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V-Type Nerve Agent Detection Using a Carbon Nanotube-Based Amperometric Enzyme Electrode

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An enzyme electrode for the detection of V-type nerve agents, VX (*O*-ethyl-*S*-2-diisopropylaminoethyl methylphosphonothioate) and R-VX (*O*-isobutyl-*S*-2-diethylaminoethyl methylphosphonothioate), is proposed. The principle of the new biosensor is based on the enzyme-catalyzed hydrolysis of the nerve agents and amperometric detection of the thiol-containing hydrolysis products at carbon nanotube-modified screen-printed electrodes. Demeton-S was used as a nerve agent mimic. 2-(Diethylamino)ethanethiol (DEAET) and 2-(dimethylamino)ethanethiol (DMAET), the thiol-containing hydrolysis product and hydrolysis product mimic of R-VX and VX, respectively, were monitored by exploiting the electrocatalytic activity of carbon nanotubes (CNT). As low as 2 μM DMAET and 0.8 μM DEAET were detected selectively at a low applied potential of 0.5 V vs Ag/AgCl at a CNT-modified mediator-free amperometric electrode. Further, the large surface area and the hydrophobicity of CNT was used to immobilize organophosphorus hydrolase mutant with improved catalytic activity for the hydrolysis of the P−S bond of phosphothiolester neurotoxins including VX and R-VX nerve gases to develop a novel, mediator-free, membrane-free biosensor for V-type nerve agents. The applicability of the biosensor was demonstrated for direct, rapid, and selective detection of V-type nerve agents' mimic demeton-S. The selectivity of the sensor against interferences and application to spiked lake water samples was demonstrated.

Chemical warfare agents have long been considered a "poor man's atomic bomb". The limited capability of antiterrorist groups to detect such weapons, the low cost and low technology required to develop chemical weapons, their extremely frightening image, and the overall efficiency of such weapons make them weapons of choice for terrorists. Sarin, soman, *O*-ethyl-*S*-(2-diisopropylaminoethyl) methylphosphonothioate (VX) and *O*-isobutyl-*S*-2-diethylaminoethyl methylphosphonothioate (R-VX), belonging to the organophosphorus (OP) group, are highly toxic nerve agents. Among these lethal chemicals, the V-type nerve agents are more persistent and one of the most toxic man-made compounds.[1,2] After the September 11, 2001 attacks in the United States, there is increased threat of the use of chemical warfare agents for terrorist purposes considering that the nerve gases sarin and VX have been used previously by terrorists in Japan.[3] Additionally, the International Chemical Weapons Convention requires the destruction of these chemical warfare agents by the extended deadline of 2012.[4] Analytical tools are therefore needed urgently for detection of these lethal chemicals.

Current analytical techniques such as gas and liquid chromatography, are very sensitive and reliable, but cannot be carried out in the field, are time-consuming and expensive, and have to be performed by highly trained technicians.[5,6] Biological methods, such as immunoassays and inhibition of acetylcholinesterase activity, for OP determination have also been reported.[5] Despite the promise of immunoassay techniques, since these methods require long analysis time (1−2 h) and extensive sample handling (several washing steps), they are unsuitable for online monitoring of detoxification processes. Similarly, analytical devices based on acetylcholinesterase (AChE) inhibition, although sensitive, have limitations of poor selectivity, tedious and time-consuming protocol, and nonsuitability for real-time monitoring.

The V-type nerve agents VX and the Russian R-VX upon hydrolysis produce thiol moieties such as 2-(diisopropylamino)-ethanethiol (DIPAET), and 2-(diethylamino)ethanethiol (DEAET), (Chart 1).[1,7,14] The hydrolysis can be achieved either chemically or enzymatically. Organophosphorus hydrolase (OPH), an orga-

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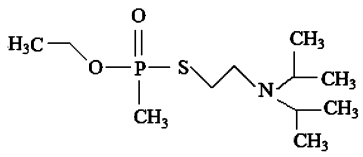
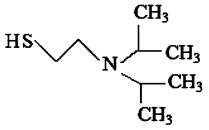
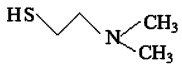
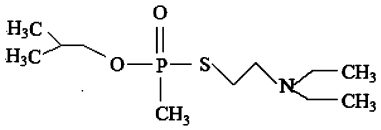
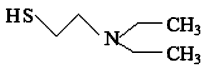
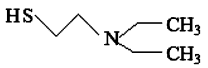
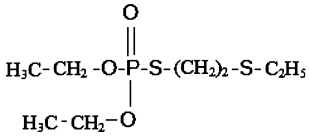
Chart 1. Structure of the V-Type Nerve Agents, Their Thiol-Containing Hydrolysis Products, and Hydrolysis Product Mimics and Demeton-S[13]

CWAs	Actual hydrolysis product	Hydrolysis product mimic examined
VX 	DIPAET 	DMAET 
R-VX 	DEAET 	DEAET 

The mimic of the V-type CWAs

Demeton-S 

nophosphotriester-hydrolyzing enzyme (discovered in soil microorganisms), has been shown to effectively hydrolyze a number of OP compounds, such as parathion, paraoxon, and demeton-S as well as chemical warfare agents such as sarin, soman, and VX at mild conditions.[8−10] However, the hydrolysis rates by OPH vary widely with hydrolysis at diffusion-controlled rates for paraoxon ($k_{cat} > 3800\ s^{-1}$ and $k_{cat}/K_M = 5.5 \times 10^7\ M^{-1}\ s^{-1}$) to several orders of magnitude lower for phosphothiolesters such as demeton-S ($k_{cat} = 1.25\ s^{-1}$ and $k_{cat}/K_M = 1600\ M^{-1}\ s^{-1}$), VX ($k_{cat} = 0.3\ s^{-1}$ and $k_{cat}/K_M = 750\ M^{-1}\ s^{-1}$), and malathion ($k_{cat} = 0.0001\ s^{-1}$ and $k_{cat}/K_M = 0.001\ M^{-1}\ s^{-1}$).[11,12] Utilizing the knowledge of the OPH crystal structure, an OPH mutant (with the histidine 254 substituted by arginine and histidine 257 substituted by leucine) with a 4- and 20-fold, respectively, improved activity for VX and demeton-S, a commonly employed mimic of V-type nerve gases, was recently reported.[13]

Thiol-containing compounds are known to undergo electrochemical oxidation at a very high potential at conventional electrodes, which results in nonspecificity. This necessitates the use of different strategies for electrochemcial detection such as the use of electron-transfer mediators, substrate oxidation, pulsed electrochemical detection, use of mercury electrodes, and derivatization.[15] Carbon nanotubes (CNT) are an important class of material due to their unique electronic, metallic, and structural characteristics.[16] The tremendous importance of CNT for sensor applications has led to wide research activities in this area.[17,18] It has been demonstrated that CNT have a stable electrochemical behavior and catalyze the electrochemical reactions of dopamine, epinephrine, and ascorbic acid,[19] NADH,[20] phenolic compounds,[21] hydrogen peroxide,[22] and thiol compounds such as cysteine, gluathione, and thiolcholine.[23,24] Additionally, the high surface area possessed by CNT and the acidic sites created after purification with oxidizing acids have been used for the immobilization of biological molecules[25−27] and biosensor applications.[28−30]

In this note, we report a microfabricated biosensor device based on the combination of OPH hydrolytic activity for phosphothiolester nerve agents and CNT electroactivity toward thiols for direct, sensitive, selective, and rapid detection of V-type nerve agents. Demeton-S was used as a V-type nerve agent mimic. The use of OPH is extremely attractive for biosensing of OP compounds that act as substrates for the enzyme as opposed to inhibitors in the case of AChE-based biosensors.[32] Because OPs

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act as substrates for OPH, biosensors based on OPH offer a simple, rapid, and selective monitoring of OP compounds and, being self-renewable, are ideal for online monitoring and control of detoxification processes.[33] The use of CNT facilitated the detection of hydrolysis products of V-type nerve agents (Since the hydrolysis product of VX, DIPAET, is not commercially available, 2-(dimethylamino)ethanethiol (DMAET) was used as a simulant.[14]) at a lower applied potential as compared to the previously reported amperometric detection of these compounds that required derivatization and a higher operating potential.[31] To our knowledge, this is the first illustration of the use of CNT for the amperometric detection of the thiol-containing degradation products of the V-type nerve agents and the detection of the V-type nerve gases' mimic by amperometric enzyme electrode.

EXPERIMENTAL SECTION

Reagents. Neat demeton-S and malathion were purchased from Supelco (Bellefonte, PA). *N*,*N*-Dimethylformamide (DMF) was purchased from Acros Organics. All other chemicals and reagents were of analytical grade and were prepared with double-distilled, deionized water. Water sample from Lake Elsinore, CA, was provided by Prof. Michael Anderson (University of California, Riverside). Acid-purified multiwall CNTs (MWCNTs) and carbon screen-printed electrodes (SPEs) (2 mm × 4 mm working area) were prepared according to the methods described elsewhere.[34−36]

Apparatus. Amperometric measurements were performed using a voltammetric analyzer (Bioanalytical Systems, model LC-4C) coupled to a chart recorder (model BD 112, Kipp and Zonen). All experiments were conducted in a three-electrode electrochemical cell with a working volume of 2 mL (50 mM phosphate buffer, pH 7.4 containing 0.1 M KCl) with the SPE modified with CNT as the working electrode, Ag/AgCl reference electrode (BAS, MF 2063), and platinum wire auxiliary electrode. For amperometric measurement, the working electrode was operated at desired potential and the transient currents were allowed to decay to a steady-state value; a magnetic stirrer and a stirring bar provided the convective transport.

Procedure. For the modification of the electrode with CNT, 2 mg of acid-purified MWCNTs were suspended in 1 mL of DMF with the aid of ultrasonic agitation to obtain a black suspension. A film of MWCNT was cast on the surface of a SPE by dropping 15 μL of this solution on the electrode surface. The electrode was then kept in an oven at 80 °C for 30 min under vacuum to evaporate the solvent.

To immobilize the enzyme on the electrode surface, 10 μL of purified mutant OPH solution was dropped on the CNT-modified electrode surface and allowed to dry at room temperature under a current of air. The electrode was then rinsed twice with phosphate buffer (pH 7.4) to remove the loosely attached enzyme molecules and MWCNT.

The mutant OPH was generated by site-directed mutagenesis. In short, site-directed mutagenesis was performed using the manufacturer's protocol (QuickChange Site-Directed Mutagenesis Kit, Stratagene) directly in the expression vector pJK33 template DNA. The basic procedure utilizes a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. Twenty nanograms of template DNA was hybridized with two mutagenic primers (125 ng), each complementary to opposite strands of vector. Primers were extended during temperature cycling (denaturation 30 s at 95 °C, followed by 18 cycles of 30 s at 95 °C, 60 s at 55 °C, 3 min 51 s at 68 °C, followed by final extension 10 min at 68 °C) by 2.5 units of *PfuUltra* DNA polymerase (Stratagene). Following temperature cycling, the polymerase chain reaction (PCR) circular nicked DNA product was treated with *Dpn*I restriction endonuclease, which is specifically targeted to methylated DNA and thus removes from PCR product parental nonmutated DNA template and selects mutation possessing synthesized DNA. The nicked vector DNA containing desired mutations was transformed into *Escherichia coli* XL-1 Blue competent cells. Protein purification was carried out according to the protocol described by Mulchandani et al.[37] and stored frozen at −80 °C until use. The kinetic parameters, k_{cat} and K_M of the purified mutant OPH were determined, using demeton-S as substrate at pH 7.4, and found to be comparable to the values reported by Di Sioudi et al.[13]

Safety Considerations. The stock solutions of thiol compounds, demeton-S, and other OP compounds should be handled in the fume hood. Inhalation or contact should be avoided by the use of appropriate safety wear.

RESULTS AND DISCUSSION

The V-type chemical warfare agents and their insecticide analogues such as demeton-S and malathion, which contain a P−S bond, are poorly degraded by native/wild-type OPH, the enzyme most suitable for their hydrolysis.[13] The approach widely used for the detection of these compounds hence has utilized the inhibition of the enzyme AChE.[38] Because of the inherently time-consuming and tedious protocol combined with the nonrenewable nature of the sensor, it is unsuitable for rapid, continuous/real-time monitoring. Thus, this important class of compounds poses an analytical challenge. The availability of the OPH variant with improved catalytic activity for hydrolysis of OPs with P−S bonds has provided an opportunity for developing a simple, selective, rapid, and direct sensor for these analytically challenging compounds.

As a first step toward realization of the biosensor for direct determination of V-type nerve agents, the amperometric detection of hydrolysis product DEAET and mimic DMAET was investigated at a MWCNT-modified screen-printed electrode. Hydrodynamic voltammetric (HDV) studies were performed to determine the optimum potential for the operation of the sensor and the benefit of MWCNT modification over a bare screen-printed electrode. Figure 1A shows the hydrodynamic voltammograms of 250 μM DEAET at a bare carbon screen-printed electrode (trace 1) and at an electrode modified with MWCNT (trace 2). At the MWCNT-

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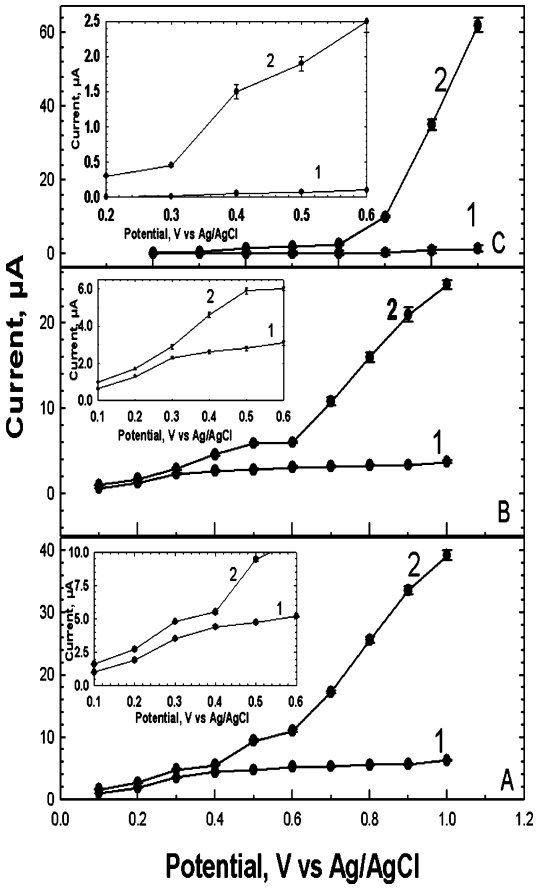

Figure 1. Hydrodynamic voltammogram for 250 μM DEAET (A) and DMEAT (B) and ex situ generated OPH mutant catalyzed hydrolysis products of demeton-S (C) at unmodified screen-printed electrode (trace 1) and MWCNT-modified screen-printed electrode (trace 2). Measurement conditions: 50 mM phosphate buffer containing 0.1 M KCl, pH 7.4. Insets: Enlarged portion of the hydrodynamic voltammogram between 0.1 and 0.6 V vs Ag/AgCl. Each point represents an average of three measurements, and the error bars represent ±1 SD.

modified electrode, there was a small increase in current between 0.1 and 0.4 V, followed by a short plateau between 0.4 and 0.6 V, and then a marked increase when the potential was increased further. In comparison, at the unmodified electrode a substantially lower response was obtained throughout the potential range studied. The higher response can be attributed to the significant catalytic current obtained at the MWCNT-modified electrode due to the electrocatalytic activity of MWCNT toward DEAET. HDV studies with DMAET showed that MWCNT exhibited similar electrocatalytic activity (Figure 1B). This can facilitate facile amperometric detection of these compounds at a MWCNT-modified electrode with a decrease in overpotential. Operation of a sensor at a low potential is analytically more desirable, and it reduces the possible electrochemical interferences. A modest potential of 0.5 V versus Ag/AgCl, at which the amperometric current at the MWCNT-modified electrode was still almost 2-fold higher than at the unmodified electrode, was chosen to demonstrate the applicability of the sensor for the sensitive detection of these chemicals.

Figure 2 displays the current−time traces for DEAET (A) and DMAET (B) at the MWCNT-modified electrode. Well-defined current signals that reached steady-state current in less than 3 min are obtained with micromolar increments in the concentration of the thiols. The calibration plots (Figure 2, inset 2) for the amperometric detection of both the thiols exhibited a linear dependence on concentration over a wide dynamic range for DEAET and DMAET, respectively, with corresponding sensitivity of 59.7 (correlation coefficient 0.9988) and 52.8 μA/mM (correlation coefficient 0.9964). Excellent lower detection limits of 0.8 μM DEAET and 2 μM DMAET can be estimated from the signal-to-noise characteristics (S/N = 3) of the response to 0.8 μM DEAET and 2 μM DMAET (Figure 2, inset 1). The sensor-to-sensor reproducibility was very good (less than 4% RSD, n = 5). The limit of detection of the electrode developed in this work was superior to the 8 μM for DEAET and 5 μM for DMEAT at a screen-printed thick-film carbon electrode reported at 800 mV versus Ag/AgCl.[31] Additionally, the detection protocol is simpler as compared to the electrochemical detection or detection using laser-induced fluorescence achieved after a tedious and time-consuming derivatization step to convert thiols to isoindole derivatives by reacting with amino acid and *o*-phthaldialdehyde.[14,31]

The excellent analytical characteristics of the CNT-modified amperometric electrode to the DEAET and DMAET provide an ideal platform for the construction of an enzyme electrode based on mutant OPH for direct and rapid detection of V-type nerve gases. To demonstrate this application, the thiol sensor was modified with a variant of OPH and applied for the detection of the V-type nerve gas mimic demeton-S. A mutant OPH showing 20-fold better activity than the wild-type OPH was generated by site-directed mutagenesis described in the Experimental Section. Hydrodynamic voltammetric studies with thiols generated *ex situ* from hydrolysis of demeton-S showed that, similar to DMAET and DEAET, the amperometric current response for the thiol of enzyme-catalyzed hydrolysis of demeton-S at the CNT-modified electrode was 30-fold higher than at an unmodified electrode even at an applied potential of as low as 0.4 V versus Ag/AgCl (Figure 1C). The significantly higher amplification of the response of the CNT-modified SPE over the unmodified SPE to the thiol released from the enzyme-catalyzed demeton-S hydrolysis compared to that observed for DMAET and DEAET is attributed to the differences in the nature of these thiols. Similar differences in the electrochemical[15,23,31,46] or fluorescence signal[14] signal for different thiols have been reported in the literature. Operating at this new lower potential, the enzyme electrode prepared by immobilizing the OPH variant by simple adsorption on CNT exhibited well-defined current signals that reached steady state in less than 3 min (Figure 3, trace A). The CNT−enzyme electrode had a wide dynamic linear range up to 85 μM with a sensitivity of 8 μA/mM (correlation coefficient 0.9925) and a limit of detection (S/N = 3) of 1 μM, which corresponds to 258 ppb (μg/L) (Figure 3 inset).

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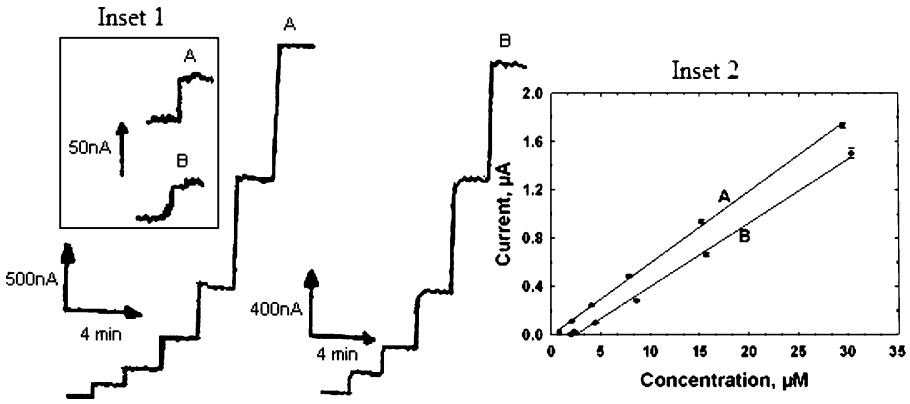

Figure 2. Current−time traces of the screen-printed electrode modified with acid-purified MWCNT to 2, 4, 8, 14, 28, and 56 μM DEAET (A) and 4, 8, 14, 28, and 56 μM DMAET (B). Also shown: (inset 1) the response for 0.8 μM DEAET (A) and 2 μM DMAET(B) and (inset 2) the resulting calibration curves for DEAET (A) and DMAET (B). Measurement conditions: applied potential 0.5 V vs Ag/AgCl, pH 7.4 phosphate buffer containing 0.1 M KCl. Each point represents an average of three measurements, and the error bars represent ±1 SD.

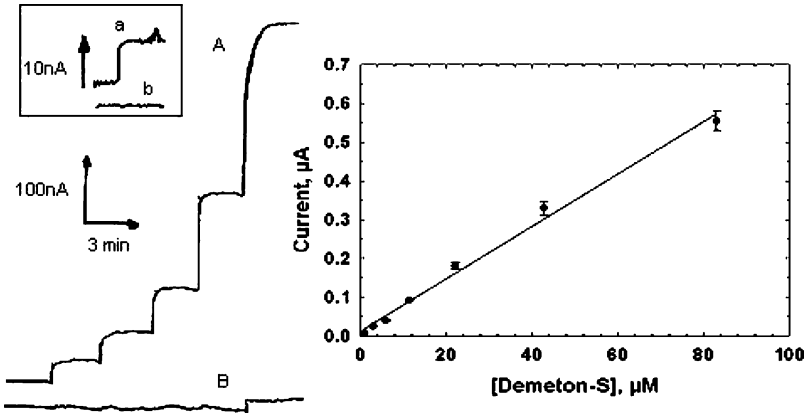

Figure 3. Current−time traces of screen-printed electrodes modified with OPH mutant (B) and with acid-purified MWCNT/OPH mutant (A) to 5, 10, 20, 40, and 80 μM demeton-S. Inset shows the response of the sensor at 1 μM demeton-S with (a) and without (b) CNT. Also shown is the calibration curve for demeton-S. Measurement conditions: applied potential 0.4 V vs Ag/AgCl, pH 7.4 phosphate buffer containing 0.1 M KCl. Each point represents an average of three measurements, and the error bars represent ±1 SD.

Control experiments performed at a bare screen-printed electrode, i.e., without the use of CNT (Figure 3, trace B), resulted in no well-defined signal near the detection limit and up to a concentration of 48 μM demeton-S. These experiments demonstrate the crucial role played by CNT in improving the detection limit even at the reduced overpotenital used for the amperometric detection. The electrode was used repeatedly for multiple analyses over the course of the investigation. The simple one-step measurement, short response time, and repeated use are the inherent benefits of the present electrode over the inhibition-based electrodes. The electrodes also showed good storage stability, retaining 96% of the original response up to 7 days when stored at 4 °C, and there was a very good electrode-to-electrode reproducibility (6% RSD, $n = 5$). The good electrode-to-electrode reproducibility and stability demonstrated the reproducibility of simple adsorption protocol for immobilization of CNT on SPE and OPH on CNT, a strong binding between them, and stable enzyme activity. These are in agreement with the literature reports.[19,23,39−42]

Several commonly used pesticides, sevin and sutan, belonging to the carbamates family, and paraoxon, methyl parathion, diazinon, and malathion, belonging to the OPs family, were evaluated for their interference effect. As shown in Figure 4, minimum interference was observed from these compounds even at very high concentration. The high discriminating ability of the new

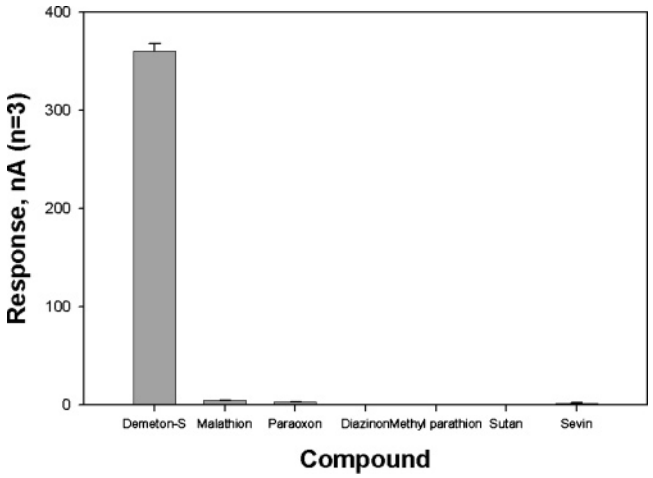

Figure 4. Selectivity of a screen-printed electrode modified with acid-purified MWCNT and OPH mutant. Current response to 50 μM target at applied potential 0.4 V vs Ag/AgCl, pH 7.4 phosphate buffer containing 0.1 M KCl. Each point represents an average of three measurements, and the error bars represent ±1 SD.

enzyme electrode against the interferences can be explained as follows. Sutan and sevin are not recognized by OPH and hence not hydrolyzed. Paraoxon, methyl parathion, and diazinon have a

Table 1. Measurement of Concentration (μM) of Demeton-S in Lake Water

in the spiked lake water	measured by the biosensor
2	1.9 ± 0.1 ($n = 3$)
20	19 ± 1.2 ($n = 3$)

P−O bond and hence no thiol is produced on the OPH-catalyzed hydrolysis of these nerve agents. The insignificant interference from malathion can be attributed to a very low activity (k_{cat} = 0.0001 s^{-1}) and selectivity (k_{cat}/K_M = 0.001 M^{-1} s^{-1}) of OPH for this phosphothiolester. The excellent selectivity of the new enzyme electrode is a significant benefit over AChE-based biosensors, which are unable to differentiate between organophosphates and other neurotoxic compounds[43] and the potentiometric OPH-based biosensors that are unable to differentiate between subclasses of OPs.[32] This will reduce "false positives" in the field application of the sensor.

The feasibility of application of the biosensor for determination of OPs in real samples was tested. Water from Lake Elsinore, CA, was filtered with a 0.22-μm filter and its pH adjusted to 7.4 from 9.2 using concentrated HCl (pH was adjusted to prevent demeton-S autohydrolysis). The water was then spiked with demeton-S and analyzed using the biosensor. As shown in Table 1, the results obtained (concentrations of demeton-S in the analyzed samples were determined using the calibration curve in Figure 3) were in good agreement with the amount spiked, indicating the selectivity of the biosensor against coexisting interfering electroactive compounds and demonstrating the validity of the newly developed biosensor for environmental matrixes. Since the signal is based on the electrooxidation of thiol products, interference from thiols and other electroactive compounds beyond the ones in the Lake Elsinore water tested in this work can be expected. Such contribution of electrooxidizable species can be accounted by measuring and subtracting the response of an enzyme-free CNT-modified screen printed electrode incorporated on the same chip.

CONCLUSIONS

A simple, direct, rapid, and selective detection of V-type nerve agent hydrolysis products and the nerve agent mimic, demeton-S, was demonstrated. As desired for field operations, the coupling of OPH with CNT-modified SPE leads to a fast, sensitive, and low-cost detection of toxic OP compounds. While the biosensor sensitivity was satisfactory for monitoring and control of the detoxification process, a higher sensitivity will be desirable for homeland security application. An ~3-fold improvement in the sensitivity at the cost of selectivity can be achieved by using a higher overpotential/working potential (~800 mV) for amperometric detection. A further enhancement in the sensitivity can be achieved by employing a OPH mutant/variant with lower K_M, higher bimolecular rate constant, or both. The advancements in enzyme engineering and *in vitro*-directed evolution techniques have made these goals possible. While the biosensor developed in the present work has been demonstrated for the detection of a V-type nerve agent mimic, demeton-S, it can also be applied for the detection of the other important phosphothioester nerve agents, such as malathion, acephate, ethyl azinophosm and phosalone, that are widely used agricultural and industrial insecticides and hence of environmental concern. A sensitive sensor for these compounds will however require OPH mutant-(s) with good hydrolytic activity toward these nerve agents generated using either site-directed or directed evolution mutagenesis techniques.[44,45] Combining these single-use devices with hand-held (battery-operated) instruments should further facilitate the field screening of OP nerve agents and provide the necessary warning/alarm in the case of military or terrorist attacks. An additional application of the carbon nanotube-modified thiol sensor will be in a simplified detection of the native thiols or generated by on-chip enzyme-catalyzed hydrolysis postseparation by capillary electrophoresis on a microchip without derivatization for detection and identification/fingerprinting of the phosphothiolester analytes in the sample.

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