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**BIOSENSOR SYSTEM FOR CONTINUOUS
MONITORING OF ORGANOPHOSPHATE AEROSOLS
(POSTPRINT)**

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Biosensor system for continuous monitoring of organophosphate aerosols

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Abstract

An enzyme-based monitoring system provides the basis for continuous sampling of organophosphate contamination in air. The enzymes butyrylcholinesterase (BuChE) and organophosphate hydrolase (OPH) are stabilized by encapsulation in biomimetic silica nanoparticles, entrained within a packed bed column. The resulting immobilized enzyme reactors (IMERs) were integrated with an impinger-based aerosol sampling system for collection of chemical contaminants in air. The sampling system was operated continuously and organophosphate detection was performed in real-time by single wavelength analysis of enzyme hydrolysis products. The resulting sensor system detects organophosphates based on either enzyme inhibition (of BuChE) or substrate hydrolysis (by OPH). The detection limits of the IMERs for specific organophosphates are presented and discussed. The system proved suitable for detection of a range of organophosphates including paraoxon, demeton-S and malathion.
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Keywords: Enzyme immobilization; Butyrylcholinesterase; Organophosphate hydrolase; Biosensor; Impinger; Aerosol sampling

1. Introduction

Organophosphates (OPs) are used throughout the world as pesticides and insecticides. OPs are also toxic to many other organisms, including humans, due to their often irreversible inhibition of essential enzymes of the central nervous system. The potency of OPs has also led to their use as chemical warfare agents (Bajgar, 2004; Wiener and Hoffman, 2004). Currently, the detection of OP exposure is mostly retrospective, because many OPs exist as atmospheric particulate matter not easily detectable by human senses. The threat of terrorist activity is often cited to emphasize the importance of sampling and analysis of airborne contaminants, yet less sinister circumstances can cause public health issues as well. For example, the ventilation of buildings using outdoor air creates a situation whereby building occupants

can be exposed to OPs both by incidental release (e.g. pesticide spraying) as well as a direct targeted attack. Therefore, there is an urgent need for aerosol sampling devices that provide an early warning of OP chemical agent release before the resultant contamination of a building's air supply.

Detection of OPs in air is currently performed by chromatography coupled with mass selective detectors or various types of spectroscopy (Staaf and Ostman, 2005; Bjorklund et al., 2004; Sanchez et al., 2003). Such techniques are time consuming, expensive and often require highly trained personnel, making them impractical for continuous monitoring. The enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and organophosphate hydrolase (OPH) have received much attention as alternatives for the detection of OPs and have been demonstrated in a range of amperometric, potentiometric, conductometric and optical formats (Andrescu and Marty, 2006; Walker and Asher, 2005; Mulchandani et al., 2001, 1998; Simonian et al., 2001; Wang et al., 2003; Singh et al., 1999). Detection using BuChE is based upon enzyme inhibition; OPs block the enzyme activity of BuChE leading to a decrease in response proportional to OP concentration. In contrast, detec-

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tion using OPH is based upon hydrolysis of OP substrates. OPH hydrolyzes a range of OPs including pesticides (e.g. parathion and malathion) and chemical warfare agents (e.g. soman, sarin and VX) (Dumas et al., 1989; Di Sioudi et al., 1999; Lai et al., 1995; Rastogi et al., 1997). The products of hydrolysis can be monitored spectrophotometrically or electrochemically. Because OPs are substrates for OPH, catalysis leads to a direct determination of analyte and the signal generated is directly proportional to the concentration of OP.

A primary limitation of many biologically based detection systems is the instability of the biological component. We have recently demonstrated that silicification provides a biocompatible and simple method for enzyme immobilization (Luckarift et al., 2004). Silicification refers to the process that certain organisms use to form hard silica skeletons by deposition of inorganic minerals. The silicification process can be adapted *in vitro* to entrap enzymes of interest within the resulting silica matrix. The silica immobilization method proved suitable for the encapsulation of butyrylcholinesterase (BuChE) with high retention of enzyme activity (>90%). Further modification of the procedure allowed the entrainment of the silica-immobilized enzymes within a fixed bed column; making them amenable to continuous flow systems (Luckarift et al., 2006). In this study, we compare the applicability of silica-immobilized BuChE and OPH for enzyme-based detection of OP contaminated air. The system provides the potential for development of continuous air monitoring devices for detecting OP contamination and integration with an alarm system. A system is envisaged for example whereby an air conditioning supply could be controlled in response to a positive signal.

2. Materials and methods

2.1. Materials

Butyrylcholinesterase (E.C.3.1.1.8; Equine serum, ≈50% protein and activity of 1200 Units/mg protein) was purchased from Sigma–Aldrich and dissolved in cholinesterase specific buffer (Luckarift et al., 2004). The synthetic peptides R5 (SSKSGSYSGSKGSKRRIL), and the R5-His-tagged variants were purchased from New England peptide (Gardner, MA). Demeton-S was obtained from ChemService (West Chester, PA). All other chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO). OPH was kindly provided by Dr. James Wild of Texas A and M University.

2.2. Air sampler design

A schematic diagram of the sampling system is illustrated in Fig. 1. Liquid samples were aerosolized using a Micro-Mist nebulizer manufactured by Hudson RCI. The nebulizers were loaded with 1 ml of solution and operated at an air flow rate of approximately 4 l/min for 4 min. At the end of 4 min, the nebulizer was switched off and the residual volume of sample remaining in the nebulizer was measured to determine the volume of aerosolized sample. Downstream of the nebulizer, aerosolized samples were diluted with approximately 8 l/min of

filtered room air. The combined flow of sample and diluent air was collected in a SKC BioSampler® impinger (SKC, Eighty Four, PA). This device employs three glass jets to impact a gas or aerosol sample into a liquid reservoir. The airflow through the impinger jets was maintained at sonic speed by providing a stable flow rate of 12 l/min. At this gas flow rate, the impinger has been documented by its manufacturer to have an aerosol collection efficiency of 80% for 0.3 μm particles to ~100% for particles greater than 1.0 μm.

Here, the liquid reservoir in the impinger was composed of phosphate buffer (25 mM, pH 7.0, 10 mM MgSO₄, 126 μM 5,5'-dithiobis (2-nitro-benzoic acid) [Ellman's reagent]) and was continuously circulated at a flow rate of ~20 ml/min using a piston pump (Fluid Metering Inc. Model QV). After the aerosolized sample was entrained into the circulating liquid stream, the mobile phase passed through a passive debubbling device to remove air bubbles. The bubble-laden flow was diverted to waste while the bubble-free flow was directed through the IMER columns and into a single wavelength absorbance detector. The flow rate was maintained at 2 ml/min by a second piston pump positioned between the debubbler and the IMER columns so that the sample was under positive pressure as it was pushed through the column and negative pressure as it was pulled through the detector. Downstream of the detector, the sample was diverted to waste.

The relationship between the concentration of a compound in the air and that in the impinger liquid can be expressed as $C_A = C_l(Q_l/Q_A)$. Where C_A is the atmospheric concentration of the organophosphate of interest, C_l is the concentration in the impinger liquid, Q_l is the liquid flow rate through the impinger and Q_A is the air flow rate within the impinger. Hence for a measured liquid concentration the atmospheric concentration can be determined.

2.3. IMER preparation and chromatography conditions

Immobilized enzyme reactor columns (IMERs) were prepared as described previously (Luckarift et al., 2006), with either BuChE (0.6 mg) or OPH (0.66 mg). In preliminary experiments, IMERs were connected to an Agilent 1100 series liquid chromatography system and liquid samples (20 μl) were injected directly via an auto injection system. For aerosol sampling, the IMER columns were integrated with a BioSampler® Impinger and attached to a Hewlett Packard 1050 series single wavelength detector (Fig. 1). Phosphate buffer (25 mM, pH 7.0, 10 mM MgSO₄, 126 μM 5,5'-dithiobis (2-nitro-benzoic acid) [Ellman's reagent]) was used as the mobile phase throughout unless otherwise stated.

2.3.1. OPH-IMERs

OPH-catalyzed hydrolysis of OPs was monitored continuously at 412 nm for malathion and demeton-S and 400 nm for paraoxon. Ellman's reagent within the mobile phase reacts with free thiols generated by hydrolysis of malathion or demeton-S to produce a detectable chromophore at 412 nm. Detection of paraoxon was by direct determination of the hydrolysis product, *p*-nitrophenol (PNP) at 400 nm. Signal intensity for all substrates

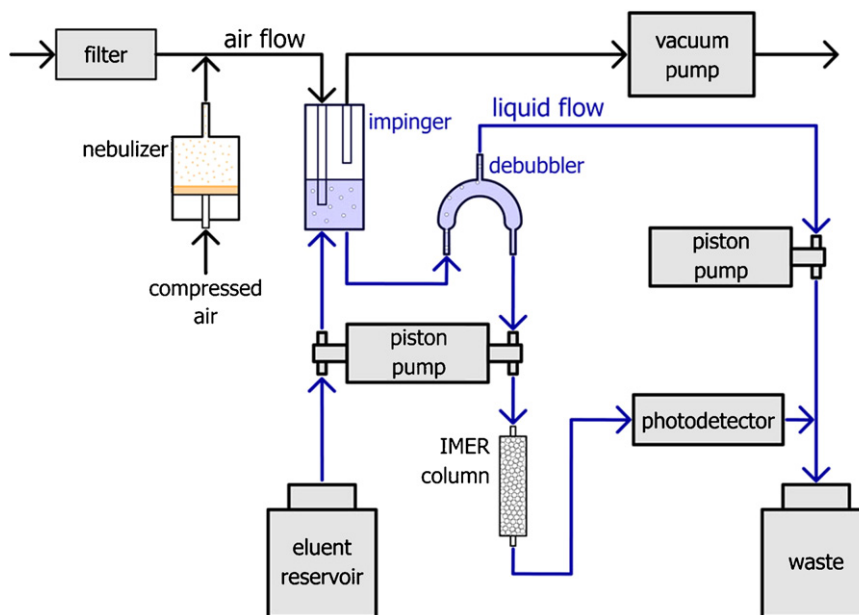


Fig. 1. Schematic for the laboratory characterization of an organophosphate measurement system.

was recorded as the product peak area. Goodness of fit (r^2) for linear regression lines was determined using GraphPad Prism software (v3.02).

2.3.2. BuChE-IMERs

Butyrylthiocholine iodide (BuCh-I) (20 μ M in phosphate buffer) was pumped continuously through the column and product was monitored continuously at 412 nm. The degree of inhibition was determined by calculating the change in peak height compared to initial steady state absorbance for a range of OP concentrations (0.1–5 mM).

2.3.3. BuChE reactivation

After incubation with OP, BuChE-IMERs were reactivated by flowing four column volumes (20 ml) of pyridine-2-aldoxime (PAM) (5 mM) through the column at 4 ml/min, followed by four column volumes of buffer (containing 126 μ M Ellman's reagent and 20 μ M BuCh-I) at 4 ml/min. Long-term reactivation studies were performed on an Agilent 1100 series liquid chromatography system with buffer as one mobile phase and the PAM reactivator (5 mM) as a second mobile phase.

3. Results and discussion

3.1. Enzyme based IMER biosensors

Immobilized enzyme reactor (IMER) biosensors were constructed with either OPH or BuChE as the immobilized enzyme component as described previously for BuChE (Luckarift et al., 2006). Briefly, IMERs are prepared using silica-encapsulation *in situ* via histidine-tag attachment of the silica/enzyme nanocomposites to a packed column by metal affinity binding. Effective loading and retention of the enzyme activity was achieved. The protein concentration of the eluate indicated greater than

90% loading efficiency; in agreement with previous studies for BuChE (Luckarift et al., 2006). The IMER columns gave stable and reproducible conversion of substrate over a 24 h period. In addition, the IMER columns could be stored at 4 °C and reused over a period of 5 days with no loss in activity (data not shown).

3.2. Detection of OPs by OPH-catalyzed hydrolysis in liquid

Detection using the biosensor containing OPH is based on the hydrolysis of OP substrates and the concentration of product formed. Three OP pesticides were selected as representative substrates; paraoxon is representative of OPs with phosphotriester bonds (P–O bond), demeton-S and malathion are representative of phosphonothioate pesticides (P–S bond). Initially the IMERs containing silica-immobilized OPH were tested by direct injection of paraoxon in the liquid phase. OPH catalyzed the hydrolysis of paraoxon to yield p-nitrophenol (PNP) and pro-

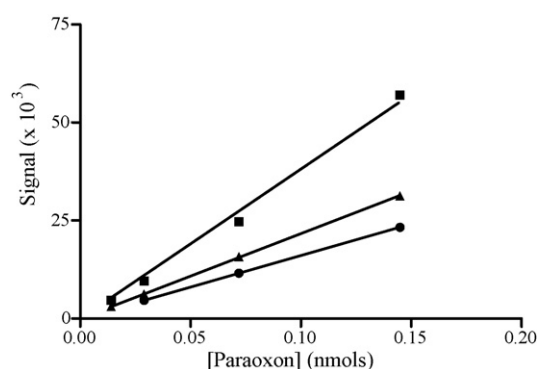


Fig. 2. OPH catalyzed hydrolysis of paraoxon as a function of flow rate. Paraoxon was injected to an OPH column ($n=1$) and signal intensity (peak area at 400 nm) of the hydrolysis product was measured at a range of flow rates: (■) 1 ml/min; (▲) 1.5 ml/min; (●) 2 ml/min.

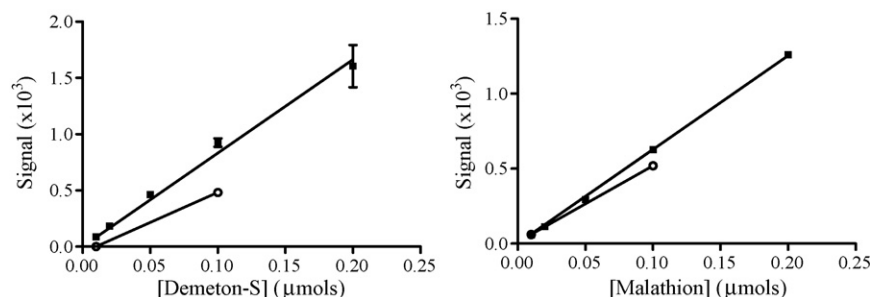


Fig. 3. Detection of demeton-S and malathion by OPH-catalyzed hydrolysis. Substrates (demeton-S and malathion) were injected into OPH columns and signal intensities of hydrolysis products were measured compared to an (enzyme-free) control column. Ellman's reagent was present in the mobile phase (126 μM). OPH-IMER column (■), Enzyme-free control column (○). Data points are mean ± S.E. ($n = 3$).

duced a concentration-dependent response to paraoxon at a range of flow rates (Fig. 2). A more rapid signal response was observed by increasing the liquid flow rate through the impinger, but there was a concurrent decrease in signal intensity and hence the sensitivity of the system resulting from the reduced residence time. Detection of paraoxon in the absence of the OPH-IMER was minimal (less than 10% relative signal intensity).

Detection of hydrolysis products by spectrophotometric analysis is limited to substrates that produce a strong chromophore. Inclusion of Ellman's reagent within the reaction, however, enhances the detection of free thiol groups generated by the hydrolysis of P–S bonds (as in demeton-S and malathion) (Lai et al., 1995; Rastogi et al., 1997). Detection of demeton-S produced a linear response in the range 0.01–0.2 μmol ($r^2 = 0.9816$) and the signal intensity was significantly enhanced compared to that in an enzyme-free column (Fig. 3). Malathion was also detectable following OPH catalysis and produced a linear response in the same substrate concentration range (0.01–0.2 μmol; $r^2 = 0.9998$), but the signal was not significantly different from an enzyme-free control at low concentrations (Fig. 3).

3.3. Aerosol sampling system configuration and characterization

The applicability of the IMERs connected to an aerosol sampling system was investigated. Initially, a control column containing no enzyme was connected to the system shown in the schematic (Fig. 1) to determine the efficiency and reproducibility of aerosol collection. The impinger device entrains contaminant particles into a fixed volume of buffer. The liquid flow rate into the impinger was controlled at a rate that could be adjusted to vary the collection efficiency and dilution factor. Aerosol samples collected within the impinger were pumped through the IMER columns and into a single-wavelength detector. PNP was selected as a model compound for calibration because it contains a strong chromophore that can be detected at 400 nm. The response for PNP was linear in the range 0.1–5 μmol ($r^2 = 0.994$). The response time was approximately 6 min and was a function of the combined residence time of the impinger, debubbler and IMER column. System parameters such as the impinger liquid flow rate can be modified to change the response time of the instrument.

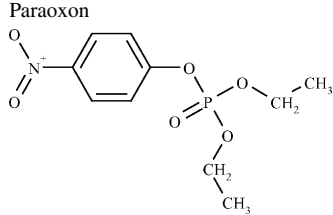
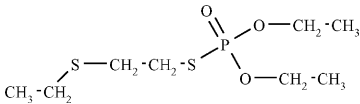
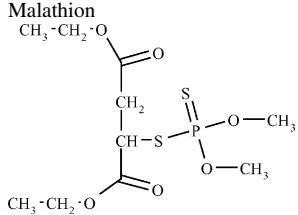
3.4. Detection of OPs in air by OPH-catalyzed hydrolysis

The OPH-IMER was connected to the impinger aerosol sampling system to determine the efficiency and reproducibility of the system during continuous operation. Paraoxon was aerosolized via an atomizer at a range of concentrations and the corresponding signal demonstrated first order reaction kinetics in the range 0–2.5 ppm ($r^2 = 0.994$). The signal intensity for detection of PNP as a product of OPH hydrolysis was in agreement with the signal observed for PNP provided directly to the system, confirming complete hydrolysis of paraoxon at the concentrations tested (Fig. 4a). The lower detection limit for paraoxon was equivalent to an ambient concentration of $\sim 0.52 \mu\text{mol}/\text{m}^3_{\text{AIR}}$ (using the equation described previously). Malathion and demeton-S produced lower responses than paraoxon (Table 1A). The lower detection limits for both demeton-S and malathion was an ambient concentration of $\sim 21 \mu\text{mol}/\text{m}^3_{\text{AIR}}$. The sensor responses of various OP substrates varied considerably due to variations in the substrate specificity of OPH as noted previously (Di Sioudi et al., 1999; Dumas et al., 1989) and the chromogenic intensity of hydrolysis products when complexed with Ellman's reagent. The K_m value for OPH catalyzed hydrolysis of demeton-S, for example, is approximately 10-fold higher than for paraoxon (Lai et al., 1995).

3.5. Detection of OPs by BuChE inhibition in air

The activity of BuChE can be determined by monitoring the hydrolysis of butyrylthiocholine iodide (BuCh-I) and measuring the concurrent formation of product upon reaction with Ellman's reagent to produce a detectable yellow anion (Luckarift et al., 2006 and references therein). OPs inhibit the hydrolysis of BuCh-I producing a decrease in signal intensity that is directly proportional to inhibitor concentration. Buffer was pumped continuously through the impinger at a fixed flow rate (20 ml/min) to give a constant substrate concentration of BuCh-I (20 μM), which yielded steady state continuous hydrolysis of BuCh-I to thiocholine. Paraoxon, demeton-S and malathion were investigated as representative cholinesterase inhibitors. All substrates were delivered to the aerosol sampling system via an atomizer and the inhibition of the steady state signal was measured. The inhibition of BuChE by paraoxon produced a second order kinetic response within the range 5–50 ppb

Table 1
Signal intensity for a range of OPs in the aerosol sampling system

	Paraoxon 		Demeton-S 		Malathion 	
	513 ppb	2.6 ppm	513 ppb	2.6 ppm	513 ppb	2.6 ppm
(A) OPH-IMER column						
Signal	2329 ± 346	12254 ± 402	40.5 ± 23	187.2 ± 44	30.1 ± 1.8	ND
Intensity ^a	100%	100%	1.7%	1.5%	1.3%	–
	Paraoxon		Demeton-S		Malathion	
	26 ppb	52 ppb	52 ppb	260 ppb	52 ppb	260 ppb
(B) BuChE-IMER column						
Signal	15.45 ± 2.475	51.55 ± 5.445	1.1†	2.7 ± 0.44	1.9†	ND
Intensity ^a	100%	100%	2.1%	–	3.7%	–

All values are mean ± S.E. ($n = 3$). Where noted (†), values are ($n = 1$). ND: not determined.

^a Signal intensity relative to Paraoxon.

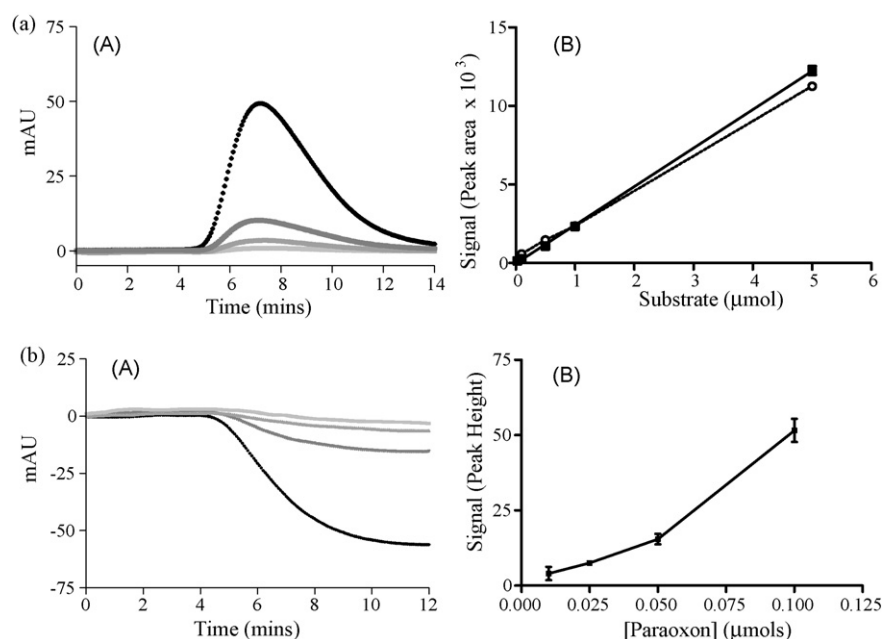


Fig. 4. (a) Detection of paraoxon by hydrolysis using the aerosol sampling system. (A) OPH-catalyzed hydrolysis of paraoxon as a function of paraoxon concentration. (B) Linear correlation of substrate concentration to signal (peak area); *p*-nitrophenol (PNP) was detected directly through a blank column (○) or as a product of paraoxon hydrolysis by OPH-IMER (■). Data points are mean $\pm$ S.E. ($n=3$). (b) Detection of paraoxon by inhibition using the aerosol sampling system. (A) BuChE inhibition by paraoxon as a function of paraoxon concentration. (B) Correlation of substrate concentration to signal (decrease in peak height). Data points are mean $\pm$ S.E. ($n=3$).

(Fig. 4b). The signal response was reproducible with standard deviations of $<10\%$ and was reproducible among three independent OPH-IMERs (data not shown). Inhibition of BuChE by paraoxon was linear at low concentrations but became saturated and lost linearity at concentrations above 25 ppb paraoxon. The lower detection limit for paraoxon based upon inhibition was approximately 5 ppb, equivalent to an ambient concentration of $\sim 0.21 \mu\text{mol}/\text{m}^3_{\text{AIR}}$. The sensitivity of the BuChE-IMER was in the order paraoxon $>$ malathion $>$ demeton-S (Table 1B). The sensitivity for the BuChE-IMER is in agreement with known inhibition of acetylcholinesterase enzymes by OPs (Simonian et al., 2001).

3.6. Biosensor reactivation and stability

BuChE becomes inactivated when exposed to OPs (Gulla et al., 2002; Mulchandani et al., 2001). In order to evaluate the performance utility of the BuChE-IMER it was necessary to reactivate the column and determine the recovered activity. Following inhibition of the BuChE-IMER, the column was treated with pyridine-2-aldoxime (PAM); a reactivator of OP-inhibited cholinesterase enzymes (Kovarík et al., 2004; Gulla et al., 2002). BuChE was irreversibly inhibited to some extent by paraoxon and demeton-S and there was a continuous decrease in enzymatic activity during repeated use of the IMER columns for inhibition experiments, even with use of the reactivator (Fig. 5). Loss of activity, however, was only detected when a saturating substrate concentration was used for activity measurements. At lower substrate concentrations, enzyme activity ($>70\%$) was retained providing reproducible and stable activity measurements for the entirety of the experiment (Fig. 5).

Despite the need to reactivate the BuChE biosensor, both OPH and BuChE biosensors were reusable over a number of samples with no loss in activity in agreement with the stability of the IMER columns demonstrated in previous studies (Luckarift et al., 2006). Detection of malathion and demeton-S resulted in low signals and caused persistent inhibition of the BuChE-IMER (data not shown). As a result, estimation of the detection level of these simulants by inhibition was limited to a small number of assays.

An important characteristic of both the OPH and BuChE biosensors was the stability and reproducibility of the signal response over successive samples (Supplemental Fig. 1). The

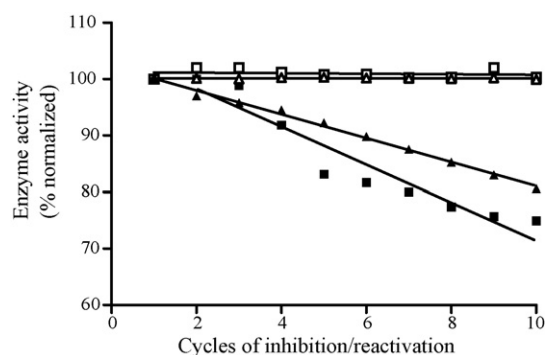


Fig. 5. Reactivation of BuChE following inhibition by demeton-S and paraoxon. Paraoxon and demeton-S were injected to BuChE columns ($20 \mu\text{l}$ of 5 mM). Following reactivation with pyridine-2-aldoxime (four column volumes of 5 mM at 4 ml/min), BuChE-I was supplied at high/saturated ($20 \mu\text{l}$ of 20 mM) and low ($2 \mu\text{l}$ of 20 mM) substrate concentrations to measure retention of enzyme activity. Paraoxon: [high BuChE-I] (▲), [low BuChE-I] (△); Demeton-S: [high BuChE-I] (■), [low BuChE-I] (□). Data points are mean ($n=2$).

stability of the signal also indicates no significant carryover of sample between subsequent analyses, which has been observed as a problem of inhibition-based biosensors.

4. Conclusion

The integration of IMER columns with an aerosol sampling system proved suitable for the detection of OPs and provided reliable and reproducible data for OPs including paraoxon, demeton-S and malathion. In the case of the OPH-IMERs, a difference between sensor responses with various OP substrates is due to variations in the substrate specificity of OPH (Di Sioudi et al., 1999; Dumas et al., 1989). The sensitivity of the systems for demeton-S and malathion differed by an order of magnitude relative to detection of paraoxon for both OPH and BuChE detection systems. Increased sensitivity and a correspondingly lower detection limit for the OPH-IMER can potentially be attained by utilizing variants of OPH with altered substrate preferences. For example, OPH has low catalytic activity towards demeton-S. Genetically modified OPH, however, shows increased catalytic activity towards P–S bonds which could be utilized to enhance the biosensor response (Simonian et al., 2004).

For a continuous air-sampling detector it is not necessary to immediately identify the agent, but simply to provide an early warning. The single wavelength absorbance detector used in this study limits the detection range of specific organophosphates. The addition of a high-pressure liquid chromatography column and spectral analysis in-line with the current detection system would allow the discrimination and quantification of specific OPs detected by the system. Such integration would provide a detection response that could be followed by a more detailed examination of the nature of the contaminant. The system could, therefore, warn against any compound that can act as a cholinergic neurotoxin, irrespective of its origin. This system is adaptable, for example, to monitoring pesticide concentrations in public water supplies or integration with the HVAC intake to a building.

The sensitivity and detection limits demonstrate the potential of the system as a proof of concept, but further optimization of the system will be required to enhance applicability. The system has inherent limitations, for example the OPH-IMER does not require a continuous supply of enzyme substrate, but the BuChE-IMER does. The detection of OPs by the aerosol sampling system is however, simple, rapid and directly applicable to kinetic response measurements. With additional optimization, the biosensor system described may find application for real-time detection of chemical agents under environmental conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2007.04.023.

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