Microchip enzymatic assay of organophosphate nerve agents

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Abstract

An on-chip enzymatic assay for screening organophosphate (OP) nerve agents, based on a pre-column reaction of organophosphorus hydrolase (OPH), electrophoretic separation of the phosphonic acid products, and their contactless-conductivity detection, is described. Factors affecting the enzymatic reaction, the separation and detection processes have been assessed and optimized. The complete bioassay requires 1 min of the OPH reaction, along with 1–2 min for the separation and detection of the reaction products. The response is linear, with detection limits of 5 and 3 mg/l for paraoxon and methyl parathion, respectively. Compared to conventional OPH-based biosensors, the OPH-biochip can differentiate between the individual OP substrates. The attractive behavior of the new OPH-based biochip indicates great promise for field screening of OP pesticides and nerve agents. The study demonstrates also for the first time the suitability of the contactless-conductivity detection for on-chip monitoring of enzymatic reactions.

Keywords: Organophosphate; Nerve agents; Enzymatic assay

1. Introduction

Organophosphate (OP) compounds are among the most toxic substances and are thus commonly used as pesticides and nerve gases. The high toxicity of OP compounds has generated urgent needs for fast-responding analytical systems for their on-site environmental monitoring and security screening. Early work focused on the developments of biosensors for screening OP substances [1]. These include enzyme-inhibition biosensors based on the inhibition of acetylcholine esterase (AChE) [2] and non-inhibition devices [1,3,4]. The inhibition bioassay lacks the necessary selectivity since AChE is inhibited by a wide range of toxic substances. The non-inhibition based biosensors rely on the use of organophosphorus hydrolase (OPH), which hydrolyzes OP pesticides and nerve agents to generate an acid and alcohol. While offering a fast response, such enzyme biosensors cannot discriminate among individual OP substances.

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attractive performance characteristics of the new OPH-based CE/conductivity microchip bioassays are reported in the following sections.

2. Experimental

2.1. Reagents

Paraoxon, parathion, and methyl parathion were purchased from Supelco (Bellefonte, PA). Stock solutions (1000 mg/l) of these pesticides were prepared in acetonitrile. Histidine (His), 2-(N-morpholino)ethanesulfonic acid (MES), sodium chloride, ammonium nitrate and potassium permanganate were purchased from Sigma. Stock solutions (1000 mg/l in acetonitrile) of 2,4,6-trinitrotoluene (TNT), 1,3-dinitrobenzene (DNB), and 2,4-dinitrotoluene (DNT) were obtained from Radian International (Austin, TX). Organophosphorus hydrolase (OPH; 2700 IU/mg of protein) activity enzyme stock solution was purchased from BioMerieux (Marcy l’Etoile, France). Organophosphorus hydrolase (OPH; 2700 IU/mg of protein) activity enzyme stock solution was purchased from BioMerieux (Marcy l’Etoile, France).

2.2. Apparatus

The PMMA microchips were manufactured at the Institute für Mikrotechnik Mainz (IMM, Mainz, Germany) and were described earlier [10]. The plastic PMMA microchip (70 mm × 24 mm) consisted of a 50 mm long separation channel (between the injection cross and the channel outlet reservoir) and 18 mm long injection channel (between the sample and unused reservoir). The two channels crossed each other halfway between the sample and the unused reservoir and 9 mm from the run buffer reservoir. The width and depth of the channels were 50 μm each. The homemade high-voltage power supply had an adjustable voltage range between 0 and +5000 V. A Plexiglas holder was fabricated for accommodating the separation chip. Short pipette tips were inserted into each of the four holes on the PMMA chip for introducing the individual solutions.

2.3. Electrode fabrication

A schematic of the experimental set up is shown in Fig. 1. The rectangular-shaped electrodes (0.8 mm × 24 mm) were fabricated from two 10 μm-thick aluminum-foil strips. The electrodes were fixed around two sides of a 1 mm-thick, 26 mm × 10 mm sized PMMA plate using a “quick-setting” epoxy with a spacing of 800 μm distance between them. The electrodes were placed in an “anti-parallel” orientation to minimize the stray capacitance between them. The detector plate was then equipped by two polyvinyl chloride (PVC) “clip-like” open rectangular holder plates to hold the microchip. This particular design does not require any microfabrication efforts to for the monolithic integration of the conductivity detector electrodes on the PMMA microchip and be easily replaced between microchips. The 125 μm thick cover plate of the chip was thus mechanically pressed towards the detection electrodes without any use of an adhesive. The exact details of the detector design were published recently [13,14]. Thin copper wires were attached to the electrodes on top of the detector plate using a conducting epoxy (Chemtronic, Kenesaw, GA, USA). The length of the wires was minimized to prevent induction of electric noise, and they were tin-soldered to the detector electronics. The detector was attached to the PMMA chip, 48 mm from the injection cross (3 mm from the ground reservoir).

2.4. Electronic circuit

The electronic circuitry of the contactless-conductivity detector was designed in accordance to a previously reported scheme [10]. All the electronic components were purchased locally. The circuit was completed by adding a passive RC filter (time constant, 0.01 s) followed by a voltage follower (LF 356) to the circuit output. This modification allows convenient interface to the data acquisition system. A HP 8116A function generator (Hewlett-Packard, Palo Alto, CA, USA) was used for generating the sinusoidal signal (usually with a frequency of 200 kHz with peak-to-peak amplitude of 10 V). The circuit board and other electronic equipment were placed in a shielding box for protection from external electric fields. The box was placed as close to the microchip (via copper wires for connection) as possible. To further minimize the noise, the chip (along with the printed electronic board) was secured from possible mechanical vibrations onto a chemical stand.

2.5. Electrophoretic procedure

The channels of the plastic chip were treated before use by rinsing with deionized water for 10 min. Reservoirs (a), (b), and (d) (Fig. 1) were filled with the electrophoretic run
buffer solution, while the sample reservoir (c) was filled with the sample solution (containing 60 U/ml OPH). After a short (60 s) reaction time, the products were injected into injection channel by applying a potential of −1000 V for 1 s between the sample (c) and the outlet (d) reservoirs. This drove the enzymatic product “plug” into the separation channel through the intersection. The analytical separation proceeded by switching the high-voltage contacts to the run-buffer reservoir, with other reservoirs grounded (a) and the outlet reservoir (d) floating.

2.5.1. Safety considerations
The high-voltage power supply should be handled with extreme care to avoid electrical shock. Parathion, methylparathion and paraoxon are very toxic substances and should be handled with extreme care. Skin or eye contact and accidental inhalation or ingestion should be avoided.

3. Results and discussion
The new OPH-based assay on CE/conductivity microchip relies on the reaction of OPH with the OP nerve agents in the sample reservoir, injection and separation of phosphonic acid reaction products, and their conductivity detection (Fig. 1). The enzymatic reaction was accomplished in the sample reservoir of the microchip system. The organophosphate nerve agents were added to the sample reservoir (containing the OPH enzyme) and were hydrolyzed to the corresponding p-nitrophenol and esters of phosphonic acid.

The reaction products were then injected into the separation channel, separated and detected by the CCD detector. Electropherograms for the individual substrates, methyl parathion, parathion and paraoxon (50 mg/l each), following their on-chip enzymatic reaction, are displayed in Fig. 2 (A, B, and C, respectively). Well-defined peaks, with favorable signal-to-noise characteristics, are observed at migration times of 97 (A), 117 (B) and 128 (C). High efficiency separations were indicated from sharper peaks with half-peak widths of 4.6 (A), 3.8 (B) and 4.4 (C). Being a contactless detector, variables such as detector geometry is not effected by the separation voltage. Fig. 2D shows the electropherogram for a mixture containing methyl parathion, parathion and paraoxon (along with OPH). The reaction products are well resolved and detected within ca. two min. The migration times are in agreement with those observed (in A–C) for the individual pesticides. As expected, no response is observed when the same sample mixture was analyzed without OPH (E).

Variables affecting the performance of the OPH-based CE microchip assay were examined and optimized. Fig. 3 displays the influence of the OPH activity upon the response for 50 ppm paraoxon. The conductivity response (of the reaction product) increases nearly linearly upon increasing the OPH activity up to 60 U/ml, then more slowly, and levels off above 80 U/ml (Fig. 3). All subsequent work utilized 80 U/ml OPH in the sample reservoir. We examined the effect of the pre-column reaction time using 80 U/ml OPH. The response increased nearly linearly with the time up to 60 s and then levelled off (not shown). A 60 s period was used in all subsequent work. We also assessed the influence of the pH of the run buffer; a MES/His buffer (pH 6.1) offered the best compromise between sensitivity and linearity, along with effective separation of the reaction products. This pH also minimized an interference of the p-nitrophenol product (whose pK_a is 7.15). Note that the optimal pH for the OPH reaction alone is 8.0 [1].

The influence of the separation voltage on the OPH enzymatic assay of 50 mg/l each of (a) methyl parathion, (b) parathion, and (c) paraoxon is shown in Fig. 4. The total time
Fig. 4. Effect of the separation voltage upon the enzymatic assay of selected nerve agents, 50 mg/l each of: (a) methyl parathion, (b) parathion and (c) paraoxon. Separation voltage, (A) $-700\,\text{V}$; (B) $-1000\,\text{V}$; (C) $-1500\,\text{V}$; (D) $-2000\,\text{V}$; and (E) $-2500\,\text{V}$. Other conditions, as in Fig. 2.

required for separating the reaction products decreases from 170 s (at $-700\,\text{V}$; A) to 80 s (at $-2500\,\text{V}$; B). Most favorable peak profiles and baseline characteristics are observed using a separation voltage of $-1000\,\text{V}$ (C); as expected, sharper peaks were observed upon increasing the separation voltage. The plate numbers ($N$) for methyl parathion are 36700, 49600, 41900, 32700, and 29650 m$^{-1}$ at $-700\,\text{V}$, $-1000\,\text{V}$, $-1500\,\text{V}$, $-2000\,\text{V}$, and $-2500\,\text{V}$, respectively. Since the OPH reaction products (phosphonic acids) are injected (as opposed to the injection of nerve agents and subsequent micellar electrokinetic separation [7]), the separation efficiency obtained were found to be very satisfactory and compare favourably with that obtained for analogous non-enzymatic assay [15]. Notice also that the separation voltage has a negligible effect upon the baseline current.

The OPH-biochip operation results in a well-defined concentration dependence. The concentration dependence is examined in Fig. 5 for solutions containing increasing levels of methyl parathion (10–50 mg/l, A–E) in the presence of a fixed (20 ppm) level of paraoxon. The methyl parathion peak (a) increases linearly with the concentration. Such linearity is indicated from the resulting calibration plot (shown also as inset). Such plot has a slope of 1.36 mV/ppm, with correlation coefficient of 0.992. Note also that the increasing levels of methyl parathion concentration have negligible effect upon the paraoxon peak. This is in contrast to the cross reactivity common to OPH biosensors. The data of Fig. 5A (lower trace) indicate detection limits of 3 mg/l methyl parathion and 5 mg/l paraoxon. Practical environmental applications may require lower detection limits; further improvements in the detectability may be achieved with the use of high-voltage CCD detection [11] and/or integration of an on-chip preconcentration function. Good precision is another attractive feature of the new OPH-biochip protocol. The precision was examined from a series of eight repetitive injections of a sample mixture containing 50 mg/l parathion and paraoxon. Reproducible signals were obtained with R.S.D. values of 2.1% (parathion) and 4.2% (paraoxon) for the peak heights.

It is often useful for various environmental and security screening applications to combine the measurements of OP nerve agent and various inorganic ions. For example, Fig. 6D demonstrates the simultaneous measurements.

Fig. 5. Electropherograms for mixtures containing increasing levels of methyl parathion (a) in steps of 10 ppm (A–E) and in the presence of 20 mg/l paraoxon (b). Also shown (inset) is the resulting calibration plot. Operation conditions, as in Fig. 2.

Fig. 6. Electropherograms for a mixture containing: (a) 50 mg/l of methyl parathion; (b) parathion and (c) paraoxon before (A) and after (B) the OPH enzymatic reaction; electropherograms showing the enzymatic assay of the same nerve agents in the presence of 20 mg/l of 2,4-dinitrobenzene and 2,4,6-trinitrotoluene nitroaromatic explosives (C), and in the presence of 300 ppm of chloride (d) and perchlorate (e) explosive-related anions (D). Operation conditions, as in Fig. 2.
of three OP compounds (a–c) with explosive-related anions such as chloride (d) and perchlorate (e). The complete assay requires less than 2.5 min (or 3.5 min including the time required for enzymatic reaction), and the inorganic explosive-related ions do not affect the quantitation of the OP nerve agents (B versus D). Neutral nitroaromatic explosives, such as 2,4-dinitrobenzene and 2,4,6-trinitrotoluene, are not detectable with the conductivity detector and are not affecting the response of the OP neurotoxins (C versus B). A dual conductivity/amperometric detector [13] should offer simultaneous measurements of organic-explosives and OP compounds.

In conclusion, the results presented above demonstrate that the coupling of on-chip OPH reactions with electrophoretic separation and CCD detection of the reaction products results in a powerful tool for screening OP nerve agents. Compared to OPH-based biosensors, the new biochip route permits convenient discrimination among individual OP compounds. For the first time, a CCD detector has been shown useful for monitoring enzymatically-generated products. The new microsystem offers promise for field screening of OP pesticides and nerve agents, with advantages of speed/warning, efficiency, portability, sample size, and cost.

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References