Environ. Sci. Technol. **2008,** *42,* 2688–2693

Carbon Nanotube-Based Electrochemical Sensor for Assay of Salivary Cholinesterase Enzyme Activity: An Exposure Biomarker of Organophosphate Pesticides and Nerve Agents

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Received September 17, 2007. Revised manuscript received November 15, 2007. Accepted December 4, 2007.

Certain saliva enzymes may be useful biomarkers for detecting exposures to organophosphate pesticides and chemical nerve agents. In this regard, saliva biomonitoring offers a simple and noninvasive approach for rapidly evaluating those exposures in real time. An electrochemical sensor coupled with a microflow injection system was developed for a simple, rapid, and sensitive characterization of cholinesterase (ChE) enzyme activities in rat saliva. The electrochemical sensor is based on a carbon nanotube (CNT)-modified screen-printed carbon electrode (SPE), which is integrated into a flow cell. Because of the excellent electrocatalytic activity of the CNTs, the sensor can detect electroactive species that are produced from enzymatic reactions with extremely high sensitivity and at low potentials. The electrochemical properties of acetylcholinesterase (AChE) enzymatic products were studied using a CNT-modified SPE, and the operation parameters such as the applied potential and substrate concentration were optimized to achieve the best performance. The AChE enzyme activity was further investigated using the CNT-based electrochemical sensor with commercially available purified AChE and ChE in saliva obtained from nave rats. It is found that the calibration curve is linear over a wide range of AChE concentrations from 5 pM to 0.5 nM, and the sensor is very sensitive with the detection limit down to 2 pM. The dynamics of the ChE enzyme activity in saliva with organophosphate pesticides was further studied using this sensor. The results show that the senor can be used to characterize salivary enzyme activity and to detect the exposure to organophosphate compounds. This new CNT-based electrochemical sensor thus provides a sensitive and quantitative tool for noninvasive biomonitoring of the exposure to organophosphate pesticides and nerve agents.

Introduction

Organophosphate (OP) pesticides and chemical nerve agents have the potential to cause serious health threats to humans and animals. These compounds have been extensively used as insecticides in modern agriculture, and certain extremely toxic OP compounds have been developed as chemical warfare agents (*1, 2*). The acute toxicity of an exposure to these OP chemicals stems from the fact that they target a number of important proteins, including a group of hydrolytic enzymes, such as acetylcholinesterase (AChE), which is particularly critical for central and peripheral nervous-system functions. The OP agents irreversibly phosphorylate AChE serine residue, required for its catalytic activity, forming a stable complex that inhibits enzyme activity (*3, 4*). Therefore, this enzyme activity and other cholinesterase (ChE) targets such as butyrylcholinesterase (BuChE) can serve as a biomarker for detecting the exposure to these OPs and for estimating the potential for adverse effects. Because of the potential for widespread toxicity of OPs, the development of a rapid, sensitive, and inexpensive method to detect OP pesticides and nerve agents that threaten living organisms is crucial.

Biomonitoring is an efficient approach for detection and evaluation of exposure to OP (*5*). It primarily focuses on measuring OP concentrations or their metabolites in blood and urine or, alternatively, measuring ChE activities in blood (*6–9*). Measuring OPs or the associated metabolites is very complicated because it requires sophisticated analytical methods, such as liquid chromatography coupled with mass spectrometry (LC-MS), gas chromatography (GC)-MS, or high-pressure liquid chromatography (HPLC)-MS (*7, 9–11*). Although these methods are sensitive and accurate, they are very expensive, time-consuming, and laboratory-oriented, and they require a pretreatment of the sample and highly qualified technicians. For the alterative approach to measure ChE activities, a blood assay is usually used, but it is invasive and time-consuming, and the results are not immediately available (*6, 12*). In contrast, a saliva ChE enzyme-activity assay offers a simpler approach for biomonitoring OP because of the convenience of sampling. Moreover, it benefits greatly from less interference because saliva contains fewer proteins than blood. An assay for salivary ChE activity has recently been reported, and it shows potential for biomonitoring exposures to OPs (*13–15*).

The ChE activities are conventionally measured with the Ellman assay using Ellman reagent (*13, 16*). Some alternative approaches, such as radiometric assay (*14*), immunoassay (*17*), fluorescence (*18, 19*), chemiluminescence (*20, 21*), or mass spectroscopy (*22*), have been reported for sensitive detection of enzyme activities. However, these assays are tedious, time-consuming, or both, and they require expensive and sophisticated instruments, which makes these methods unsuitable for rapid and onsite applications. Therefore, it is imperative to develop simple, rapid, and sensitive biomonitoring methods for onsite and emergency use.

Electrochemical techniques combined with a flow-injection system offer a simple and inexpensive approach for rapid and onsite biomonitoring of enzyme activities (*23*). They have been widely used in various chemo/biosensors because of their simplicity, low cost, and high sensitivity, and particularly, because the device can be miniaturized (*23–29*). The sensitivity of these types of chemo/biosensors can be further enhanced by using various nanotechnology-based amplifications (*30, 31*). Carbon nanotubes (CNTs) represent a new class of nanomaterials and are composed of graphitic carbons with one or several concentric tubules. Recent studies have demonstrated that CNTs can improve the direct electron transfer reaction of some biomolecules (*32–34*) on an electrode surface, this is attributed to their unique electronic structure, high electrical conductivity, and redox-active sites.

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10.1021/es702335y CCC: $40.75
Published on Web 02/23/2008

CNTs can greatly decrease the over potential of oxidizing or reducing some enzymatic products, for example, hydrogen peroxide (*28, 35, 36*) and thiocholine (*26, 37, 38*), because of their electrocatalytic effects. Although some biosensors that are based on AChE-modified electrodes or CNT-coated electrodes for the detection of organophosphate compounds have been reported recently (*27, 37*), the use of CNTs as a transduction material for biomonitoring OP exposure via detection of the ChE enzyme activities in real biological samples such as saliva has not been reported.

In this paper, we report the development of a CNT-based amperometric sensor with a flow injection system for characterization of enzyme activities in rat saliva samples. The flow-injection system provides great advantages for onsite, real-time, and continuous detection of biomolecules and requires only a small volume of the sample. The sensor takes advantage of the electrocatalytic properties of CNTs, which makes it feasible for a sensitive electrochemical detection of the products from enzymatic reactions at low potential. It offers a new biomonitoring approach for a rapid, inexpensive, and highly sensitive detection of exposures to OP compounds.

Experimental Section

Chemicals. Human acetylcholinesterase (500 units/mg protein, AChE) from human erythrocytes, acetylthiocholine (ATCh), phosphate-buffer saline (PBS), and acetone were purchased from Sigma (St. Louis, MO). Multiwall CNTs (0.5−2 mm in length and 20 nm in diameter) were obtained from Cheap Tubes, Inc. (Brattleboro, VT). 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.

Modification of CNT Film on a Screen-Printed Carbon Electrode (SPE). A 10 μL aqueous suspension containing 50 μg mL^{-1} of CNT diluted from stock solution (5 mg mL^{-1}) was cast onto the surface of the carbon working electrode of the SPE and allowed to dry naturally. Before use, the modified electrode was carefully rinsed with water to remove unbound CNTs and dried under an N_2 stream.

Generation of Enzymatic Product Thiocholine. A 5 mM quantity of acetylthiocholine in 50 mM of PBS buffer solution (pH 8) was mixed with different concentrations of AChE in 50 mM of PBS buffer (pH8) or with diluted saliva solutions at a volume ratio of 1:1. The mixture was incubated for a designated time period (e.g., 10 min). The reaction solution was then detected on the electrode surface with electrochemical techniques.

Incubation of the Saliva Sample with Paraoxon Solutions and Analysis. The saliva sample was diluted 10 times with 50 mM PBS buffer, and then the paraoxon stock solution (7.0 mM) was prepared in acetone. A series of dilutions of the paraoxon stock solution was prepared with 50 mM PBS buffer, and two concentrations of diluted paraoxon (0.7 and 7 nM) were chosen to mix with a 10-fold diluted saliva sample (1:1 v/v). The mixture was incubated for different time periods, for example, 5, 10, 30, 60, or 120 min. Then 100 μL of the above mixed solution was subsequently reacted with an equal volume of 5 mM ATCh for 10 min; 40 μL of the reaction solution was sequentially injected into the sensing system, and the $i-t$ curves were recorded.

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 laboratory-built flow-through electrochemical cell (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^{-1}. The laboratory-built microelectrochemical cell was constructed by sandwiching the SPE between a plastic base and a plastic cover with two holes for the inlet and outlet, respectively. A Teflon gasket with a flow channel was mounted between the SPE and the cover to form a flow cell, in which the SPE was exposed to the cell for the electrochemical detection (Figure 1B).

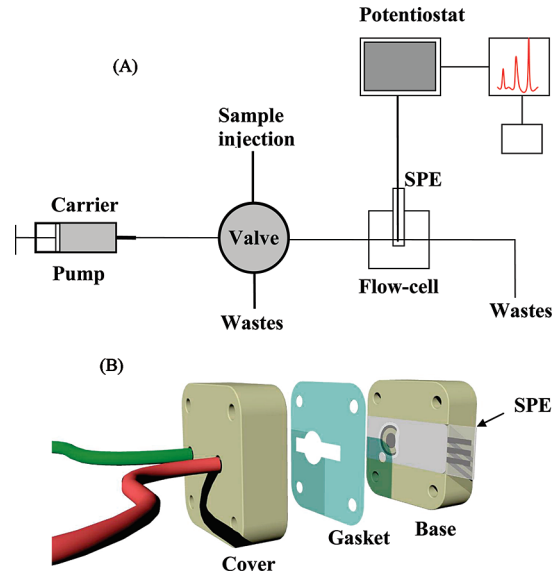

FIGURE 1. (A) Schematic diagram of the flow-injection sensing system. (B) 3-D image of the electrochemical flow-cell.

Electrochemical Detection. All electrochemical experiments were carried out with an electrochemical analyzer CHI 660 (CH Instruments, Inc., Austin, TX) connected to a laptop computer. A disposable SPE (Alderon Biosciences) was employed that consisted of a CNT-modified electrode as the working electrode, an Ag/AgCl, and a carbon ring as the reference- and counterelectrodes, respectively. A sensor connector was used to connect the SPE to the electrochemical analyzer. Square wave voltammetric (SWV), cyclic voltammetric (CV), and amperometric measurements were performed with the SPE. CV measurement was performed with the potential range of 0−1.0 V and a 100 mV/s scanning rate. For SWV measurements, the potential was scanned from 0 to 1.0 V with a step of 4 mV, an amplitude of 25 mV, and a frequency of 15 Hz. A 0.05 M PBS buffer solution (pH 8.0) was used as the supporting electrolyte for electrochemical experiments. The amperometric measurements were conducted at different potentials (e.g., 0.15 V), and all potentials were referred to the Ag/AgCl reference.

Results and Discussion

We developed a CNT-based electrochemical sensor coupled with a flow-injection system for measurement of ChE activities in biological fluids such as saliva (Figure 1). The assay of enzyme activities in saliva is based on the excellent electrocatalytic properties of CNTs, which makes enzymatic products detectable at low potentials with extremely high sensitivity. The amount of enzymatic products that are generated from ChE enzyme depends on the enzyme activity. Therefore, this sensor can measure ChE activities in saliva by sensitively detecting the electroactive enzymatic products. The detection principle of the sensor is based on the following reaction:

$$\text{acetylthiocholine} + H_2O \xrightarrow{\text{ChE}} \text{thiocholine} + \text{acetate} \quad (1)$$

The ChE enzyme in saliva can hydrolyze acetylthiocholine to thiocholine, and the latter can be detected at the CNT-

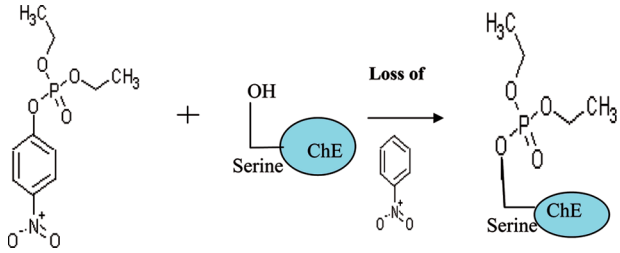

FIGURE 2. Schematic illustration of the process of OPs and nerve agents binding to ChE.

modified SPE by application a low potential (0.15 V). The oxidation of thiocholine on electrode surface involves the following process:

$$\mathrm{RSH} \xrightarrow{-e^-} \mathrm{RSH}^{+\bullet} \xrightarrow{-H^+} \mathrm{RS}^{\bullet} \rightarrow \mathrm{RSSR} \qquad (2)$$

The procedure to test enzyme activities in saliva is quite simple: the diluted solution of the saliva is mixed with the substrate (acetylthiocholine) for a few minutes; then the mixture is injected into the flow cell, and the generated electrochemical signals are subsequently recorded. The signal results from the oxidation of thiocholine, an enzymatic product, and the signal magnitude reflects enzyme activities in the saliva.

The process of the OPs and nerve agents binding to the ChE is shown in Figure 2. Here, paraoxon is chosen as a model OP. This mechanism and process of the OP binding to ChE is similar to the literature pervious reported by Thompson (*17*). Following exposure, the OPs will bind to the serine of peptide located at the active site of the ChE enzyme and release the nitrobenzene group, while phosphorylating the enzyme (ChE-OP), which leads to inhibiting the activity and biological functions of the enzymes. Therefore, the ChE activity in saliva is a good biomarker for monitoring the OP exposures (*15*).

Electrochemical Characteristics of Thiocholine at the CNT-Modified SPE. The electrochemical characteristics of the enzymatic product thiocholine on a bare and CNT-modified SPE were investigated. Figure 3A shows the typical SWVs of acetylthiocholine and thiocholine on a bare SPE in a PBS buffer (pH8). No obvious redox peaks in the PBS (black line a) and ATCh (blue line b) were observed on the bare carbon electrode. A broad peak was observed at 0.85 V (curve c), which is attributed to the oxidation of thiocholine on the bare carbon electrode. No obvious peaks were observed for the AChE solution without ATCh (data now shown). Figure 3B shows the hydrodynamic voltammograms of thiocholine at a carbon and a CNT-modified carbon electrode, respectively. With a desired potential applied to the working

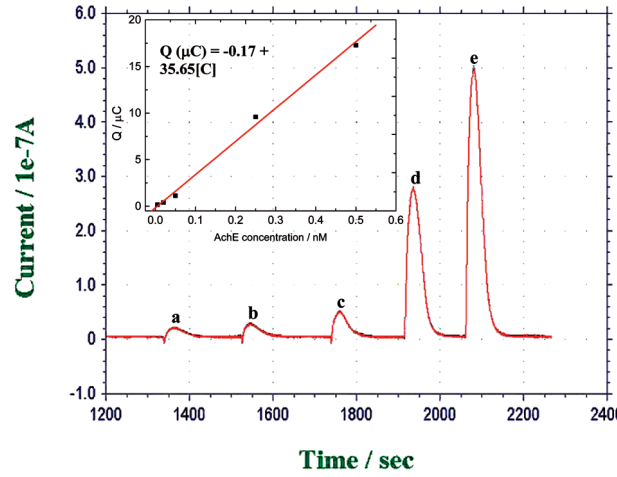

FIGURE 4. *i*−*t* Curve obtained at the sensor with different dilutions of human AChE. Sequential injection of PBS buffer solutions containing ATCh and (a) 5 pM AChE; (b) 0.020 nM AChE (c), 0.05 nM AChE, (d) 0.25 nM AChE, and (e) 0.5 nM AChE. Incubation time: 10 min; 5 mM of ATCh used. Inset is the plot of charges vs different concentrations of AChE.

electrode, the transient currents that decayed to a steady-state value were recorded. The response to the PBS buffer solution that contained ATCh but no AChE was subtracted from the response to one that contained both ATCh and AChE. It can be seen that the peak current at a bare SPE is negligible when the applied potential is from 0 to 0.3 V. Thereafter, the current increases slowly with the potential from 0.3 to 0.8 V, and then it goes down as the potential increases. However, the current at a CNT-modified SPE increases with the applied potential from 0.1 V and reaches a plateau at 0.5 V. It starts to level off when the potential is between 0.5 and 0.6 V, and then decreases rapidly with a higher potential. The current response at the CNT-modified SPE is much higher than that at the bare SPE when the potential is less than 0.8 V, and the peak potential shifts negatively at the CNT-modified SPE, which indicates that CNTs decrease the overpotential of thiocholine oxidation. The results have revealed that CNTs can electrochemically catalyze the oxidation of the enzymatic product thiocholine and greatly enhance the amperometric signal. The decrease of the overpotential is beneficial for avoiding interferences from the biological matrixes.

Operational conditions for the sensor such as the substrate concentration and incubation time for the CNT-based sensor were studied with solutions that contain commercial AChE and ATCh. Through the optimal experiments, the parameters

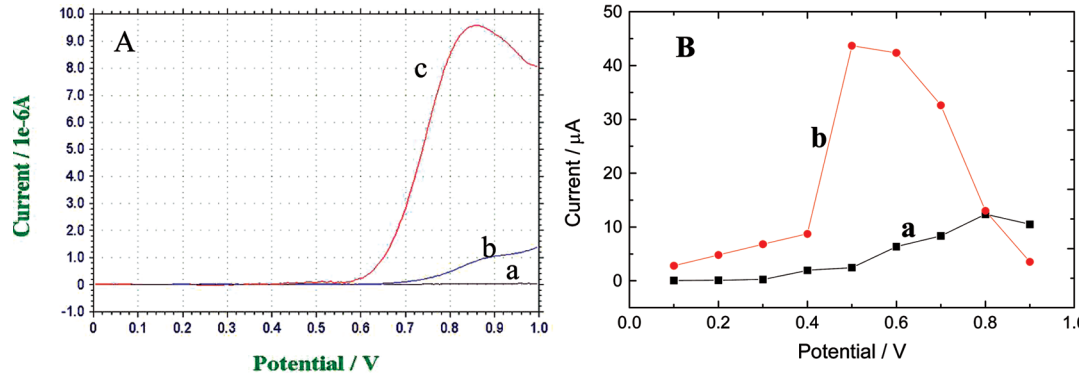

FIGURE 3. (A) SWVs of thiocholine and acetylthiocholine at carbon electrode. Black curve a: PBS buffer; blue curve b: acetylthiocholine; red curve c: thiocholine; (B) hydrodynamic voltammogram of thiocholine obtained at (a) unmodified SPE and (b) CNT-modified SPE. Thiocholine was obtained by 5 nM AchE interaction with 5 mM ATch for 10 min. The solutions were prepared with 0.05 M phosphate buffer (pH 8.0). The linear baseline correction was conducted with the software for Figure 3A.

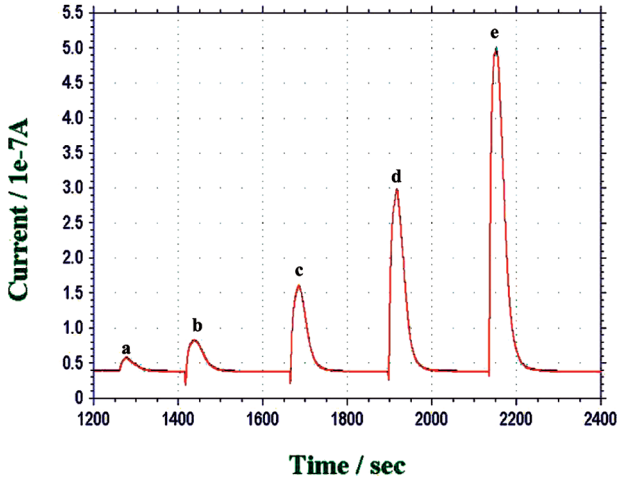

FIGURE 5. *i*–*t* Curve obtained at the sensor with different dilutions of the saliva sample from rats: (a) 5-fold diluted saliva in PBS buffer without ATCh, (b) 40-fold diluted saliva in PBS buffer with ATCh, (c) 20-fold diluted saliva in PBS buffer with ATCh, (d) 10-fold diluted saliva in PBS buffer with ATCh, and (e) 5-fold diluted saliva in PBS buffer with ATCh. ATCh concentration: 5 mM. Incubation time: 10 min.

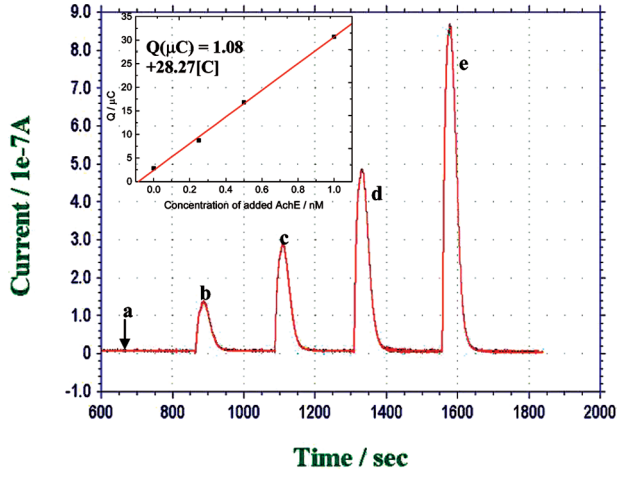

FIGURE 6. Amperometric responses from adding different concentrations of AChE in 20-fold diluted saliva at the sensor: (a) 20-fold diluted saliva in buffer without ATCh, (b) 20-fold diluted saliva after addition of 0 nM AChE and ATCh, (c) 20-fold diluted saliva after addition of 0.25 nM AChE and ATCh, (d) 20-fold diluted saliva after addition of 0.5 nM AChE and ATCh, (e) 20-fold diluted saliva after addition of 1.0 nM AChE and ATCh. Incubation time: 10 min; 5 mM ATCh used. The inset is the plot of the charges vs different concentrations of added AChE.

that were chosen for the sensor are as follows: applied potential 0.15 V, 5 mM ATCh, and incubation time 10 min. A 0.15 V potential for amperometric experiments was chosen to achieve a balance between good sensitivity and less interference (see Supporting Information).

Characterization of AChE Activity. The characteristics of the enzyme-activity assay with this sensor were examined with commercially available human AChE. Figure 4 shows the typical *i*–*t* curves that were obtained using different AChE concentrations incubated with an equal volume of ATCh (5 mM) for 10 min. A plug of 40 μL testing solution was injected when the baseline became stable. It can be seen from Figure 4 that the peak current increases with an increase of the AChE concentration, which indicates that the response is resulted from the oxidation of thiocholine that was generated by the reactions between AChE and ATCh in the solution.

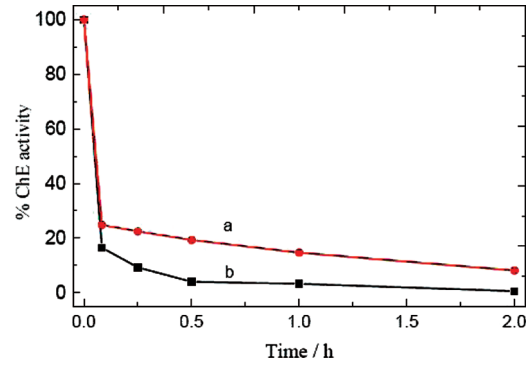

FIGURE 7. Inhibition of salivary enzyme activity with the incubation of different concentrations of paraoxon for different periods: 10-fold diluted saliva is used. The red curve is for 0.7 nM paraoxon and the black curve for 7.0 nM paraoxon. The incubation time period with ATCh (5 mM) is 10 min.

The inset shows the plot of charge versus AChE concentration. It can be seen that the charge increases linearly with increasing AChE concentrations, and the linear range is from 5 pM to 0.5 nM. On the basis of the signal-to-noise characteristics of the data ($S/N = 3$), the detection limit of this biosensor was estimated to be about 2 pM, which is comparable to that of colorimetric methods (*39, 40*).

Measurement of the ChE Enzyme Activity in Saliva. The saliva fluid was collected from Sprague–Dawley male rats and serially diluted with a PBS buffer. The diluted saliva solutions were incubated with the solution containing the same concentration of ATCh for 10 min and subsequently injected into the flow cell for electrochemical measurements. Figure 5 shows the electrochemical signal with different dilutions of the saliva sample. It can be seen that the electrochemical signals decrease with increasing dilutions of saliva samples. The signal from 5-fold diluted saliva without ATCh (peak a) was much smaller than that from the equally diluted saliva with ATCh (peak e), which indicates that the signal from electroactive substances in the matrix is very little, and the background signal becomes negligible with more dilutions. These results confirmed that the change in the electrochemical signal with the dilution of saliva is resulted from the change of the enzyme concentration in the test solution. Even in a 40-fold dilution of the saliva sample, the enzyme activity was still detectable (peak b). These results demonstrate that the developed sensor is sensitive enough to characterize the enzyme activity in saliva samples.

The ChE enzyme concentration in the saliva was measured by adding standard AChE to the saliva sample. A 20-fold diluted saliva sample, spiked with 0, 0.25, 0.5, and 1 ng mL^{-1}AChE, was used for the measurements. Figure 6 shows the amperometric response of the saliva samples spiked with a different standard AChE. It can be seen that the signal from the saliva without ATCh incubation is negligible (curve a), and the amperometric signal increases with higher spiked concentrations of AChE. The inset is a plot of the charge versus concentrations of the spiked ACHe. The Charges Q were obtained by the subtraction of blank peak of ATCh in PBS buffer. It can be seen that the relationship between them is linear. The regression equation is Q (μC) = 1.08 + 28.27[C], and the correlation coefficient = 0.999. From this equation, the concentration of ChE in the 20-fold diluted saliva sample was calculated to be 0.038 nM. Therefore, the concentration of ChE in the original saliva is about 0.7 nM. The successful detection of the saliva samples with the electrochemical sensor demonstrates its promise for sensitive assays of ChE enzyme activities in biological fluids.

The reproducibility of the flow-injection sensor for the detection of enzyme activity was further investigated with saliva samples. The average relative standard deviation (RSD) for the responses is ≤8%, which indicates that the response

of the sensor is reproducible (see Supporting Information). Although this sensing system can apply for most OPs and nerve agents, chemicals containing thiol, for example, thiophosphate may generate confounding electrochemical signals. Therefore, the electrochemical properties of thiol-contained OPs should be further evaluated to justify if these types of OPs can be applied with this sensing system.

Dynamics of Salivary Enzyme Activities with Organophosphate Pesticides. To explore the feasibility of the CNT-based sensor for detection of OP exposure, we examined the salivary ChE enzyme activity after an incubation of saliva with OP chemicals. The paraoxon and the 10-fold diluted saliva sample were chosen for this study. Figure 7 shows ChE enzyme activity with different concentrations of paraoxon with increasing incubation time. It can be seen from this figure that the enzyme lost almost 97% activity after 0.5 h incubation with 7 nM paraoxon and 80% after 0.5 h incubation with 0.7 nM paraoxon. Thereafter, the enzyme activity that corresponds to 0.7 nM paraoxon continues to decrease through 2.0 h post-treatment, while the activity that corresponds to 7 nM paraoxon was nearly completely inhibited within 0.5 h. This demonstrates that the amperometric sensor with a flow injection system can monitor salivary ChE enzyme activities and detect the exposure to OP pesticides and nerve agents. Future studies are planned to evaluate the sensor performance in saliva following in vivo exposure of rats to environmentally relevant doses of OP insecticides.

In summary, we have developed a CNT-based electrochemical sensor and demonstrated that this sensor is simple, inexpensive, and sensitive for detection of ChE enzyme activities in saliva with a low detection limit and good reproducibility. The high sensitivity of this biosensor stems from the unique electrocatalytic properties of CNTs and the inherent high sensitivity of the electrochemical techniques. This technique may be easily extended to detect ChE enzyme activities in other biological samples, such as plasma and whole blood. This CNT-based sensor coupled with a portable electrochemical analyzer shows great promise for in-field and point-of-care diagnosis for OP and carbamate pesticides, as well as nerve-agent exposures.

Acknowledgments

The work was done at Pacific Northwest National Laboratory (PNNL) and supported partially by CDC/NIOSH Grant R01 OH008173-01 and grant number U01 NS058161-01 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke, NIH. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. The research described in this paper was performed at the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DOE's Office of Biological and Environmental Research and located at PNNL. PNNL is operated by Battelle for DOE under Contract DE-AC05-76RL01830. The authors would like to acknowledge Dr. Guodong Liu for the helpful discussion during this work.

Supporting Information Available

Detailed description on sampling of saliva from rats, optimization of experimental parameters, and reproducibility data are available free of charge via the Internet at http://pubs.acs.org.

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ES702335Y