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Amperometric Thick-Film Strip Electrodes for Monitoring Organophosphate Nerve Agents Based on Immobilized Organophosphorus Hydrolase

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An amperometric biosensor based on the immobilization of organophosphorus hydrolase (OPH) onto screen-printed carbon electrodes is shown useful for the rapid, sensitive, and low-cost detection of organophosphate (OP) nerve agents. The sensor relies upon the sensitive and rapid anodic detection of the enzymatically generated *p*-nitrophenol product at the OPH/Nafion layer immobilized onto the thick-film electrode in the presence of the OP substrate. The amperometric signals are linearly proportional to the concentration of the hydrolyzed paraoxon and methyl parathion substrates up to 40 and 5 μM, showing detection limits of 9×10^{-8} and 7×10^{-8} M, respectively. Such detection limits are substantially lower compared to the $(2-5) \times 10^{-6}$ M values reported for OPH-based potentiometric and fiber-optic devices. The high sensitivity is coupled to a faster and simplified operation, and the sensor manifests a selective response compared to analogous enzyme inhibition biosensors. The applicability to river water sampling is illustrated. The attractive performance and greatly simplified operation holds great promise for on-site monitoring of OP pesticides.

Organophosphate (OP) compounds have found wide applications as pesticides and insecticides in agriculture and as chemical warfare agents in military practice. In view of major concerns regarding the toxicity of these compounds, there are urgent needs for innovative devices for monitoring OP pesticides and chemical warfare agents. Such devices would facilitate the detection of OP pesticides in our water and food sources, provide an early warning of potential terrorist attack, and facilitate effective monitoring of detoxification processes. Rapidly responding field-deployable analytical tools should offer the desired timely protective measure in the case of a sudden OP contamination or attack, while avoiding errors and the cost of laboratory-based measurements.

Biosensor technology is well-suited to satisfy the demands for on-site environmental monitoring and the rapid detection of chemical warfare agents.[1] In particular, inhibition enzyme electrodes, based on the modulated biocatalytic activity of acetylcholinesterase (AChE), have been widely used for monitoring OP pesticides.[2−4] Despite of their high sensitivity, such indirect inhibition biosensors suffer from major drawbacks, including poor selectivity, irreversible response, or a multistep protocol (involving a substrate addition and incubation). Thus, a faster, simpler, direct, and selective biosensor protocol is highly desired for meeting the needs of on-site monitoring of OP pesticides and chemical warfare agents.

This note describes a microfabricated device, based on the enzyme organophosphorus hydrolase (OPH), for the amperometric biosensing of OP nerve agents. Organophosphorus hydrolase, an organophosphotriester-hydrolyzing enzyme (discovered in soil microorganisms), has been shown to effectively hydrolyze a number of OP pesticides, such as parathion and paraoxon, as well as chemical warfare agents, such as sarin and soman.[5,6] The use of OPH is extremely attractive for biosensing of OP compounds that act as substrates for the enzyme (rather than exerting an inhibitory action). Recently, we took advantage of the unique biocatalytic activity of OPH and developed potentiometric and fiber-optic biosensors for monitoring OP compounds.[7−10] The sensors relied on the immobilization of purified OPH or recombinant *Escherichia coli* cells, expressing OPH, onto a pH glass electrode or a fiber-optic transducer, and then monitoring the protons or chromophoric product, respectively, released during the biocatalytic hydrolysis of the OP substrate. These OPH-based biosensors offer a simple, rapid, and selective monitoring means for OP compounds, and they are ideal for on-line monitoring and control of detoxification processes. Their micromolar ((2−5) ×

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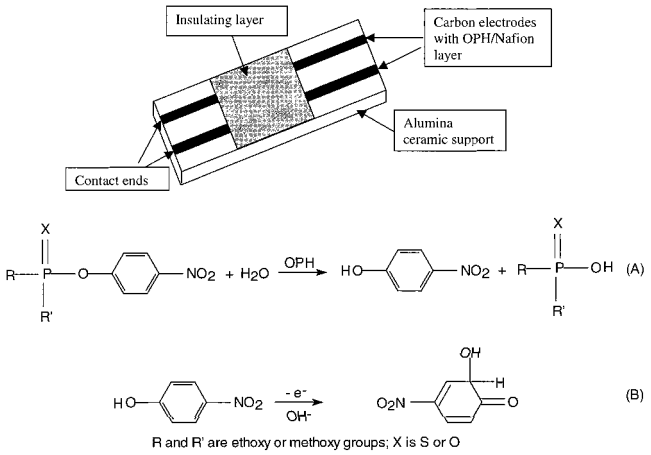

Figure 1. Schematic drawing of the OPH-modified screen-printed electrode and reaction scheme of OPH-catalyzed hydrolysis of organophosphate nerve agent (A) followed by electrooxidation of the liberated *p*-nitrophenol (B).

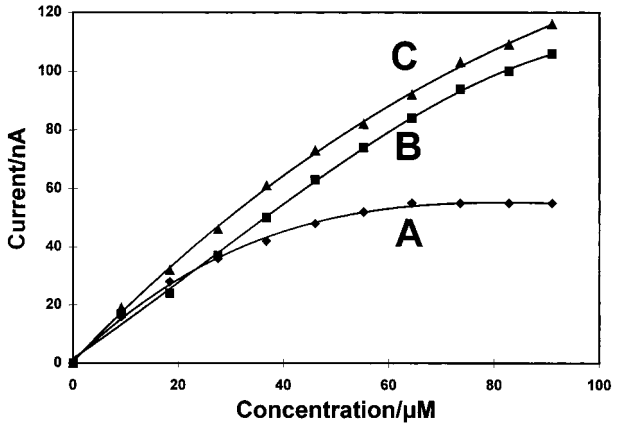

Figure 2. Effect of OPH surface loading upon the response to paraoxon over the 1×10^{-5}–9×10^{-5} M range. OPH loading: 540 (A), 1080(B), and 1620(C) IU. Operating potential, +0.85 V; electrolyte, phosphate buffer (0.05 M, pH 7.4); stirring rate, 600 rpm.

10^{-6} M) detection limits, however, are not sufficient for most practical environmental applications. In the following sections, we will illustrate the suitability of OPH for amperometric biosensing of some OP nerve agents. The new device, which relies on the anodic detection of the *p*-nitrophenol hydrolysis product (Figure 1), greatly lowers the detection limit compared to the potentiometric and fiber-optic counterparts. As desired for rapid and sensitive field testing, such amperometric transduction of the OPH recognition is combined with single-use thick-film strip electrodes, and it offers the instantaneous detection of submicromolar OP concentrations.

EXPERIMENTAL SECTION

Apparatus. Amperometric experiments were performed with the BAS CV-27 voltammetric analyzer (Bioanalytical Systems, W. Lafayette, IN), in conjunction to the BAS *X*−*Y*−*t* recorder. The screen-printed working enzyme electrode, Ag/AgCl (3M NaCl) reference electrode (BAS RE-1), and platinum wire counter electrode were added to the 10-mL cell (model CV-2, BAS) through holes in its Teflon cover. A magnetic stirrer and stirring bar provided the desired convective transport.

Electrode Fabrication. A semiautomatic screen printer (model TF-100, MPM Inc., Franklin, MA) was used for fabricating the strip transducers. The carbon ink (Electrodag 440B, Acheson, Ontario, CA) was printed on a 1.33 in. × 4.00 in. laser scribed alumina ceramic substrate (Coors Ceramics, Golden, CO), through a patterned stencil to yield 10 strips (3.3 × 1.0 cm each) with a 30 × 2 mm printed carbon area. The ink drying was carried out subsequently at 120 °C for 30 min. An insulating layer was then printed to cover most of the printed carbon area, exposing a 5 × 2 mm working electrode area and a similar area (on the opposite side) that served as an electrical contact.

The enzyme, OPH, was immobilized by casting a 20-μL droplet, containing a certain amount of OPH (usually 10 μL of 108 IU/μL) in Nafion (1%, in ethyl alcohol) onto the printed carbon surface, and allowing the solvent to evaporate. The enzyme-modified strip was subsequently dried completely at room temperature and kept in a refrigerator (at 4 °C) until use.

Reagents. OPH (7250 IU/mg of protein, 15 mg of protein/mL; activity measured using paraoxon as substrate) was produced and purified according to the methods described by Mulchandani et al.[7] Paraoxon and methyl parathion were obtained from Sigma Chemical Co. (St. Louis, MO) and Supelco Inc. (Belefonte, PA), respectively, and *p*-nitrophenol was purchased from Aldrich. The Rio Grande river water was collected at Las Cruces, NM.

Procedure. All experiments, unless stated otherwise, were performed by applying a potential of +0.85 V onto the OPH-modified printed strip electrode and allowing the transient background current to decay to a steady-state value, prior to additions of the OP substrate. A stirred solution (300 rpm) was employed during the amperometric experiments, while a quiescent solution was used during the chronoamperometric testing. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Pure OPH, isolated and purified from recombinant *E. coli* cells,[7] was used throughout this study, in a one-step immobilization process, involving the casting of a mixed Nafion/OPH solution onto a screen-printed carbon electrode. Exposure of the OPH-modified strip to the OP substrate leads to its biocatalytic (hydrolytic) conversion to a readily detectable *p*-nitrophenol product (Figure 1). The anodic signal, arising from the oxidation of the liberated *p*-nitrophenol, is proportional to the level of the hydrolyzed substrate. The optimization and characterization of the new OPH biosensor are described below in conjunction with the model OP agents paraoxon and methyl parathion.

OPH Immobilization. A rapid and effective immobilization of OPH onto the thick-film transducer can be achieved by casting a mixed OPH/Nafion solution onto its surface. As expected, the surface loading of OPH has a profound effect upon the sensor's performance (Figure 2). The strip coated with 540 IU of OPH displays linearity up to 2×10^{-5} M paraoxon, with a leveling off above 4×10^{-5} M (Figure 2A). This profile reflects a biocatalytic-limited response associated with the thin enzyme layer (calculated $K_m = 40.2$ μM). Different trends are observed for the 1080- and 1620-IU OPH loadings (Figure 2B and C, respectively). These loadings display linearity up to 4×10^{-5} M, with a slight curvature at higher levels. Such profiles offer convenient quantitation over the entire concentration range and reflect the mass transport limitation imparted by the increased enzyme activity, thicker

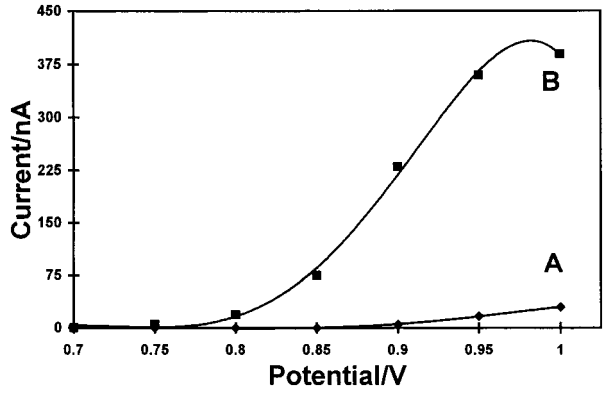

Figure 3. Hydrodynamic voltammograms for 2.3×10^{-5} M paraoxon at the Nafion-coated carbon strip (A) and the OPH-based amperometric biosensor (B). OPH surface loading, 1080 IU. Other conditions, as in Figure 2.

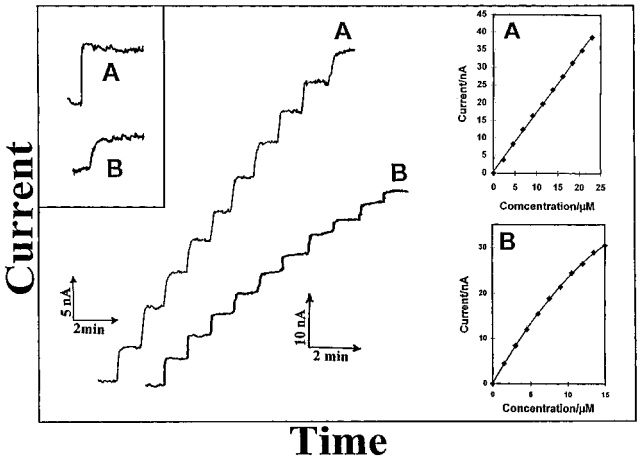

Figure 4. Current–time amperometric response to successive 2.3×10^{-6} M increments in the level of paraoxon (A) and 1.5×10^{-6} M increments in the level of methyl parathion (B). Also shown (insets) are the resulting calibration plots and the response for 4×10^{-7} M paraoxon (A) and 1.5×10^{-7} M methyl parathion (B). Other conditions, as in Figure 2B.

enzyme layers, and/or Nafion barrier properties. Overall, the sensitivity of the new OPH biosensor corresponds to a tradeoff between higher biocatalytic activity and mass transport limitations. Thus, for further studies we selected a surface loading of 1080 IU of OPH.

Optimal Operating Potential. Figure 3 shows a hydrodynamic voltammogram (HDV) for 2.3×10^{-5} M paraoxon over the 0.7–1.0-V potential range at Nafion-coated (A) and OPH-modified (B) carbon strips. The oxidation of the enzymatically liberated *p*-nitrophenol starts at +0.75 V, with the response rising sharply up to +0.95 V, and then levels off thereafter. This HDV profile mirrored the HDV profile for *p*-nitrophenol (data not shown). All subsequent work was performed at +0.85 V due to the small signals observed for the direct oxidation of paraoxon at the "enzyme-free" strip using potential higher than +0.9 V.

Performance Characteristics. Figure 4 displays current–time amperometric recordings obtained with the thick-film OPH strip for paraoxon (A) and methyl parathion (B). Well-defined current signals are observed for these micromolar increments in the OP substrate concentration. No response was observed for analogous measurements at the bare (enzyme-free) electrode (data not shown). The resulting calibration plots (also shown in Figure 4) display high linearity for paraoxon (slope, 1.67 nA/μM; correlation coefficient, 0.999), and a curvature above 5×10^{-6} M in the case of methyl parathion (slope of initial linear portion, 2.83 nA/μM). Another experiment involving 10 additions of 1×10^{-5} M paraoxon yielded linearity up to 4×10^{-5} M, with a slight curvature thereafter. The data of Figure 4 also demonstrate that the OPH/carbon-strip electrode offers attractive dynamic properties, with ~10 s required to attain steady-state currents. Such a response is significantly faster than that of current inhibition (AChE)-based amperometric OP biosensors[2–4] which require addition of the substrate and an incubation period.

Extremely low detection limits of 7×10^{-8} M methyl parathion and 9×10^{-8} M paraoxon can be estimated from the signal-to-noise characteristics (S/N = 3) of the response to 1.5×10^{-7} M methyl parathion and 4×10^{-7} M paraoxon (Figure 4, inset). Such values are significantly lower than the micromolar ($(2-5) \times 10^{-6}$ M) values reported for OPH-based potentiometric and fiber-optic biosensors.[7–10] The lower (nM) detection limits of AChE inhibition biosensors are attained following prolonged incubation period of 10–30 min.[2,3] Further improvements in the sensitivity and detectability of the amperometric OPH bioelectrode may be achieved through an electrocatalytic (accelerated) detection of the *p*-nitrophenol product. Alternatively, the device may be combined with common sample preparation/extraction (enrichment) protocols.

The response of OPH/carbon-strip biosensor is highly reproducible; a relative standard deviation of 1.9% characterized 12 repetitive chronoamperometric measurements of 4.6×10^{-6} M paraoxon (not shown). This precision implies no problem with electrode poisoning that can be attributed to the oxidation of the *p*-nitrophenol product. Hence, in addition to single-use applications, the new warning device can serve as a reusable biosensor. This is not the case with inhibition OP biosensors that suffer from irreversible response.

The specificity of the OPH recognition, coupled with the permselectivity and protection action of the host Nafion polymer, can facilitate assays of relevant environmental samples. Figure 5 displays the chronoamperometric response for a river water sample containing increasing levels of paraoxon in 2×10^{-6} M steps (a–j). Despite the use of an untreated sample (and no deliberately added electrolyte), the sensor responds favorably to these micromolar concentration changes. Such well-defined signals yield a linear calibration plot (see inset), with a sensitivity of 1.45 nA/μM and correlation coefficient of 0.998. This sensitivity is quite similar to that observed in Figure 4A for synthetic sample. The response for the unspiked sample (dotted line) indicates the absence of coexisting interfering electroactive species. Since the signal transduction is based on the electrooxidation of *p*-nitrophenol product, interference from phenolic and other electroactive pollutants can be expected. Such contribution of oxidizable constituents can be addressed by measuring (and subtracting) the response of an "OPH-free" carbon strip (coprinted on the same chip). Alternatively, the coimmobilization of *p*-nitrophenol monooxygenase may also be used to alleviate potential interferences relative to the conversion of *p*-nitrophenol product to hydroquinone (that can be detected at very low potentials).[11] Signifi-

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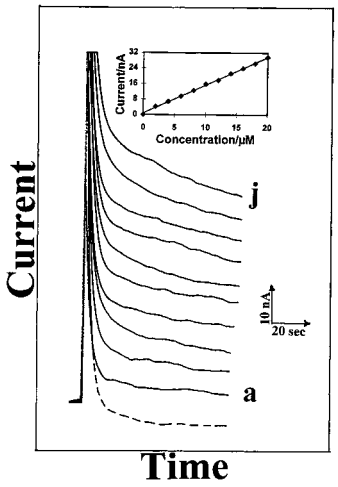

Figure 5. Assay of a river water sample. Chronoamperometric response to 2×10^{-6} M increments in the level of paraoxon (a−j), as well as for the unspiked sample (dotted line). Potential step to +0.85 V using a quiescent solution. Other conditions, as in Figure 2B. The resulting standard additions plot is shown as an inset. Currents were sampled at 60 s following the potential step.

cantly lower degree of selectivity is common with inhibition-based OP enzyme electrodes that are affected by the presence of numerous coexisting neurotoxins such as carbamate pesticides and heavy metals.

CONCLUSIONS

The suitability of OPH for amperometric biosensing of OP nerve agents has been illustrated for the first time. The enzyme has been immobilized onto a disposable screen-printed carbon transducer that offers favorable anodic detection of the liberated *p*-nitrophenol product. While the amperometric concept has been illustrated in connection with the biosensing of paraoxon and methyl parathion, it is also valid for other OP pesticides, such as parathion, fenitrothion, and ethyl *p*-nitrophenyl thiobenzenephosphonate, that upon hydrolysis produce *p*-nitrophenol and could be readily extended to the rapid detection of OP pesticides and OP chemical warfare agents. When integrated with any chromatographic separation (normal laboratory HPLC or microfabricated laboratory-on-chip device), the OPH-modified SPE could be used to determine individual OP nerve agent concentrations. As desired for field operations, the coupling of OPH with SPE leads to a fast, sensitive, and low-cost detection of toxic OP compounds. Combining these single-use devices with hand-held (battery-operated) instruments should further facilitate the field screening of OP pesticides and provide the necessary warning/alarm in the case of military or terrorist attacks. The advances in enzyme engineering have provided a means for engineering OPH variants with a high specificity for a given OP compound.[12] Using these variants, it will be possible to develop a microarray biosensor capable of finger-printing the OP profile of an analyte sample. Ongoing efforts in our laboratories are aimed at developing such pocket-size OP meters, as well as fast-responding OPH-based remote in-site sensors and gas (OP vapor) microdetectors, aimed at addressing the needs of various environmental and defense scenarios.

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