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Biomonitoring of Organophosphorus Agent Exposure by Reactivation of Cholinesterase Enzyme Based on Carbon Nanotube-Enhanced Flow-Injection Amperometric Detection

Dan Du,[†,‡] Jun Wang,[‡] Jordan N. Smith,[‡] Charles Timchalk,[‡] and Yuehe Lin*[,‡]

Key Laboratory of Pesticide and Chemical Biology of Ministry of Education, College of Chemistry, Central China Normal University, Wuhan 430079, P. R. China, and Pacific Northwest National Laboratory, Richland, Washington 99352

A portable, rapid, and sensitive assessment of subclinical organophosphorus (OP) agent exposure based on reactivation of cholinesterase (ChE) from OP-inhibited ChE using rat saliva (in vitro) was developed using an electrochemical sensor coupled with a microflow-injection system. The sensor was based on a carbon nanotube (CNT)-modified screen printed carbon electrode (SPE), which was integrated into a flow cell. Because of the extent of interindividual ChE activity variability, ChE biomonitoring often requires an initial baseline determination (noninhibited) of enzyme activity which is then directly compared with activity after OP exposure. This manuscript describes an alternative strategy where reactivation of the phosphorylated enzyme was exploited to enable measurement of both inhibited and baseline ChE activity (after reactivation by an oxime, i.e., pralidoxime iodide) in the same sample. The use of CNT makes the electrochemical detection of the products from enzymatic reactions more feasible with extremely high sensitivity (5% ChE inhibition) and selectivity. Paraoxon was selected as a model OP compound for in vitro inhibition studies. Some experimental parameters, e.g., inhibition and reactivation time, have been optimized such that 92–95% of ChE reactivation can be achieved over a broad range of ChE inhibition (5–94%) with paraoxon. The extent of enzyme inhibition using this electrochemical sensor correlates well with conventional enzyme activity measurements. On the basis of the double determinations of enzyme activity, this flow-injection device has been successfully used to detect paraoxon inhibition efficiency in saliva samples (95% of ChE activity is due to butyrylcholinesterase), which demonstrated its promise as a sensitive monitor of OP exposure in biological fluids. Since it excludes inter- or intraindividual variation in the normal levels of ChE, this new CNT-based electrochemical sensor thus provides a sensitive and quantitative tool for point-of-care assessment and noninvasive biomonitoring of the exposure to OP pesticides and chemical nerve agents.

Organophosphates (OP) including chemical nerve agents and pesticides represent a diverse group of highly toxic compounds.[1,2]

The acute toxicity resulting from OP exposure stems from the fact that they readily inhibit important cholinergic enzymes, such as acetylcholinesterase (AChE), through an attack on the serine residue of AChE, forming a phosphorylated adduct. When the function of AChE is inhibited, accumulation of acetylcholine will result in overstimulation of corresponding muscarinic and nicotinic cholinergic receptors, which may lead to serious health problems or even death.[3,4] Because of the widespread use of OPs as pesticides across the world and the increased threat to peoples' health resulting from the potential use of chemical nerve agents in terrorist attacks or military activities, there is a need to develop fast, sensitive, and field-deployable screening technology for quick response to exposure to OPs to facilitate triage. Second, a rapid screening technology can also be exploited for real-time biological monitoring of workers that are involved in the manufacture and/ or application of OP insecticides. At present, detection and evaluation of OP exposure is generally performed at dedicated centralized laboratories using large, automated analyzers such as liquid or gas chromatography coupled with mass spectrometry (HPLC/GC/MS),[5–8] requiring sample transportation and processing which ultimately increases the waiting time for results. Rapid near-patient or field biomonitoring tools for the first responders is highly desirable to facilitate rapid screening to initiate appropriate treatments.

Currently, three approaches have been developed for biomonitoring of OP exposure: (1) assay of enzyme activity,[9,10] (2) measurement of metabolites,[11–13] and (3) detection of

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* To whom correspondence should be addressed. E-mail: yuehe.lin@pnl.gov. Phone: 01-509-371-6241.

† Central China Normal University.

‡ Pacific Northwest National Laboratory.

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phosphorylated adducts.[14−16] Although the measurement of metabolites is a sensitive and accurate method for detection and identification of OPs, it is not suitable for rapid detection or field biomonitoring because this approach generally involves a laboratory based analysis, such as GC/LC/MS. The phosphorylated complex (i.e., adducts) may also serve as an indicator of OP exposures; however, a challenge may lie in the current unavailability of recognition elements or appropriate receptors, i.e., antibodies, for specifically targeting the phosphorylated enzyme. Enzyme activity assays have been extensively used for screening of OP exposure in a number of applications including occupational and environmental health and military screening. Cholinesterase (ChE) enzyme activity measurements from blood (and potentially saliva) are a good biomarker to evaluate OP exposure. The assays are relatively simple, as numerous biomonitoring methods have been developed to measure the enzyme activity of cholinesterase including, but not limited to, Ellman assay,[17] fluorescence assay,[18] Michel (ΔpH) ChE assay,[19] radioactive assay,[20] and Walter Reed Army Institute of Research (WRAIR) assay.[21,22] Most recently, enzyme activity kits (Test-Mate assay) for fast screening OP exposure have been developed and are commercially available.[23] However, this kit and the other available screening methods have an inherent disadvantage since a control or baseline is needed because the measured value of enzyme activity has to be compared with the unexposed normal (baseline) values. To circumvent this problem, a statistically derived value of enzyme activity measured from a large sample size of population generally serves as the control. However, these methods are not accurate because of the large variability in baseline values derived from the variation of enzyme activity between individuals (e.g., sex, age, ethnicity, etc.) and the deviation of measurement methods from different laboratories.[24] In general, considering inter- and intraindividual variations in the normal levels of ChE, the exposure that results in inhibition of less than about 20% (especially if clinical symptoms are absent) may not be easily detectable or provide reliable evidence for current available screening methods unless recent control values of that particular individual are available.[24,25] In a worse case situation, these methods may provide ambiguous results. Moreover, biomonitoring of individuals with a low level of OP exposure (<10% inhibition) may be problematic without a pre-exposure baseline enzyme determination with these methods.

It was found that some compounds such as nucleophiles[26−30] or fluoride ions[31−34] can reactivate the phosphorylated complex yielding a restored enzyme activity. This process is called "reactivation". On the basis of the reactivation, a number of assay methods such as measuring released OP from phosphorylated enzyme (fluoride ions-induced reactivation) using MS have been developed for detection and identification of exposure to OPs.[31,32] In parallel, oxime-induced reactivation has been used to evaluate drug efficacy and for therapeutic intervention as a treatment for OP poisoning. The most commonly used pyridinium oxime is pralidoxime iodide (2-PAM).[27] Current biomonitoring methods for OP exposures ignore evaluation of the restored enzyme activity via reactivation. However, if the activity of phosphorylated enzyme could be regenerated completely to its original state and activity determined, this restored enzyme could serve as its own control or baseline. Therefore, a double test of enzyme activity in biological samples before and after reactivation could be exploited to quantify the extent of enzyme activity/inhibition.

In this article, we report a novel approach combining the advantage of enzyme reactivation and signal enhancement of the nanomaterials for fast, inexpensive, noninvasive, baseline-free, and field-deployable biomonitoring of OPs using saliva samples. Rat saliva was utilized as an initial biological matrix for evaluation, since our research group has previously characterized rat saliva ChE activity; it is readily obtainable (i.e., noninvasive) and under well controlled experimental conditions appears to parallel the response of plasma ChE.[35,36] Different from the available optical method, this approach is based on a flow-injection amperometric sensor, of which the carbon nanotube-screen printed electrode is integrated in the flow cell. In this approach, a double-test mode detects the variation of enzyme activity before and after reactivation. This method provides advantages since a control sample is not needed and it excludes inter- or intraindividual variation in the normal levels of ChE. Therefore, this measurement is accurate and reliable. Furthermore, this assay is highly sensitive and selective because the use of carbon nanotubes makes the

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electrochemical detection of the products from enzymatic reactions more feasible at low potentials. In general, this method is inexpensive, sensitive, portable, noninvasive and provides real-time results. It is anticipated that this novel sensor method will open up a new avenue for rapid point-of-care (POC) screening and early assessment of OP exposures providing for early clinical intervention in the case of severe exposures and rapid identification of victims in a terrorist attack.

EXPERIMENTAL SECTION

Reagents. Human acetylcholinesterase (500 U/mg proteins, AChE) from human erythrocytes, acetylthiocholine (ATCh), pralidoxime iodide (2-PAM), phosphate-buffer saline (PBS), acetone, and pilocarpine were purchased from Sigma (St. Louis, MO). Paraoxon was obtained from Chem Service (Chester, PA). All the chemicals were of analytical reagent grade and were used as received. All solutions were prepared with distilled water that was purified with the Nanopure system (Barnstead, Dubuque, IA), and the purified water was used throughout the experiments.

Animals. All animal handling was done in accordance with protocols established in the NIH/NRC Guide and Use of Laboratory Animals and was reviewed by the Institutional Animal and Care Use Committee of Battelle, Pacific Northwest Division. Sprague−Dawley male rats were purchased from Charles River Laboratories (Raleigh, NC) and were 10 weeks old at the time of sacrifice. These animals were acclimated for 1 week to the laboratory conditions. Prior to their use, the animals were housed in solid-bottom cages with hardwood chips under standard laboratory conditions and given free access to water and food (Purina Rodent Chow).

Rats were anesthetized with isoflurane, and once under anesthesia, the jugular vein was cannulated, and a tracheotomy was performed to facilitate respiration and anesthesia during saliva collection. Rats were then infused with 1 mg/mL of pilocarpine in physiological saline via the jugular vein cannula at a rate of 3 mL/h for 2 h. Pilocarpine induced salivation and saliva was collected continuously using glass capillary tubes draining into 2 mL microcentrifuge vials. Saliva samples were stored at −80 °C until the time of analysis.

Generation of Paraoxon−AChE Adducts. The paraoxon stock solution was prepared in acetone and diluted to different concentrations with 20 mM PBS (pH 8.0). Then 50 μL of a series of dilutions of the paraoxon (final concentration was 0.1, 0.5, 1, 5, 10, 25, and 50 nM, respectively) was dispensed into 50 μL of AChE (final concentration was 3 nM) or saliva samples which were 3-fold diluted with 20 mM PBS buffer (pH 8.0) for incubation of 30 min.

Reactivation of AChE and Analysis. The degree of reactivated AChE was measured as an increase of the peak current (i). A volume of 50 μL of the above mixed solution was subsequently reacted with an equal volume of 5 mM 2-PAM for 15 min. After regeneration, 50 μL of ATCh (final concentration was 5 mM) was added into the mixed solution for reaction of 1 min, then 40 μL of the final reaction solution was sequentially injected into the sensing system, and current−time (i−t) curves were recorded. In the control, 50 μL of AChE (final concentration was 3 nM) or the diluted saliva samples were reacted with an equal volume of ATCh for 1 min and then 40 μL of the final reaction solution was injected into sensing
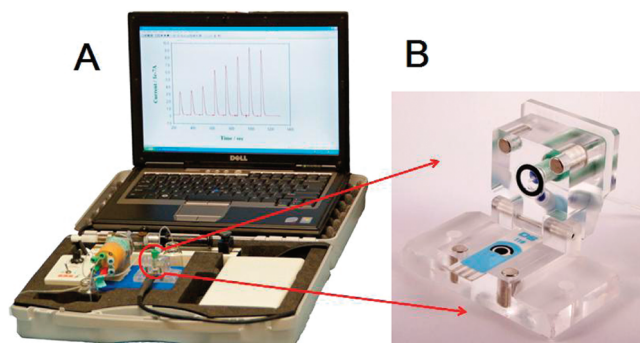
Figure 1. (A) The entire analysis system and (B) the part of the sensing area.

system. The inhibition efficiency calculated by the control (I%), inhibition efficiency calculated by reactivation (I_r%), and reactivation efficiency (R%) were taken as an output parameter and calculated with eqs 1, 2, and 3, respectively.

$$I\% = (i_0-i_t)/i_0 \times 100 \qquad (1)$$

$$I_r\% = (i_r-i_t)/i_r \times 100 \qquad (2)$$

$$R\% = (i_r-i_t)/(i_0-i_t) \times 100 \qquad (3)$$

where i_0 is from the activity of the controlled intact enzyme, i_t is from the activity of the inhibited enzyme, and i_r is from the activity of the reactivated enzyme.

Flow Injection Sensing System. The laboratory-built flow injection system consisted of a carrier, a syringe pump (model 1001, BAS), a sample injection valve (Valco Cheminert VIGI C2XL, Houston, TX), and a flow-through electrochemical cell (Dropsens, Spain). The entire sensing system is held in a small case (Figure 1A). The total carrier volume was 10 mL, and the sample volume was 40 μL. All flow injection analyses were conducted at a flow rate of 200 μL/min. The microelectrochemical cell was constructed by sandwiching the carbon nanotube-screen printed electrode (CNT/SPE) in a plastic cell that was held tightly in place by magnetic forces using a built-in magnet. The cover has two holes for the inlet and outlet, respectively. A plastic ring was mounted between the SPE sensing area and the cover to form a flow cell, in which the SPE was exposed to the cell for the electrochemical detection (Figure 1B).

Electrochemical Detection. All electrochemical experiments were carried out with a portable electrochemical analyzer CHI 1324 (CH Instruments, Inc., Austin, TX) connected to a laptop computer. A disposable CNT/SPE (Dropsens, Spain) was employed as the working electrode, a Ag/AgCl and a carbon ring as the reference and counter electrodes, respectively. A sensor connector was used to connect the CNT/SPE to the electrochemical analyzer. Cyclic voltammetric (CV) measurements were performed with the glassy carbon (GC) electrodes and CNT modified GC (CNT/GC) electrodes, with the potential range of −0.1 to 0.8 V and a 50 mV/s scanning rate. A 0.02 M PBS buffer solution (pH 8.0) was used as the supporting electrolyte for electrochemical experiments. The amperometric measurements were conducted at 0.30 V, and all potentials were referred to the Ag/AgCl reference electrode.

Scheme 1. Schematic Illustration of OP-AChE Formation and the AChE Regeneration Process by the Reactivator
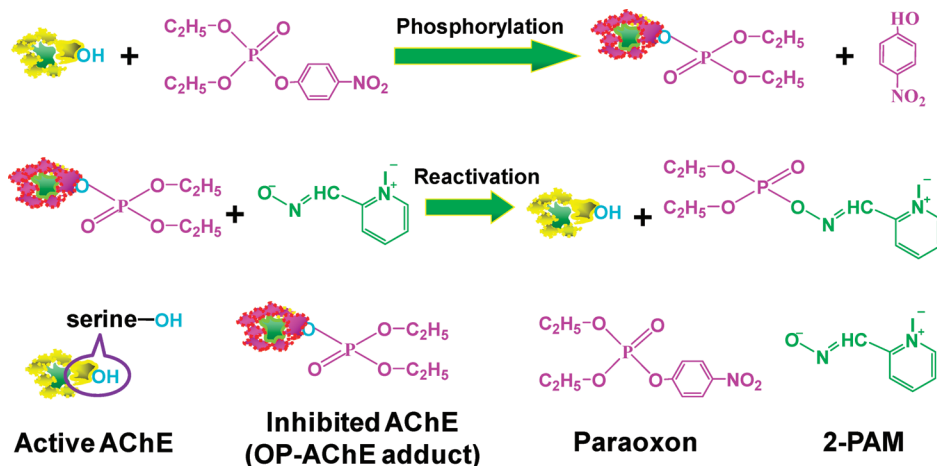
Safety Considerations. Paraoxon is highly toxic and the wastes containing these compounds were collected in a specific container. Appropriate personal protective equipment was utilized to minimize skin and eye contact and the potential for accidental inhalation or ingestion.

RESULTS AND DISCUSSION

Electrochemical Characteristics of AChE Reactivation at the CNT Modified Electrode. The process of OPs binding to the AChE and regeneration by 2-PAM is shown in Scheme 1. AChE can hydrolyze ATCh to produce thiocholine, which is electroactive and detectable at the CNT/SPE by application of a low potential. Therefore, the magnitude of signal from thiocholine at the sensor correlates with enzyme activity. Motivated by this reactivation principle, we developed a CNT-based electrochemical sensor for measurement of regeneration of the inhibited ChE activity, indicating low OP exposure (before showing symptoms) in biological fluids such as saliva.

The cyclic voltammograms of ATCh and enzymatic product thiocholine were investigated on a bare and CNT modified electrodes in a PBS buffer (pH 8), as shown in Figure 2A. No detectable redox peaks of ATCh were observed on the bare carbon electrode (curve a) and CNT modified carbon electrode (curve b). However, when AChE was added into the detection solution, an obvious oxidation peak was observed on both bare (curve c)

and CNT modified electrodes (curve d). No peaks were observed for the AChE solution without ATCh (data not shown). Obviously, this peak is attributed to the oxidation of thiocholine, hydrolysis product of the ATCh substrate which was catalyzed by active AChE. Furthermore, the oxidation peak current on the CNT modified electrode was much higher than that on the bare electrode. The peak of thiocholine on the CNT/SPE (curve d) appeared at 390 mV while 590 mV on the bare electrode (curve c). The peak potential shifted negatively 200 mV. These results have revealed that CNT can greatly enhance the amperometric signal of the enzymatic product and decrease the overpotential of thiocholine oxidation. The decrease of the overpotential is beneficial for avoiding interferences from the electroactive species in biological matrixes.

Cyclic voltammetric measurements were further used to understand the effects of OP exposure and reactivation on AChE activity, as shown in Figure 2B. When 25 nM paraoxon was dispersed in AChE solution for reaction of 30 min before adding ATCh, the peak current greatly decreased (curve c) compared to AChE in the absence of paraoxon (curve a). Paraoxon as a model OP clearly reduced the enzymatic activity of AChE. However, after addition of 5 mM 2-PAM to the inhibited AChE solution for 15 min, the peak current regained nearly completely to control levels (curve b), indicating that the inhibited AChE had been completely reactivated by 2-PAM. With this reactivation procedure, the degree of enzyme regeneration is related to the extent of paraoxon inhibition. Therefore, a double test of enzymatic activities, the first without and the second with the presence of a reactivator in the sample is a simple and effective way for monitoring AChE activity and subsequently OP exposures.

Optimization of Inhibition and Reactivation Time. As shown in Figure 3, the impact of inhibition time on AChE activity at different paraoxon concentrations was examined. With increasing incubation time, the inhibition efficiency ($I\%$) increased fast in the first 20 min and reached a plateau within 30 min, which was then maintained for several hours. As anticipated with increasing OP concentration, the extent of AChE inhibition increased. At all concentrations of OP, the maximum inhibition was achieved within 30 min. Thus, 30 min was used as the time frame for measuring maximum inhibition and for adding the reactivating agent.
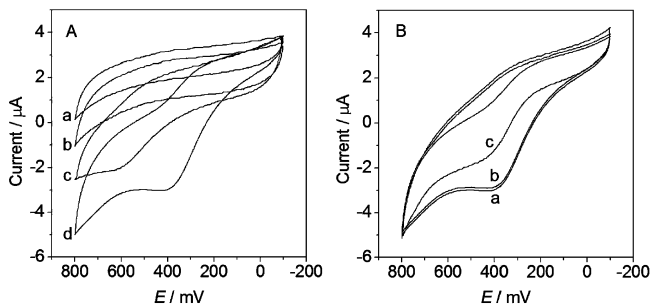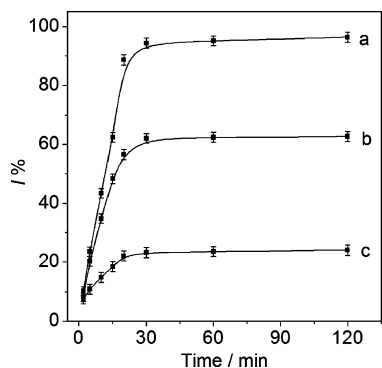

Figure 2. (A) Cyclic voltammograms of ATCh at (a) bare and (b) CNT modified electrodes in PBS buffer and at (c) bare and (d) CNT modified electrodes in PBS containing 3 nM AChE. (B) Cyclic voltammograms of ATCh at CNT modified electrodes (a) in PBS containing 3 nM AChE and (b) in PBS containing 5 mM 2-PAM for reaction of 15 min after dispersing 25 nM paraoxon in 3 nM AChE for 30 min and (c) in PBS dispersing 25 nM paraoxon in 3 nM AChE for 30 min without adding 5 mM 2-PAM.

Figure 3. Effect of inhibition time on inhibition efficiency by adding (a) 50, (b) 10, and (c) 1 nM paraoxon to 3 nM AChE.
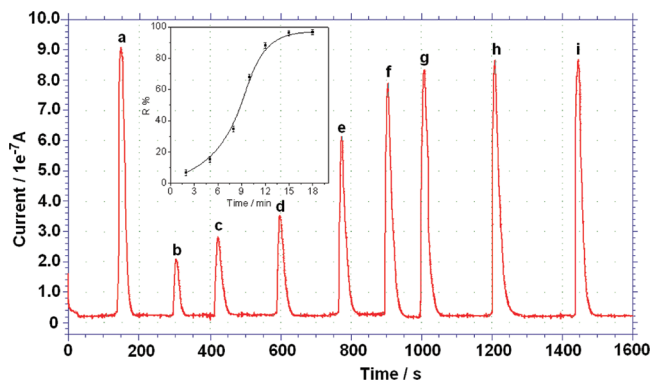

Figure 4. Amperometric responses of 3 nM AChE in PBS containing 5 mM ATCh (a) before paraoxon exposure and after 25 nM paraoxon inhibition of 30 min followed by treating with 5 mM 2-PAM for (b) 0, (c) 2, (d) 5, (e) 8, (f) 10, (g) 12, (h) 15, and (i) 18 min. Inset: plot of reactivation efficiency vs different incubation time.

One of the most influential parameters in double-test enzymatic activity is the reactivation time for regeneration of inhibited enzyme. Figure 4 shows the typical $i–t$ curves that are obtained after different incubation time with 2-PAM. The intact AChE displayed the maximum response to ATCh (curve a), and the current reduced greatly after exposure to 25 nM paraoxon for 30 min (curve b). In the presence of 2-PAM (5 mM), the peak currents increased with increasing incubation time (curves c–i), indicating that the inhibited AChE was regenerated from phosphorylated enzymes and the restored AChE activity hydrolyzed ATCh to thiocholine producing a detectable response. As shown from the inset, the reactivation efficiency (R%) trended to a constant value after 15 min, which revealed the maximum reactivation.

Correlation between Double Test Mode and Enzyme Activity Assay with Known Controls. After exposing the enzyme to 0.1, 0.5, 1, 5, 10, 25, and 50 nM paraoxon (different OP inhibitions from 5 to 94%) for 30 min and then treating with 2-PAM for 15 min respectively, 92–95% of the enzymatic activity could be regained (Figure 5). On the basis of this nearly complete reactivation, the proposed flow-injection analysis device can be applied to detect the extent of OP inhibition. As shown in Figure 6A, the uninhibited AChE displayed the maximum peak current (peak a), and this amperometric signal was decreased as a function of paraoxon concentrations (peaks b, d, f, h, j, l, and n). The observed amperometric signal inversely correlates with the paraoxon concentration, indicating that a decrease in enzymatic
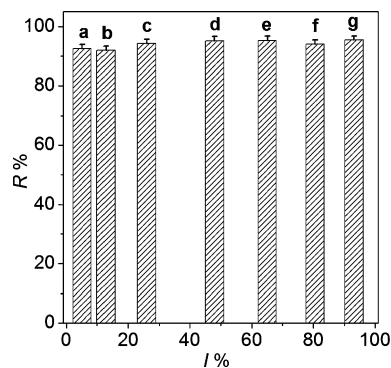
Figure 5. Reactivation efficiency of AChE treated with 5 mM 2-PAM for 15 min after paraoxon inhibition of (a) 5.11%, (b) 12.84%, (c) 25.99%, (d) 47.96%, (e) 65.05%, (f) 80.53%, and (g) 93.15%.
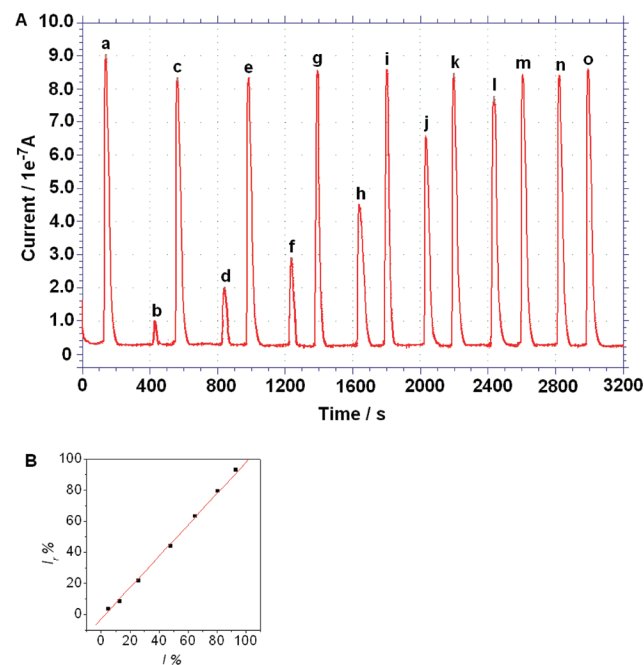

Figure 6. (A) Amperometric responses of 3 nM AChE in PBS containing 5 mM ATCh (a) before paraoxon exposure and after exposure to (b) 50, (d) 25, (f) 10, (h) 5, (j) 1, (l) 0.5, and (n) 0.1 nM paraoxon for 30 min and (peaks c, e, g, i, k, m, o) following reactivation by 5 mM 2-PAM incubation for 15 min. (B) Relationship between inhibition efficiencies calculated from reactivated AChE (I_r%) and those from control intact AChE (I%).

activity was directly related to OP exposure. However, after treatment with 2-PAM for 15 min, it can be seen that the amperometric signal increased almost to the level of the control, indicating that the inhibited AChE could be regenerated completely (peaks c, e, g, i, k, m, and o). Figure 6B showed the relationship between inhibition efficiencies calculated from reactivated AChE (I_r%) and those from control intact AChE (I%). It can be seen that the relationship is highly linear over a broad range of relative enzyme inhibitions (5–94%). The regression equation is I_r% $= -3.861 + 1.031I$%, and the correlation coefficient is 0.9993. It confirmed that the I_r% is highly correlative to I%. The variation (ΔI) ranges from ~0.2 to <5% and is shown in Table 1. Overall, these results indicate that the restored enzyme activity can serve as a baseline to quantify the extent of enzyme inhibition in a given sample. Therefore, the inhibition efficiency obtained from this double test mode can be

Table 1. Inhibition Efficiencies of $I\%$ and $I_r\%$

$I\% = (i_0 - i_t)/i_0 \times 100$	$I_r\% = (i_r - i_t)/i_r \times 100$	$\Delta I\% = I\% - I_r\%$
5.113	3.739	1.374
12.84	8.613	4.227
25.99	21.73	4.26
47.96	44.07	3.89
65.05	63.32	1.73
80.53	79.44	1.09
93.15	92.98	0.17

directly used for screening and evaluating exposures to OP insecticides and nerve agents. Since almost complete reactivation from inhibited AChE can be regenerated, control is not needed in real sample determinations. Moreover, when OP exposure results in inhibition of less than 20% and the clinical symptoms are absent, this methodology can easily detect and quantify exposures. Thus this electrochemical sensor combined with a flow-injection device represents a new method for early assessment and screening of OP exposure.

Measurement of OP Inhibition on ChE Activity in Saliva. Our research group has previously characterized saliva ChE as a potential biological matrix that could be exploited for biomonitoring for OP exposures.[35,36] It has been previously reported that rat salivary ChE active site concentration is estimated to be ~12 000 and 1000-fold less than that of the brain and plasma ChE, respectively; and using specific ChE inhibitors, butyrylcholinesterase (BuChE) accounts for 95% of the total cholinesterase activity in saliva, with a reported enzyme concentration of 1.2 ± 0.13 fmol mL^{-1} saliva.[35] Since ATCh is an excellent substrate for both AChE and BuChE enzyme activity, the assay developed with AChE can be directly exploited for BuChE activity. Hence, the OP inhibition in saliva was measured via the double determination of enzymatic activity. A 3-fold diluted saliva sample was first exposed to different concentrations of paraoxon for 30 min and then adding 5 mM 2-PAM for 15 min. On the basis of the variations of the signal, the inhibition of paraoxon can be calculated to be 90.4%, 58.7%, 36.5%, and 13.3%, respectively (Figure 7). The reproducibility was further investigated with saliva samples. The average relative standard deviation (RSD) for the response was <4%, which indicated a reproducible response (data not shown). We found that the inhibited ChE in saliva can be returned to the active enzyme completely. The successful detec-
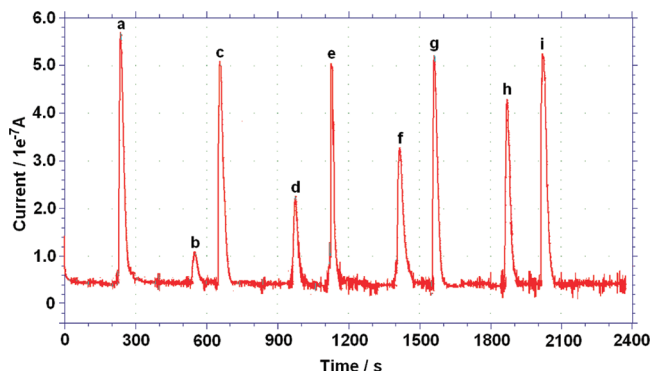

Figure 7. Amperometric responses of 3-fold diluted saliva in PBS containing 5 mM ATCh (a) before paraoxon exposure and after exposure to (b) 25, (d) 10, (f) 5, and (h) 0.5 nM paraoxon for 30 min and (peaks c, e, g, i) following reactivation by 5 mM 2-PAM incubation for 15 min.
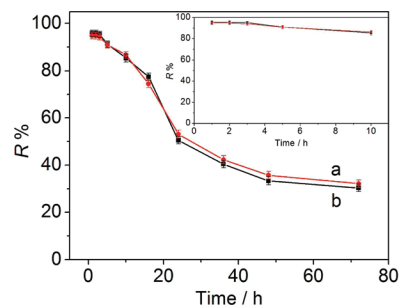

Figure 8. Reactivation efficiency of AChE by 2-PAM incubation for 15 min after exposing (a) 25 and (b) 1 nM paraoxon to AChE for different periods. Inset: enlarged plot of reactivation efficiency before 10 h exposure of paraoxon.

tion of OP inhibition efficiency in rat saliva with this flow-injection device based on the double test of enzymatic activity before and after reactivation demonstrates its promise for sensitive monitoring of OP exposure in biological fluids. Comparison with the routine approach for evaluating the decrease from an average or control value, this double-test procedure excludes considerable inter- and intraindividual variations in assessing ChE activities. Therefore, it may offer a more convenient and accurate method for monitoring occupational and nonoccupational exposures.

Aging of OP−ChE Adduct. The reactivation of inhibited ChE is dependent on both the amount of reactivator and phosphorylated enzyme.[37] The "aged enzyme" undergoes dealkylation, where, after a molecular rearrangement of the alkylphosphate group attached to the serine residue, it is resistant to reactivation.[38] To explore the feasibility of regeneration, we examined the reactivation efficiency after exposing paraoxon to AChE for a different time. As shown in Figure 8, the inhibited AChE could be restored to ~90% of the initial activity at ~5 h postincubation at two concentrations (25 nM (curve a) and 1 nM (curve b)) of paraoxon. In addition, after 10 h postincubation an additional 5% (~85% activity) of the enzyme activity was lost further. These results suggest that for insecticides like paraoxon, enzyme aging does occur and the total AChE activity may not be fully regenerated with this method. However, for pesticides that exhibit a relatively slow aging process the impact of aging on the assessment of total ChE activity may be minimal, particularly if the enzyme activity is assessed in a timely manner following an exposure.

It is known that aging of soman (chemical nerve agent) inhibited ChE is very fast (the half-life is about 10 min) but is considerably slower for the nerve agent sarin (half-life is about 10−15 h). Whereas for the nerve agent VX, enzyme aging was not observed within 24 h.[39,40] For soman, therapeutic intervention has been difficult due to this rapid aging and stabilization of the ChE adducts. In the case of other nerve agents and pesticides, reactivation of ChE is still possible hours after the intoxication.[41,42] Therefore, this sensing system of the double-test mode are most

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suitable for biomonitoring of slow or nonaging OP pesticides and nerve agents. The device is also promising for the first responders to quickly respond to nerve agent attacks where the diagnosis process for a victim needs to be done within minutes after the attacks rather than hours or days. Since some environmental sensors for directly identifying chemical agents have been developed,[43−45] the first responders could use the environmental sensors first for rapid identification of chemical agents used in a terrorist attack by analyzing environmental samples (air, soil, or water), then use the enzyme activity sensor and the biomonitoring protocol developed in this work for rapid identification of the victims of the attack for medical treatment.

CONCLUSIONS

In this article, we have developed a CNT-based amperometric sensor combined with a microflow-injection device and double test mode via regeneration of AChE from OP-inhibited saliva samples and demonstrated its low-cost, simplicity, and sensitivity for assessment of subclinical OP exposure. Compared with routine approaches for evaluating the decrease from an average or control value, this new technology based on double-test mode excludes inter- or intraindividual variation in the normal levels of ChE. It

can be easily extended to detect OP inhibition in other biological samples, such as plasma and whole blood. The high sensitivity of this biosensor stems from the unique electrocatalytic properties of CNTs and the inherent high sensitivity of the electrochemical techniques. This portable analytical device based on the CNT-sensor shows great promise for the in-field and point-of-care diagnosis of asymptomatic organophosphate and carbamate pesticide exposures, as well as chemical nerve-agent poisoning. It is promising for the first responders to use the portable device for rapid identification of the victims in a nerve agent attack for medical treatment.

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