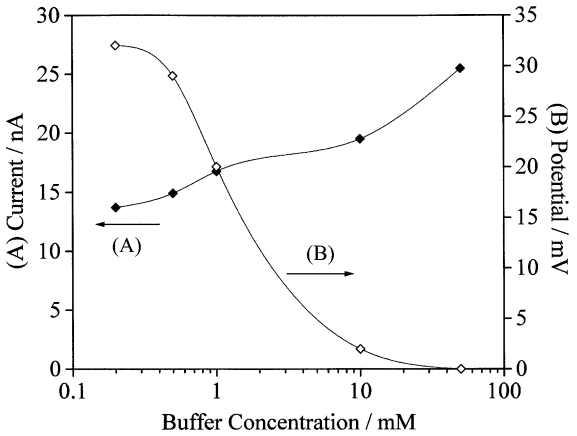

Fig. 4. Effect of the buffer concentration to the sensor signals of (A) the amperometric gold electrode and (B) the potentiometric EIS sensor. Analyte, paraoxon (100 μM); carrier solution, phosphate buffer (0.5 mM, pH 9.0)/1 mM KCl; flow rate, 1.3 ml min^{-1}; injection loop, 500 μl; applied potential (A), +0.75 V; constant capacitance (B), 22 nF.

ployed a capacitance of 22 nF and an applied potential of +0.75 V.

The buffer concentration has a profound effect upon the amperometric and potentiometric signals (Fig. 4). The two transducers display different profiles with the ampeometric response increasing slowly over the 0.2–50 mM range, while the potentiometric output decreasing rapidly (sigmoidally) over this region. As expected for the uptake of the enzymatically liberated protons, no potentiometric response was observed for buffer concentrations higher than 50 mM. A 0.5 mM buffer solution was employed in all subsequent work as a compromise between low-buffer capacity and a stable analyte solution.

Fig. 5 displays the influence of the flow rate upon the amperometric (A) and potentiometric (B) response to paraoxon. The current increases rapidly upon raising the flow rate between 0.7 and 1.15 ml min^{-1} and decreases gradually for rates higher than 1.35 ml min^{-1}. This profile reflects the forced-convection effect upon the flux of the substrate as well as the reaction time. The potentiometric output increases rapidly between 0.75 and 1.1 ml min^{-1}, then more slowly, and levels off above 1.8 ml min^{-1}. Such behavior of the potentiometric sensor reflects the flow-rate effect upon the transport of the liberated protons through the enzyme membrane to the pH-sensitive Ta_2O_5 surface. The

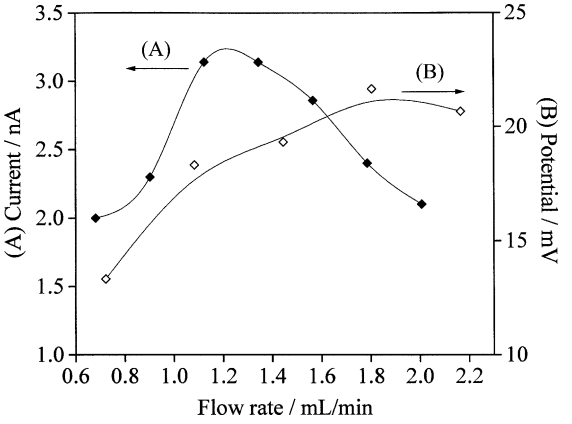

Fig. 5. Effect of flow rate on the amperometric (A) and potentiometric (B) response to paraoxon (1 μM (A) and 100 μM (B)). Injection loop, 500 μl; injections of (A) 1 μM and (B) 100 μM paraoxon; carrier solution: (A) 50 mM and (B) 0.5 mM; (A) pH 7.0 and (B) pH 9.0; phosphate buffer with (A) 10 μM and (B) 10 mM KCl (other conditions as in Fig. 2).

response time of the bare pH-transducing layer is in the order of several hundreds milliseconds [15]. Most subsequent work employed a flow rate of 1.8 ml min^{-1}.

A fast, stable and independent response to sudden changes in the level of the OP compound, with no apparent carry-over effects, is essential for their reliable monitoring. Fig. 6 displays the response of the dual biosensor flow system to a series of alternate injections of paraoxon (a) and dichlorvos (b). As expected, while both compounds are conveniently detected with the potentiometric device (B), the amperometric probe (A) responds only to the paraoxon injections. The response of both biosensors is highly reproducible and reversible, with no apparent cross reactivity. Relative standard deviations of 1.6(A,a), 3.8(B,a), and 8.8(B,b)% have thus been estimated for these amperometric and potentiometric data ($n = 15$). The response of both biosensors remained highly stable over several hours of continuous operation (not shown). Apparently no passivation of the amperometric electrode surface is occurred (despite the high anodic potential) and no loss of the biocatalytic activity of OPH is observed under these conditions.

The concentration dependence was evaluated for flow-injection potentiometric measurements of increasing levels of paraoxon and dichlorvos over the

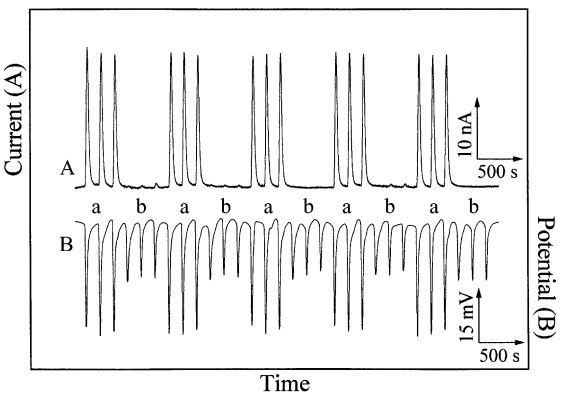

Fig. 6. Dynamic behavior of the dual-transducer amperometric (A) and potentiometric (B) system. Alternate injections of 100 μM paraoxon (a) and dichlorvos (b) solutions. Carrier solution, phosphate buffer (0.5 mM, pH 9.0)/10 mM KCl; flow rate, 1.8 ml min^{-1}; injection loop, 1000 μl; operating potential (A), +0.75 V.

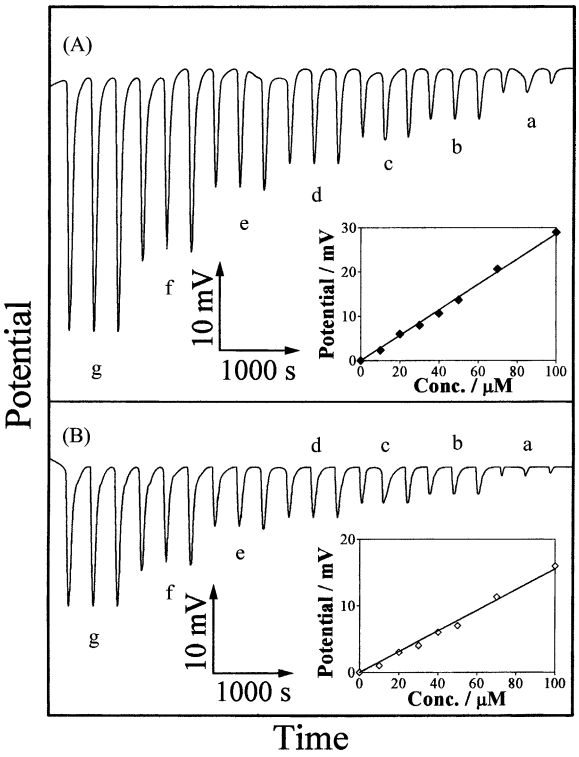

Fig. 7. Potentiometric response to injections of (A) paraoxon and (B) dichlorvos solutions of increasing concentrations: (a) 10 μM; (b) 20 μM; (c) 30 μM; (d) 40 μM; (e) 50 μM; (f) 70 μM; (g) 100 μM. Insets show the corresponding calibration plots for paraoxon (◆) and dichlorvos (◇) (other conditions as in Fig. 2).

10–100 μM range (Fig. 7A and B, respectively). The response peaks of both OP neurotoxins increase linearly with the concentration. Also shown in Fig. 7 are the resulting calibration plots. The slopes of the resulting calibration plots correspond to sensitivities of 0.286 (A) and 0.155 (B) mV/μM (correlation coefficients, 0.999 and 0.998, respectively). Detection limits of 2 μM paraoxon and 6 μM dichlorvos were estimated on the basis of the signal-to-noise characteristics of the corresponding 10 μM peaks (Fig. 7a; S/N = 3). These potentiometric detection limits are higher than those (nM) observed with inhibition-based biosensors (1–3). The amperometric detector, in contrast, displayed lower detection limits (e.g. of around 70 nM paraoxon based on the response to a 0.5 μM solution; not shown), and yielded a highly linear calibration plot for paraoxon over the 2–14 μM range (correlation coefficients, 0.999). Similar to other biosensing systems, the present one would require some sample preparation to enrich the OP compounds from environmental samples. In contrast, no such enrichment would be required for monitoring detoxification processes (where the OP concentrations are relatively high).

4. Conclusions

We described a flow-injection system with dual amperometric and potentiometric OPH biosensors for the simultaneous and rapid measurements of OP compounds. The independence of the two analytical signals obtained with the dual-transducer system was illustrated. The results indicate that the dual-transduction operation increases the information content and can be used to distinguish between subclasses of OP neurotoxins. The combined information could be used to minimize false (negative or positive) alarms in connection to a proper cross checking. Further enhancement of the information content and higher degree of discrimination could be achieved using different enzymes (OPH, AChE, etc.) or variants of the same (OPH) enzyme (of varying and tuned specificity and activity), in connection to similar or different transducers. For example, the integration of a fluoride selective electrode could distinguish the presence of neurotoxins containing P–F bonds (e.g. sarin, soman). Such integration of multiple transducers and/or enzymes should lead to the creation of powerful cross-reactive biosensor array systems (in connection to a logical signal processing). We are currently exploring such integration, along with on-line enrichment, further miniaturization and adaptation to remote (unattended) operation. Attention is also being given to possible electroactive interferences in connection to an 'OPH-free' amperometric detection. Similarly, an 'OPH-free' potentiometric transducer should be useful for addressing potential pH changes associated with real samples (1–3). These developments should facilitate the field screening and real-time analysis of OP neurotoxins in various defense and environmental scenarios. The new multi-transducer biosensing concept can be extended to other classes of target analytes in connection to the corresponding enzymes.

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