Highly Sensitive and Selective Immuno-Capture/Electrochemical Assay of Acetylcholinesterase Activity in Red Blood Cells: A Biomarker of Exposure to Organophosphorus Pesticides and...

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · DECEMBER 2011

Impact Factor: 5.33 · DOI: 10.1021/es202689u · Source: PubMed

3 AUTHORS, INCLUDING:

Dan Du
Washington State University
130 PUBLICATIONS **3,747** CITATIONS
SEE PROFILE

Yuehe Lin
Washington State University
364 PUBLICATIONS **21,422** CITATIONS
SEE PROFILE

Environmental Science & Technology

Article

pubs.acs.org/est

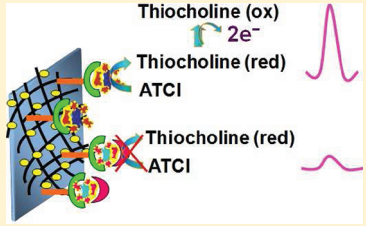
Highly Sensitive and Selective Immuno-Capture/Electrochemical Assay of Acetylcholinesterase Activity in Red Blood Cells: A Biomarker of Exposure to Organophosphorus Pesticides and Nerve Agents

Aiqiong Chen,[†] Dan Du,*[,†] and Yuehe Lin*[,‡]

[†]Key Laboratory of Pesticide and Chemical Biology of Ministry of Education, College of Chemistry, Central China Normal University, Wuhan 430079, PR China

[‡]Pacific Northwest National Laboratory, Richland, Washington 99352, United States

ABSTRACT: Acetylcholinesterase (AChE) enzyme activity in red blood cells (RBCs) is a useful biomarker for biomonitoring of exposures to organophosphorus (OP) pesticides and chemical nerve agents. In this paper, we reported a new method for AChE activity assay based on selective immuno-capture of AChE from biological samples followed by enzyme activity assay of captured AChE using a disposable electrochemical sensor. The electrochemical sensor is based on multiwalled carbon nanotubes−gold (MWCNTs−Au) nanocomposites modified screen printed carbon electrode (SPCE), which is used for the immobilization of AChE specific antibody. Upon the completion of immunoreaction, the target AChE (including active and inhibited) is captured onto the electrode surface and followed by an electrochemical detection of enzymatic activity in the presence of acetylthiocholine. A linear response is obtained over standard AChE concentration range from 0.1 to 10 nM. To demonstrate the capability of this new biomonitoring method, AChE solutions dosed with different concentrations of paraoxon were used to validate the new AChE assay method. AChE inhibition in OP dosed solutions was proportional to OP concentration from 0.2 to 50 nM. The new AChE activity assay method for biomonitoring of OP exposure was further validated with in vitro paraoxon-dosed RBC samples. The established electrochemical sensing platform for AChE activity assay not only avoids the problem of overlapping substrate specificity with esterases by using selective antibody, but also eliminates potential interference from other electroactive species in biological samples. It offers a new approach for sensitive, selective, and rapid AChE activity assay for biomonitoring of exposure to OPs.



INTRODUCTION

Organophosphorus (OP) compounds have been extensively used as pesticides, and some of them have been developed as chemical warfare agents, which may cause serious health threats to humans and animals.[1−3] The toxicity of OPs stems from the inhibition of acetylcholinesterase (AChE) to form phosphorylated adduct (OP-AChE), which leads to an inability to remove neurotransmitter acetylcholine (ATh) from receptors and subsequent impairment of numerous body functions.[4,5] Butyrylcholinesterase (BChE) is also another member of the serine hydrolase family and capable of hydrolyzing ACh.[6] It is known as pseudocholinesterase and can be inactivated by OPs, although toxicologic symptoms from OPs exposure are attributed to inhibition of AChE.[7]

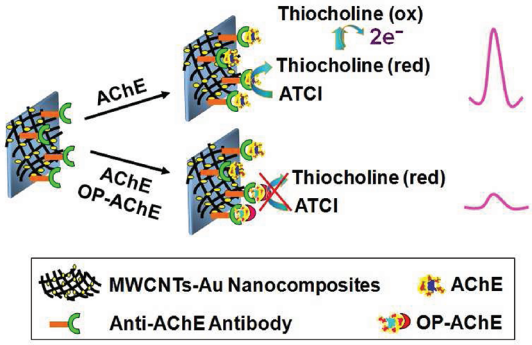
Numerous methods have been developed to measure cholinesterase (ChE) activities including Ellman colormetric assay,[8] chemiluminescence assay,[9,10] fluorescence assay,[11,12] and radioactive assay.[13] Recently, our group reported electrochemical techniques for assay of salivary ChE activity.[14,15] However, all these methods are difficult to estimate individual activity of AChE and BChE respectively by determining total enzymes activities. To help address this problem, two approaches have been reported for measuring AChE and BChE activities respectively in blood. (1) Separating blood into erythrocytes, which contain only AChE, and plasma which contains only BChE, to measure their activity individually. These methods require the separation of plasma and erythrocyte fractions for the measurement of erythrocyte AChE and plasma BChE activities.[16−18] (2) Using a BChE-specific inhibitor to measure the activity of AChE in whole blood.[19] However, the inhibitors used for completely inhibiting BChE activity also inhibited AChE activity leading to errors in reported values. Recently, Walter Reed Army Institute of Research (WRAIR) Assay[20,21] was reported for simultaneous measurement of AChE and BChE activities. It also required the use of an AChE-specific inhibitor (huperzine A) and a BChE-specific inhibitor (Iso-OMPA) to determine the sensitivity coefficients of AChE and BChE for each substrate.

Motivated by the high degree of similarity between AChE and BChE, here we use AChE specific-antibody to selectively capture target AChE (including active and inhibited) followed by electrochemical measurement of its activity. Electrochemical techniques offer a simple and inexpensive approach for rapid

Received: August 3, 2011
Revised: November 8, 2011
Accepted: December 29, 2011
Published: December 29, 2011

and onsite biomonitoring of enzyme activities.[22,23] The sensitivity of the signal can be further enhanced by using newly emerged nanoparticles.[24,25] In this paper, multiwalled carbon nanotube/gold (MWCNTs−Au) nanocomposites modified screen printed carbon electrode (SPCE) is used as sensor platform for AChE specific-antibody attachment. Upon the completion of immunoreaction, the target AChE (including active and inhibited) is captured onto the electrode surface. Electrochemical detection of AChE activity is performed in the presence of acetylthiocholine (Scheme 1). To our knowledge, this is the first report to establish immuno-capture/electrochemical platform for biomonitoring of OP exposure via detection of AChE enzyme activities in real biological samples. The novelty of this method relies on the use of antibody to selectively capture AChE for activity assay, avoiding the problem of overlapping substrate specificity with esterases in enzyme activity assay and potential interference of electroactive species in biological samples.

Scheme 1. Schematic Illustration of the Principle of Immunosensing Platform for Measurement of Enzyme Activity and OP Exposure



EXPERIMENTAL SECTION

Reagents. Human acetylcholinesterase (AChE), acetylthiocholine chloride (ATCh), mercaptoacetic acid, gold(III) chloride solution ($HAuCl_4$), trisodium citrate, Triton X-100, Tween-20, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), phosphate buffer saline (PBS), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO). Paraoxon was obtained from Chem Service (Chester, PA). Anti-AChE monoclonal antibody was purchased from Abcam Inc. (Cambridge, MA). Human red blood cells (RBCs) were ordered from Biochem. Service (Winchester, VA). Multiwalled carbon nanotubes (MWCNTs) were acquired from Nanoport Co. Ltd. (Shenzhen, China). All solutions were prepared with distilled water that was purified with the Nanopure system (Barnstead, Dubuque, IA).

Apparatus. Electrochemical measurements were performed on a CHI 660C workstation (CH Instruments Co., Shanghai, China). A disposable screen printed carbon electrode (SPCE) consisting of a carbon working electrode, a carbon auxiliary electrode, and an Ag/AgCl reference electrode was purchased from Dropsens Inc. (Spain). Electrochemical surface plasmon resonance (ESPR) equipment was the Autolab SPRINGLE system (Echo Chemie B.V., The Netherlands). Scanning electron microscopy (SEM) images were performed on a LEO 1450VP (Japan) scanning electron microscope.

Preparation of MWCNTs−Au Nanocomposites. One (1.0) mg of pretreated MWCNTs was dispersed in 1.0 mL of 0.01% $HAuCl_4$ by sonication for 5 min to get a homogeneous suspension. It was then diluted to 100 mL with doubly distilled water and heated to boiling while stirring. Afterward, 4.0 mL of 1.0% trisodium citrate was quickly added to the boiling solution which was kept heating for 5−10 min until the solution turned red and did not change. The resulting MWCNTs−Au nanocomposites were separated from mixture solution by centrifugation at 5000 rpm/min and resuspended in water.

Preparation of Electrochemical Immunosensor. Before modification, the SPCE was lightly polished with 0.05 m alumina, followed by sonication in double distilled water.[26] Then 5.0 μL of MWCNTs−Au nanocomposites (0.5 mg/mL) was dropped onto the working electrode of SPCE and allowed to dry naturally. The MWCNTs−Au nanocomposites modified SPCE (MWCNTs−Au/SPCE) was carefully rinsed and allowed to dry. Then 5.0 μL 0.02% mercaptoacetic acid was placed on the MWCNTs−Au/SPCE for 2 h to form a self-assembled layer. Prior to attaching anti-AChE antibodies, 10 μL of freshly prepared 400 mM EDC and 100 mM NHS in pH 5.2 MES buffer was placed onto the above surface and washed off after 30 min. This was immediately followed by a 1-h incubation at 37 °C with 5 μL of 1.0 mg/mL anti-AChE antibodies in pH 7.4 PBS. After washing with 0.05% Tween-20 PBS buffer, the obtained anti-AChE/MWCNTs−Au/SPCE was incubated in 3% BSA PBS solution at 37 °C for 1 h. The electrode was then washed with 0.05% Tween-20 PBS buffer before use.

Generation of Paraoxon Inhibited AChE or RBCs Solutions. RBCs-AChE was prepared according to Hammond et al.[27] with minor modifications. Briefly, RBCs were lysed in ice-cold water and the cells were then vortexed and incubated for 10 min at 4 °C. The cell membranes were isolated by 8−10 cycles of repeated centrifugation (15 000*g*, 20 min) and rinsing with PBS (10 mM, pH 7.4) until only slightly pink. The smaller dark red pellet on the bottom was non-lysed RBCs and was avoided. Paraoxon stock solution was prepared in acetone and diluted to different concentrations with 20 mM PBS. Then 10 μL of a series of paraoxon dilutions was dispensed into 40 μL of 3.0 nM AChE or solubilized RBCs ghost for incubation of 30 min and detection of enzyme activity. Pure paraoxon-AChE adduct (OP-AChE) was prepared as described before.[28] The decrease in enzyme activity was monitored until AChE inhibition was complete. This solution was dialyzed in 0.01 M PBS to remove the excess of paraoxon. The protein concentrations of AChE and OP-AChE stock solution were determined to be 0.24 and 0.22 mg/mL, respectively, by BCA method.

Electrochemical Detection. The immunosensor, anti-AChE/MWCNTs−Au/SPCE, was first incubated with 5.0 μL AChE of different concentration or the above inhibited mixture solutions for 30 min, followed by washing with 0.05% Tween-20 and PBS buffer. Then, the electrochemical detection of enzyme activity was performed in pH 7.4 PBS containing 1.0 mM ATCh. The inhibition efficiency (I %) was calculated as

$$I\% = 100 \times \%(i_{p,control} - i_{p,exp})/i_{p,control}$$

where $i_{p,\,control}$ and $i_{p,\,exp}$ are the peak currents of ATCh on the AChE-anti-AChE/MWCNTs−Au/SPCE before and after paraoxon inhibition, respectively.

dx.doi.org/10.1021/es202689u | *Environ. Sci. Technol.* 2012, 46, 1828−1833

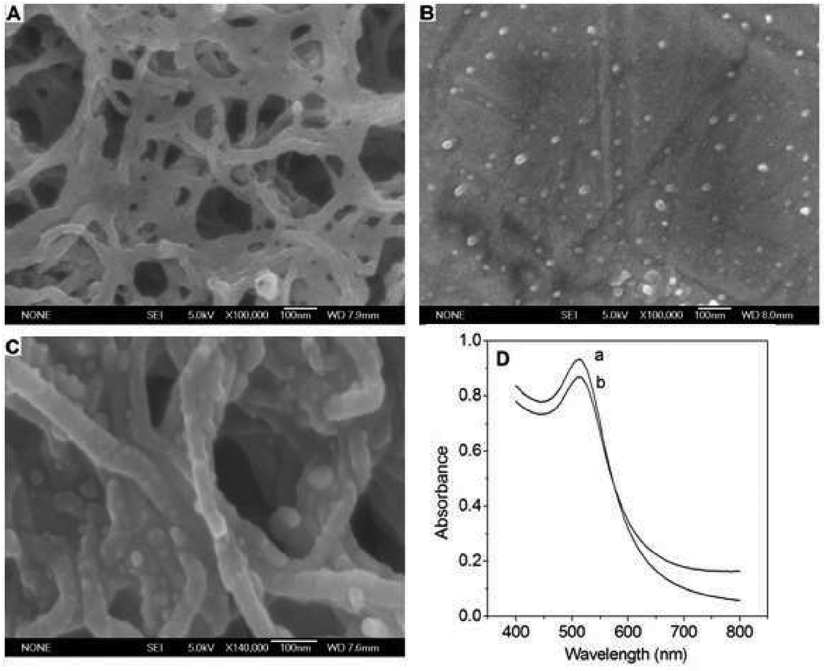


Figure 1. SEM image of (A) MWCNTs, (B) Au nanoparticles, and (C) synthesized MWCNTs–Au nanocomposites. (D) UV–vis absorption spectroscopy of Au nanopartiles (a) and MWCNTs–Au nanocomposites (b).

RESULTS AND DISCUSSION

Characterization of MWCNTs–Au Nanocomposites. Figure 1 shows SEM images of MWCNTs (A), Au nanoparticles (B), and synthesized MWCNTs–Au nanocomposites (C). One can see that MWCNTs–Au nanocomposites showed different morphology, indicating that MWCNTs have been nicely decorated with Au nanoparticles. The average size of Au nanoparticles was 15–20 nm. The formation of Au nanoparticles on the MWCNTs was further investigated using UV–vis absorption spectroscopy. As shown in Figure 1D, both Au nanopartiles (curve a) and MWCNTs–Au nanocomposites (curve b) display an absorption band at 512 nm, which is a characteristic peak of well-dispersed Au nanoparticles, indicating that Au nanoparticles have been efficiently deposited onto the MWCNTs without congregation. From the loss of intensity at 512 nm, we make a rough estimate of 95% Au nanoparticles loaded on the MWCNTs.

Electrochemical Characterization of AChE Activity on Immunosensor. The activity of AChE and enzymatic product thiocholine are investigated on anti-AChE antibody based-immunosensor. As shown in Figure 2A, no detectable redox peak was observed at anti-AChE/MWCNTs–Au/SPCE in pH 7.4 PBS containing 1.0 mM ATCh (curve a). However, after incubating with 3 nM AChE for 30 min, the resulting AChE-anti-AChE/MWCNTs–Au/SPCE showed an obvious oxidation peak at 660 mV (curve c). This peak is attributed to the oxidation of thiocholine, hydrolysis product of ATCh which is catalyzed by the captured AChE on the immunosensor. Furthermore, one can see that the oxidation current obtained on AChE-anti-AChE/MWCNTs–Au/SPCE (curve c) is much higher than that on AChE-anti-AChE/SPCE (curve b) and the peak potential shifted negatively 200 mV. This is due to the excellent conductivity of MWCNTs–Au nanocomposites which greatly facilitates electron transfer of thiocholine, electroactive product from enzymatic reactions at low potential. Square wave voltammetry (SWV) measurements were further

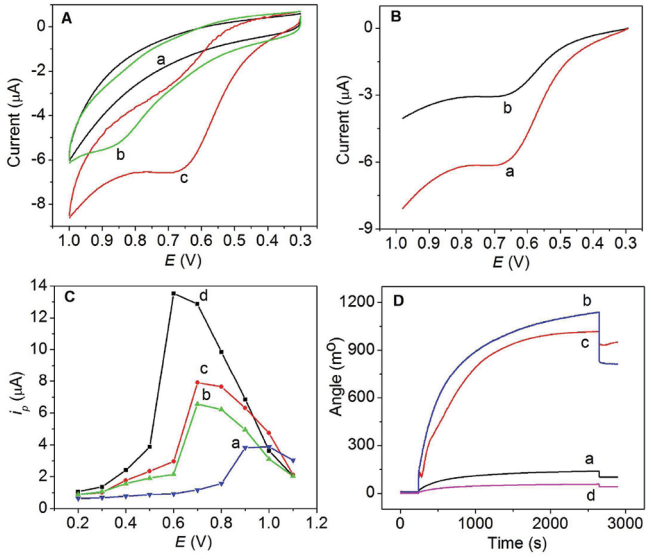


Figure 2. (A) Cyclic voltammograms obtained at anti-AChE/MWCNTs–Au/SPCE (a), AChE-anti-AChE/SPCE (b), and AChE-anti-AChE/MWCNTs–Au/SPCE (c) in pH 7.4 PBS containing 1.0 mM ATCh. (B) Square wave voltammograms of 3 nM AChE in the absence of paraoxon (a) and with 25 nM paraoxon for reaction of 30 min (b) at anti-AChE/MWCNTs–Au/SPCE. (C) Hydrodynamic voltammograms of thiocholine on SPCE (a), MWCNTs/SPCE (b), Au/SPCE (c), and MWCNTs–Au/SPCE (d). (D) SPR responses of AChE on MWCNTs–Au nanocomposites modified chip (a) and AChE (b), OP-AChE (c), and BChE (d) on anti-AChE/MWCNTs–Au nanocomposites modified chips.

used to understand OP inhibition on enzyme activity (Figure 2B). When 25 nM paraoxon was dispersed in 3 nM AChE for reaction of 30 min, the peak current decreased greatly (curve b) compared to that of 3 nM AChE in the absence of paraoxon (curve a). It is evident that paraoxon as model OP pesticide

dx.doi.org/10.1021/es202689u | Environ. Sci. Technol. 2012, 46, 1828–1833

clearly reduced AChE activity, resulting in a decreased electrochemical response.

Figure 2C shows the hydrodynamic voltammograms of thiocholine on SPCE (curve a), MWCNTs/SPCE (curve b), Au/SPCE (curve c), and MWCNTs–Au/SPCE (curve d), respectively. It can be seen that both MWCNTs/SPCE (curve b) and Au/SPCE (curve c) improved the electrocatalytic oxidation of thiocholine compared with that on SPCE (curve a), while the MWCNTs–Au/SPCE (curve d) exhibited the largest catalytic oxidation current and lowest oxidation potential, indicating that MWCNTs–Au nanocomposite is the best electrocatalyst to the oxidation of thiocholine. These data concluded that the nanocomposite with both MWCNTs and Au nanoparticles shows a synergistic effect on the electrocatalytic oxidation to thiocholine. Such synergistic effects have also been observed in quantum dots–gold nanoparticles composites[29] and prussian blue–MWCNTs nanocomposite[30,31] for improving electrocatalytic reaction.

The immune-affinities of anti-AChE antibody to both AChE and OP-AChE were studied by SPR (Figure 2D). One can see that the incubation of AChE or OP-AChE with anti-AChE/MWCNTs–Au nanocomposites modified SPR chips resulted in a SPR angle shift 1140 m° (curve b) and 1125 m° (curve c), respectively. These data illustrate that anti-AChE antibody can recognize both AChE and OP-AChE (1.0 nM) in almost the same affinity. Therefore, we may conclude that the decrease of peak current at the anti-AChE based immunosensor (Figure 2B) stems from the formation of phosphorylated adducts, rather than different immune-affinities. We further use BChE as control to demonstrate the selectivity of the AChE specific-antibody. The SPR curve shows very small angle shift (curve d). All these results demonstrate that immuno-capture approach has high selectivity to AChE. It is capable of selectively assaying AChE activity in samples. It is observed that AChE causes a perceptible SPR angle shift 126 m° on MWCNTs–Au nanocomposites modified SPR chips even without anti-AChE (curve a). The SPR angle shift can be attributed to the existence of nonspecific absorption of AChE to the chips.

Establishment of Quantitative Relationship between Enzyme Activity and Its Concentration. Standard AChE solutions were used to explore the quantitative relationship between enzyme activity and its concentration. It can be seen that the electrochemical signals increase with an increase of AChE concentrations (Figure 3A). A linear response is obtained over the concentration range from 0.1 to 10 nM, with the regression equation of i_p (μA) = 1.548 + 1.550lgc (nM) and the correlation coefficient of 0.9948 (Figure 3B). The detection limit is 0.05 nM. Although it is higher than that of our reported result of 5 pM using carbon nanotube-based electrochemical sensor for assay of salivary cholinesterase enzyme activity,[14] the sensitivity of the method developed in this work is sufficient for AChE activity assay in RBCs. Besides, this method is highly selective for AChE activity assay by using selective antibody and avoids the problem of overlapping substrate specificity with esterases and avoids the potential interferences of electroactive species in biological samples.

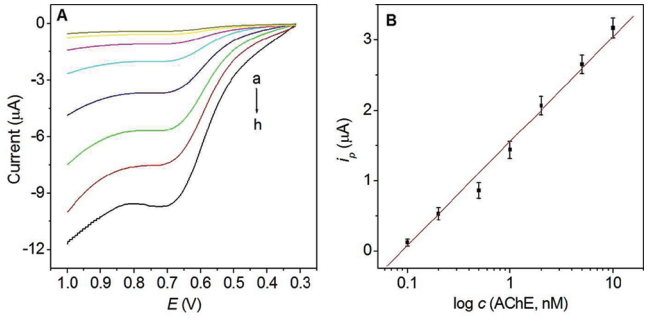


Figure 3. (A) Square wave voltammograms of AChE activity assay in pH 7.4 PBS containing 1.0 mM ATCh after immuno-capture of AChE from solution. The concentrations of AChE are 0 (a), 0.1 (b), 0.2 (c), 0.5 (d), 1.0 (e), 2.0 (f), 5.0 (g), and 10 (h) nM, respectively. (B) Calibration curve for measurement of AChE activity.

Immunosensing Platform for Measurement of AChE Activity in OP-Dosed AChE Solutions. To demonstrate the capability of this new biomonitoring method, AChE solutions dosed with different concentrations of paraoxon were used to validate the new AChE assay method. A series of different concentrations of paraoxon was incubated with 3 nM AChE for 30 min and the mixture was then incubated with anti-AChE antibody based-immunosensor followed by electrochemical detection of AChE activity. As shown in Figure 4A, the SWV signal decreased with increasing concentration of incubated

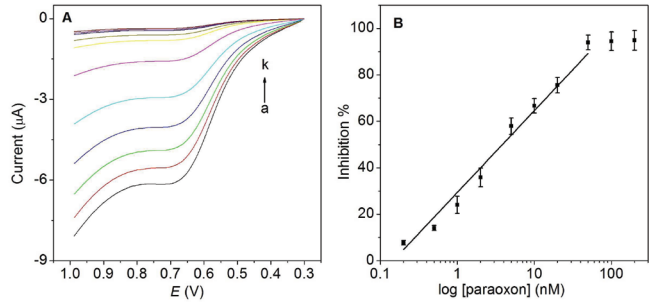


Figure 4. (A) Square wave voltammograms of AChE activity assay in pH 7.4 PBS containing 1.0 mM ATCh after immuno-capture of AChE from solution. Three nM AChE was exposed to paraoxon of 0 (a), 0.2 (b), 0.5 (c), 1.0 (d), 2.0 (e), 5.0 (f), 10 (g), 20 (h), 50 (i), 100 (j), and 200 (k) nM. (B) Plot of AChE inhibition vs paraoxon dosage.

paraoxon. The plot of AChE inhibition vs log[paraoxon] showed a wide linearity ranging from 0.2 to 50 nM (Figure 4B). These results indicate that this new AChE activity assay method can be used for the biomonitoring of exposure to OP.

The intra-assay precision of the immunosensor was evaluated by assaying one electrode for six replicate determinations, and the relative standard deviations (RSD) were less than 8%. When the immunosensor was not in use, it was stored at 4 °C. The immunosensor retained 85% of its initial response after 30-days storage. The RSD of interassay on six immunosensors was 9.3%.

Measurement of AChE Activity in Paraoxon-Dosed RBCs Samples. The new AChE activity assay method for biomonitoring of OP exposure was further validated with in vitro paraoxon-dosed RBC samples. The results are listed in Table 1. Using pre-exposure RBCs as control, the increased inhibition indicated a decrease in enzyme activity in RBCs samples, which was directly related to paraoxon exposure. It is sensitive enough to detect less than 5% inhibition. The developed immunosensor offers a new approach for rapid, selective, and sensitive assay of AChE enzyme activity for biomonitoring of exposure to OP pesticides as well as chemical nerve agents.

Table 1. Detection of AChE activity in Paraoxon-Dosed RBCs Samples

	control	sample 1	sample 2	sample 3	sample 4
added paraoxon (nM)	0	0.2	1.0	5.0	20
calculated inhibition from control	0	4.03%	23.6%	58.5%	80.6%

AUTHOR INFORMATION

Corresponding Author

*E-mail: dudan@mail.ccnu.edu.cn (D.D.); yuehe.lin@pnnl.gov (Y.L.).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (21075047), the Program for Chenguang Young Scientist for Wuhan (200950431184), and the Special Fund for Basic Scientific Research of Central Colleges (CCNU10A02005). Y.L. acknowledges the financial support by the CounterACT Program, Office of the Director, National Institutes of Health (OD) and the National Institute of Neurological Disorders and Stroke (NINDS), Grant U01 NS058161-01. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. Pacific Northwest National Laboratory is operated for DOE by Battelle under Contract DE-AC05-76L01830.

REFERENCES

(1) Quinn, D. M. Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states. *Chem. Rev.* **1987**, *87* (5), 955–979.

(2) Minton, N. A.; Murray, V. S. A review of organophosphate poisoning. *Med. Toxicol. Adverse Drug Exp.* **1988**, *3* (5), 350–375.

(3) Noort, D.; Benschop, H. P.; Black, R. M. Biomonitoring of exposure to chemical warfare agents: A review. *Toxicol. Appl. Pharmacol.* **2002**, *184* (2), 116–126.

(4) Worek, F.; Thiermann, H.; Szinicz, L.; Eyer, P. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. *Biochem. Pharmacol.* **2004**, *68* (11), 2237–2248.

(5) Lockridge, O.; Schopfer, L. M.; Masson, P. Chapter 56 in *Handbook of Toxicology of Chemical Warfare Agents*; Gupta, R. C., Ed.; Academic Press, 2009; pp 847–858.

(6) Thiermann, H.; Kehe, K.; Steinritz, D.; Mikler, J.; Hill, I.; Zilker, T.; Eyer, P.; Worek, F. Red blood cell acetylcholinesterase and plasma butyrylcholinesterase status: Important indicators for the treatment of patients poisoned by organophosphorus compounds. *Arch. Ind. Hyg. Toxicol.* **2007**, *58* (3), 359–366.

(7) Worek, F.; Aurbek, N.; Wetherell, J.; Pearce, P.; Mann, T.; Thiermann, H. Inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds: Pig versus minipig acetylcholinesterase. *Toxicology* **2008**, *244* (1), 35–41.

(8) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7* (2), 88–95.

(9) Godoy, S.; Leca-Bouvier, B.; Boullanger, P.; Blum, L. J.; Girard-Egrot., A. P. Electrochemiluminescent detection of acetylcholine using acetylcholinesterase immobilized in a biomimetic Langmuir-Blodgett nanostructure. *Sensor. Actuat. B* **2005**, *107* (1), 82–87.

(10) Sabelle, S.; Renard, P.; Mioskowski., C. Design and synthesis of chemiluminescent probes for the detection of cholinesterase activity. *J. Am. Chem. Soc.* **2002**, *124* (17), 4874–4880.

(11) Vamvakaki, V.; Fournier, D.; Chaniotakis., N. A. Fluorescence detection of enzymatic activity within a liposome based nanobiosensor. *Biosens. Bioelectron.* **2005**, *21* (2), 384–388.

(12) Maeda, H.; Matsuno, H.; Ushida, M.; Katayama, K.; Saeki, K.; Itoh, N. 2,4-Dinitrobenzenesulfonyl fluoresceins as fluorescent alternatives to ellman's reagent in thiol-quantification enzyme assays. *Angew. Chem. Int. Ed.* **2005**, *44* (2), 2922–2925.

(13) Henn, B. C.; McMaster, S.; Padilla, S. Measuring cholinesterase activity in human saliva. *J. Toxicol. Environ. Health Part A* **2006**, *69* (19), 1805–1818.

(14) Wang, J.; Timchalk, C.; Lin, Y. H. Carbon nanotube-based electrochemical sensor for assay of salivary cholinesterase enzyme activity: An exposure biomarker of organophosphate pesticides and nerve agents. *Environ. Sci. Technol.* **2008**, *42* (7), 2688–2693.

(15) Du, D.; Wang, J.; Smith, J. N.; Timchalk, C.; Lin, Y. H. Biomonitoring of organophosphorus agent exposure by reactivation of cholinesterase enzyme based on carbon nanotube-enhanced flow-injection amperometric. *Anal. Chem.* **2009**, *81* (22), 9314–9320.

(16) Tecles, F.; Ceron, J. J. Determination of whole blood cholinesterase in different animal species using specific substrates. *Res. Vet. Sci.* **2001**, *70* (3), 233–238.

(17) Kolf-Clauw, M.; Jez, S.; Ponsart, C.; Delamanche, I. S. Acetyl- and pseudo-cholinesterase activities of plasma, erythrocytes, and whole blood in male beagle dogs using Ellman's assay. *Vet. Hum. Toxicol.* **2000**, *42* (4), 216–219.

(18) Reiner, E.; Sinko, G.; Skrinjaric-Spoljar, M.; Simeon-Rudolf, V. Comparison of protocols for measuring activities of human blood cholinesterases by the Ellman method. *Arh. Hig. Rada Toksikol.* **2000**, *51* (1), 13–18.

(19) Kikuchi, T.; Okamura, T.; Fukushi, K.; Irie, T. Piperidine-4-methanthiol Ester Derivatives for a Selective Acetylcholinesterase Assay. *Biol. Pharm. Bull.* **2010**, *33* (4), 702–706.

(20) Gordon, R. K.; Haigh, J. R.; Garcia, G. E.; Feaster, S. R.; Riel, M. A.; Lenz, D. E.; Aisen, P. S.; Doctor, B. P. Oral administration of pyridostigmine bromide and huperzine A protects human whole blood cholinesterases from ex vivo exposure to soman. *Chem. Biol. Interact.* **2005**, *157–158*, 239–246.

(21) Haigh, J. R.; Lefkowitz, L. J.; Capacio, B. R.; Doctor, B. P.; Gordon, R. K. Advantages of the WRAIR whole blood cholinesterase assay: Comparative analysis to the micro-Ellman, Test-mate ChE (TM), and Michel ([Delta] pH) assays. *Chem. Biol. Interact.* **2008**, *175* (1–3), 417–420.

(22) Du, D.; Chen, S. Z; Cai, J.; Zhang, A. D. Immobilization of acetylcholinesterase on gold nanoparticles embedded in sol-gel film for amperometric detection of organophosphorous insecticide. *Biosens. Bioelectron.* **2007**, *23* (1), 130–134.

(23) Du, D.; Chen, S. Z.; Cai, J.; Song, D. D. Comparison of drug sensitivity using acetylcholinesterase biosensor based on nanoparticles-chitosan sol-gel composite. *J. Electroanal. Chem.* **2007**, *611* (1–2), 60–66.

(24) Du, D.; Zou, Z. X.; Shin, Y.; Wang, J.; Wu, H.; Engelhard, M. H.; Liu, J.; Aksay, I. A.; Lin, Y. H. Sensitive immunosensor for cancer biomarker based on dual signal amplification strategy of graphene sheets and multienzyme functionalized carbon nanospheres. *Anal. Chem.* **2010**, *82* (7), 2989–2995.

(25) Du, D.; Wang, L. M; Shao, Y. Y; Wang, J.; Engelhard, M. H.; Lin, Y. H. Functionalized graphene oxide as a nanocarrier in a multienzyme labeling amplification strategy for ultrasensitive electrochemical immunoassay of phosphorylated p53 (S392). *Anal. Chem.* **2011**, *83* (3), 746–752.

(26) Du, D.; Ding, J. W.; Cai, J.; Zhang, A. D. Electrochemical thiocholine inhibition sensor based on biocatalytic growth of Au nanoparticles using chitosan as template. *Sens. Actuators B* **2007**, *127* (2), 317–322.

(27) Hammond, P. I.; Kern, C.; Hong, F.; Kollmeyer, T. M.; Pang, Y. P.; Brimijoin, S. Cholinesterase reactivation in vivo with a novel bis-oxime optimized by computer-aided design. *J. Pharmacol. Exp. Ther.* **2003**, *307* (1), 190–196.

(28) Liu, G. D.; Wang, J.; Barry, R.; Petersen, C.; Timchalk, C.; Gassman, P.; Lin., Y. H. Nanoparticle-based electrochemical immunosensor for the detection of phosphorylated acetylcholinesterase: An exposure biomarker of organophosphate pesticides and nerve agents. *Chem.—Eur. J.* **2008**, *14* (32), 9951−9959.
(29) Du, D.; Chen, S. Z.; Song, D. D.; Li, H. B.; Chen., X. Development of acetylcholinesterase biosensor based on CdTe quantum dots/gold nanoparticles modified chitosan microspheres interface. *Biosens. Bioelectron.* **2008**, *24* (3), 475−479.
(30) Zeng, J. X.; Wei, W. Z.; Liu, X. Y.; Wang, Y.; Luo, G. M. A simple method to fabricate a Prussian Blue nanoparticles/carbon nano-tubes/poly (1,2-diaminobenzene) based glucose biosensor. *Microchim. Acta* **2008**, *160* (3), 261−267.
(31) Ricci, F.; Amine, A.; Moscone, D.; Palleschi, G. Prussian blue modified carbon nanotube paste electrodes: A comparative study and a biochemical application. *Anal. Lett.* **2003**, *36* (9), 1921−1938.