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Magnetic Electrochemical Immunoassays with Quantum Dot Labels for Detection of Phosphorylated Acetylcholinesterase in Plasma

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A new magnetic electrochemical immunoassay has been developed as a tool for biomonitoring exposures to organophosphate (OP) compounds, e.g., insecticides and chemical nerve agents, by directly detecting organophosphorylated acetylcholinesterase (OP−AChE). This immunoassay uniquely incorporates highly efficient magnetic separation with ultrasensitive square wave voltammetry (SWV) analysis with quantum dots (QDs) as labels. A pair of antibodies was used to achieve the specific recognition of OP−AChE that was prepared with paraoxon as an OP model agent. Antiphosphoserine polyclonal antibodies were anchored on amorphous magnetic particles preferably chosen to capture OP−AChE from the sample matrixes by binding their phosphoserine moieties that were exposed through unfolding the protein adducts. This was validated by electrochemical examinations and enzyme-linked immunosorbent assays. Furthermore, antihuman AChE monoclonal antibodies were labeled with cadmium-source QDs to selectively recognize the captured OP−AChE, as characterized by transmission electron microscopy. The subsequent electrochemical SWV analysis of the cadmium component released by acid from the coupled QDs was conducted on disposable screen-printed electrodes. Experimental results indicated that the SWV-based immunoassays could yield a linear response over a broad concentration range of 0.3−300 ng/mL OP−AChE in human plasma with a detection limit of 0.15 ng/mL. Such a novel electrochemical immunoassay holds great promise as a simple, selective, sensitive, and field-deployable tool for the effective biomonitoring and diagnosis of potential exposures to nerve agents and pesticides.

Neurotoxic organophosphates (OPs) have been widely used as pesticides in the agricultural industry and as chemical warfare agents.[1,2] As a result, OP contaminations have been widespread in air, water, soil, and food, such that there is a potential for human exposure. Therefore, public concern about the development of detection devices for effectively monitoring OPs and evaluating the human health risk of OP exposure has grown steadily in recent years.[3−5] Moreover, after the Tokyo subway attack in 1995,[6] the need for feasible OP detection methods has become increasingly urgent for the purpose of early warning of potential terrorist attacks and diagnostic mitigation of the effects from alleged nerve agent exposures.[2,3,5−7]

In recent decades, numerous analysis methods have been developed to assess OP exposures, and the relevance of end points to human health is of utmost concern.[3,8−10] In this regard, biomonitoring of OP exposures is recognized to be one of the best approaches.[8] The internal dosages of OP agents or their metabolites are quantitatively or qualitatively measured on the basis of our knowledge of the metabolic fate of the toxicants, thus providing an accurate evaluation of the health risk of integrated OP exposure. Unfortunately, the majority of current biomonitoring protocols for OP exposures[3,8,11,12] may still suffer from some intrinsic disadvantages of either low detection specificity and sensitivity (i.e., Ellman colorimetric assays[13,14]) or expensive analysis settings entailing well-trained personnel and inconvenience for field applications (i.e., gas or liquid chromatography coupled with mass spectrometry, GC− or LC−MS) [4,15−17]). Hence, simple, sensitive, selective, and field-deployable tools are still highly desired for biomonitoring and diagnostic evaluation of OP exposures, especially to enhance our response to a sudden

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emergency and improve our ability to medically counteract the effects.

It is generally recognized that selecting suitable biomarkers of OP exposures is of central importance for developing a biomonitoring strategy.[8,12] Biomarkers that are currently used include free OPs in blood, or their metabolites in urine, and cholinesterase (ChE) inhibition in blood.[3,8,9,12] OPs can stoichiometrically bind with ChE and inhibit enzyme activity; at the same time, they are metabolized by organophosphorus hydrolase to form inactive phosphonic acids that are then renally excreted.[8] The high reactivity of OPs with these enzymes suggests that the levels of free OPs will be inherently low (typically in the range of nanogram per liter or parts per trillion in blood[12]), such that ultrasensitive detection methodologies are thereby required. However, the formidable false positive signals might be difficult to avoid. Moreover, while the OP metabolite level in urine is also considered a sensitive indicator of OP exposure,[8,18] the fact that not all toxicant-specific metabolites are derived solely from OPs is a real concern.[12] Also, ChE inhibition as a biomarker of an OP exposure effect has historically been an important strategy, but the inhibition-based quantification can also be problematic.[12,19] For example, for a quantitative assessment, a baseline enzyme level is required to accommodate the individual fluctuations in enzyme levels. All of these factors thereby make the blood ChE measurements less viable for assessing some OP exposures.[12] Therefore, exploring selective, sensitive, and reliable alternative biomarkers is an important consideration for biomonitoring of OP exposures.

Electrochemical immunoassays with high selectivity and sensitivity have evolved rapidly over the past decades.[20−23] Their detection sensitivity may be enhanced with various nanoscale materials newly emerged, i.e., QDs,[24] for electrochemical signal amplifications.[23,25] Such a versatile analysis tool can possess some advantages over the present standard methods for assessing OP exposures that are based on GC−MS and LC−MS.[20,21,26] More importantly, their simple operation and miniaturized analysis instruments can meet the requirements of decentralized point-of-care tests or field detections.[21,22,27] Moreover, according to the biochemical mechanism widely accepted for ChE phosphorylation,[28,29] the inhibition event may produce very stable enzyme complexes with structurally precise phosphoserine esters,[30] suggesting that these products may serve as selective indicators directly correlated to the severity of OP exposures. However, a challenge may lie in the current unavailability of recognition elements or appropriate receptors, i.e., antibodies, for specifically targeting phosphorylated ChE. Although some specific antibodies against OP agents, e.g., paraoxon, have been recently developed and are commercially available for immunoassays,[31−33] they might be unable to selectively recognize modified or aged OP moieties of OP-AChE, which is addressed in this study.

In this work, we present preliminary studies to establish a novel electrochemical immunoassay of phosphorylated acetylcholinesterase (AChE) as a biomarker for biomonitoring and diagnosis of OP exposures. OP-AChE, which was prepared by incubating human AChE with paraoxon, was used as the model target. To circumvent the current limitations of OP-AChE recognition, two kinds of antibodies, antiphosphoserine polyclonal antibodies (termed as Ab_1) and antihuman AChE monoclonal antibodies (termed as Ab_2), were employed to facilitate the specific recognition of OP-AChE. Amorphous magnetic particles (MPs) with a large surface-to-volume ratio were chosen to load Ab_1 to capture the OP-AChE from the sample matrixes by binding the phosphoserine moieties, which were disclosed through reductively unfolding the protein adducts via dithiothreitol (DDT). This was followed by the second recognition of Ab_2 labeled with quantum dots (QDs) serving as the signal-amplifying tags. The sandwich immunoreaction events were subsequently quantified by square wave voltammetric (SWV) analysis. The main parameters governing the SWV responses were optimized, including the MP−Ab_1 conjugate dosage, QD−Ab_2 label concentration, and reaction time. Moreover, a blocking agent consisting of 3% bovine serum albumin (BSA) and 1% poly(ethylene glycol) (PEG) was introduced for effective minimization of nonspecific adsorptions in the immunoassays. The magnetic immunoassay was overall proofed with simple, selective, and sensitive analysis features by spiking human plasma with known OP-AChE concentrations.

EXPERIMENTAL SECTION

Reagents and Materials. The Qdot@655 antibody conjugation kit, which was purchased from Molecular Probes Inc. (Eugene, OR), includes QDs (CdS@ZnS), succinimidyl *trans*-4-(*N*-maleimidylmethyl)cyclohexane-1-carboxylate solution, DDT solution, dye-labeled marker for antibody elution, mercaptoethanol, separation media, and exchange buffer. The BioMag magnetic immobilization kit was obtained from Polysciences Inc. (Warrington, PA) with amine-terminated amorphous magnetic particles (MPs, 50 mg/mL), glutaraldehyde (GLU) coupling reagent, and glycine quenching solution. Human AChE, PEG (MW 10 kDa), BSA, hydroxylamine, phosphate buffer saline (PBS), Tris-HCl stock buffer (1.0 M), and Tween-20 were the products of Sigma-Aldrich. Ab_1 and their horseradish peroxidase (HRP) conjugate as well as Ab_2 were purchased from Abcam Inc. (Cambridge, MA). Paraoxon was

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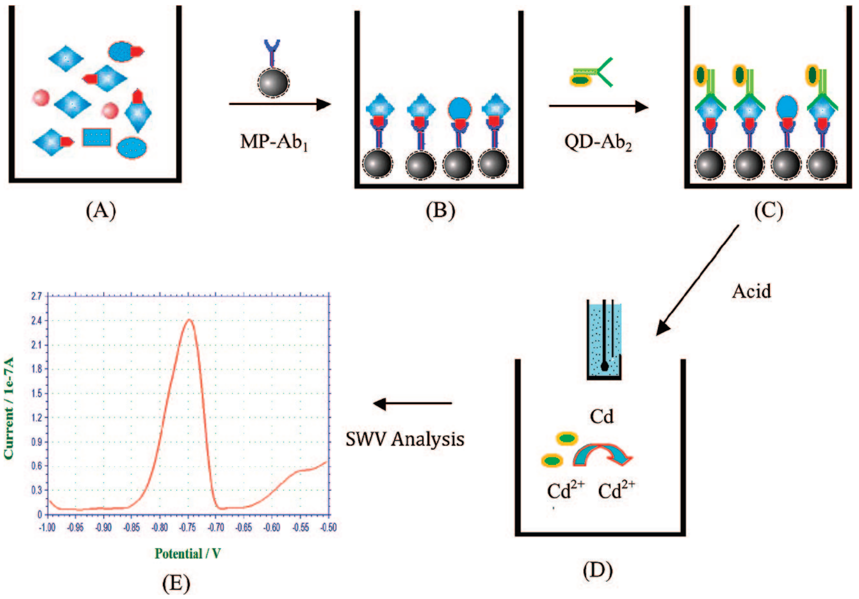

Figure 1. Schematic illustration of magnetic electrochemical immunoassays of OP−AChE: (A) plasma samples, (B) magnetic capture of OP−AChE using amorphous MP−Ab$_1$ conjugates, (C) selective recognition of bound OP−AChE using QD−Ab$_2$ labels, (D) electrochemical SWV analysis of cadmium released by acid from the captured QDs, and (E) representative SWV signal output.

bought from Chem Service, Inc. (West Chester, PA). The BSA−PEG blocking agent consists of 3% BSA and 1% PEG in Tris-HCl buffer (0.02 M), and the pH was adjusted to 7.4 with 0.1 M HCl. The washing buffer was prepared with 0.02 M Tris-HCl buffer containing 0.1% Tween-20, 0.5% BSA, and 0.15 M NaCl. All stock and buffer solutions were prepared with Nanopure-purified water. Other reagents were of analytical reagent grade.

Instruments. The MCB 1200 Biomagnetic Processing Platform was the product of Dexter Magnetic Technologies (Sigris, CA). SWV measurements were performed with an electrochemical analyzer CHI 660 (CH Instruments, Austin, TX), which is connected to a personal computer. Disposable screen-printed electrodes (SPEs), consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode, were purchased from Alderon Biosciences Inc. (Durham, NC). A sensor connector allows for connecting the SPE to the CHI electrochemical analyzer. A transmission electron microscope (TEM, Hitachi H-7000) was used to characterize the sample suspensions, each of which was dropped onto the carbonate film-coated copper grids (3-mm diameter, 200 mesh) to be dried at room temperature and then measured at 75 kV. Microplates for enzyme-linked immunosorbent assay (ELISA) were purchased from Becton (Franklin Lakes, NJ). Disposable PD-10 desalting columns packed with Sephadex G-25 medium (Amersham Bioscience Corp.) were used to purify the protein solutions. Centrifugation was performed with a Sorvall RC 26 plus (Kendro Laboratory Product). A Vortex mixer with touch on−off mixing functions (Barnstead International) was used for sample and reactant mixing.

Preparation of MP−Ab$_1$ Conjugates. The MP−Ab$_1$ conjugates were prepared following the modified protocol available in the BioMag magnetic immobilization kit. Typically, 1.0 mL of BioMag amorphous MP suspension terminated with amine groups was transferred to a 2.5-mL centrifuge tube to be washed three times with 0.5 mM PBS (pH 7.4) and then magnetically separated on the Biomagnetic processing platform. The supernatant was aspirated out, leaving the BioMag packed as a wet cake on the tube wall. Two milliliters of GLU (5%) was added to the BioMag to be vortexed and then rotated for 3 h at room temperature. Furthermore, magnetic separation was conducted to remove the unreacted GLU. After being washed four times with 0.5 mM PBS buffer, 1.0 mL of 0.5 mg/mL Ab$_1$, which were prepared in 0.5 mM PBS, were then added to the GLU-activated BioMag particles. The mixture was vortexed and further rotated overnight at room temperature, followed by magnetic separation and two washings. Subsequently, 5.0 mL of glycine solution was introduced into the coupled BioMag to be further rotated for 30 min at room temperature to quench the activated BioMag that was possibly left and then magnetically separated and washed three times. In the immunoassays, the MP−Ab$_1$ suspension was diluted with the BSA−PEG blocking agent to 1.0 mg/mL MP particle concentration, which was defined as the stock suspension of MP−Ab$_1$ conjugates.

Preparation of QD−Ab$_2$ Labels. The QD−Ab$_2$ labels were prepared following the protocol from the Qdot@655 antibody conjugation kit. Basically, 0.5 mg/mL Ab$_2$ was first reduced by DDT to obtain Fab′ fragments with free sulfhydryls by unfolding the disulfide bonds at the Fc region of the antibodies. The amine-terminated QDs (TEM image shown in the top right of Figure 2) were preactivated by succinimidyl *trans*-4-(*N*-maleimidylmethyl) cyclohexane-1-carboxylate and then added to the resultant antibody solution to be mixed and reacted for 1 h at room temperature. After being quenched by mercaptoethanol, the mixture was concentrated by ultrafiltration and purified with size-exclusion chromatography. The QD−Ab$_2$ suspension was orange-red. Each QD nanocrystal was estimated to load about eight antibody fragments or four intact antibodies[24] with the well-retained activity of the binding antigens. In the immunoassays, the QD−Ab$_2$ suspension was further diluted by the BSA−PEG blocking agent at the optimized dilution ratio of 1/120 (v/v), which was defined as the stock suspension of QD−Ab$_2$ labels.

Preparation of OP−AChE. OP−AChE was prepared by the incubation of AChE with paraoxon as a model of OPs in 50 mM

PBS buffer solution. Briefly, 1.0 mL of a PBS buffer solution containing 240 μg of human AChE was mixed with 50.0 μL of acetone containing 34 μg of paraoxon and incubated overnight. The resulting solution was purified with a PD-10 column to remove unbound paraoxon and solvent acetone. The enzyme activity of the product was further determined with the Ellman assay until it was completely inhibited.[13] Moreover, the resultant OP-AChE was concentrated with ultrafiltration to a final volume of $\sim$1.0 mL and stored at −20 °C for future use. The protein concentration of the OP−AChE stock solution was determined to be $\sim$0.2 mg/mL by spectrophotometry at 280 nm. Of note, all of the OP-AChE in the experiments referred to those that were reductively unfolded by 5.0 mM DDT for 15 min before the immunoassays, unless otherwise indicated.

ELISA for Antibody Immunoaffinities to OP−AChE. The immunoaffinities of Ab$_1$ and Ab$_2$ to OP−AChE were examined by ELISA using HRP-conjugated Ab$_1$ and Ab$_2$ whereas the comparable assays for non-DDT-unfolded OP−AChE, AChE, and BSA were conducted in parallel as the controls. A 50-μL aliquot of Ab$_2$ solution (1.0 μg/mL in the blocking solution) was first added into each well of the microplate with three replicates to be incubated overnight at 4 °C. After rinsing the plate wells three times with the washing buffer, a 250-μL aliquot of the BSA−PEG blocking agent was introduced to each well to be incubated for 1.5 h at 37 °C. Then, the plate was washed three times. Furthermore, a 50-μL aliquot of sample with the desired concentrations was added into each well to be incubated for 1 h at 37 °C, and the plates were washed three times. Following that, a 50-μL aliquot of HRP-conjugated Ab$_1$ solution (1.0 μg/mL) was added to each well, and the immunoreaction proceeded for 1 h at 37 °C. After the wells were rinsed four times, a 100-μL aliquot of the color substrate was added, and the enzyme-catalyzed reaction progressed for 10 min at room temperature. Subsequently, the enzymatic reaction was stopped by adding a 10-μL aliquot of 1.0 M HCl per well. The plate was immediately measured at 450 nm on a microplate reader, and the absorbance of each well was recorded separately.

Magnetic Electrochemical SWV Immunoassays. The 25-μL aliquots of samples, with the desired OP−AChE concentrations in Tris-HCl buffer (pH 7.4) or human plasma, were added separately into 1.5-mL plastic centrifuge tubes that were prewashed twice with the washing buffer. A 25-μL aliquot of MP−Ab$_1$ conjugate suspension (1.0 mg/mL) was then introduced into each tube to be vortexed by the touch on−off mixer, and it was further rotated on the Biomagnetic processing platform for 45 min at room temperature. The mixtures were magnetically separated simply by raising up the magnet at the platform and then rinsed twice with the washing buffer. Following the same procedure above, a 50-μL aliquot of QD−Ab$_2$ labels (1/120, v/v) was injected into each tube to be vortexed and rotated for 30 min at room temperature. The resulting mixtures were rinsed twice (the formed immunocomplex was diluted in Tris-HCl buffer for TEM measurements with the images shown in Figure 2). Moreover, a 10-μL aliquot of 1.0 M HCl was added into each tube to release cadmium ions from the captured QD labels, followed by the addition of a 50-μL aliquot of 0.20 M acetate buffer (pH 4.6) containing 10 μg/mL Hg and 0.5 μg/mL Bi. After mixing for 2 min, the BioMag was magnetically precipitated, resulting in a "wet cake" formed on the tube walls. A 50-μL aliquot of the supernatants was then drawn to be separately transferred to the surface of the SPEs, which were pretreated electrochemically by cyclic voltammetric scanning for 10 times at a potential range of 0−1.5 V in 50 mM PBS (pH 7.4). Subsequently, electrochemical SWV measurements were carried out with an in situ-plated Hg/Bi film formed on the SPEs by a 2-min accumulation at −1.4 V. The analysis parameters mainly included the applied potential range of −1.0 to 0.5 V, a step potential of 4 mV, an amplitude of 25 mV, and a frequency of 15 Hz. A baseline correction of the resulting voltammogram was performed with CHI 660A software (Note: the waste after each immunoassay should be collected in a bottle to be disposed of safely).

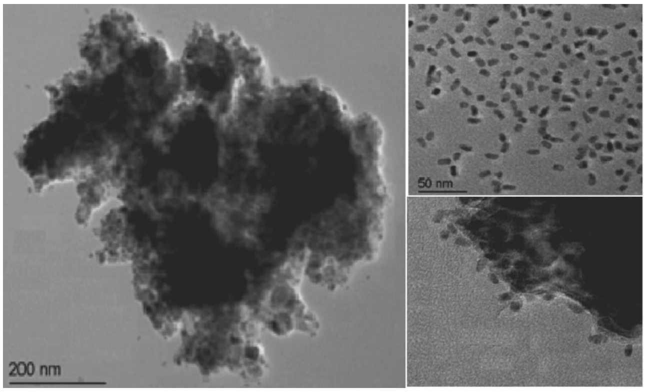

Figure 2. Typical TEM images of the formed sandwich immunocomplex, the QD tags used (top right), and part of the immunocomplex in high magnitude (bottom right).

RESULTS AND DISCUSSION

Principle of Magnetic Electrochemical Immunoassay Using QD Labels. It is recognized that OPs have the same general structure and toxicological mode of action.[28] In this work, paraoxon was used as a model of an OP nerve agent and pesticide to prepare the OP−AChE. Herein, the biochemical mechanism of OP inhibition of AChE has been well documented and understood. This process is initiated by precursory phosphorylation at the catalytic serine residue.[28−30] The phosphorylation of AChE by paraoxon is synchronous with the release of *p*-nitrophenoxy and further goes through an aging process to produce a stable, covalent phosphoserine ester bond or an OP−AChE conjugate.[30] The so formed OP−AChE can quantitatively correlate to the original OP dose, thus potentially serving as a selective OP biomarker. However, the detection of this biomarker may be problematic if specific antibodies cannot detect the modified protein. Moreover, in addition to binding with AChE at its peripheral anionic site,[34,35] OP (i.e., paraxon) is recognized mostly for phosphorylating the serine residues at the catalytic active center located at the base of a deep gorge within the proteins,[30,36] of which phosphoserine moieties are thought to be inaccessible to many traditional forms of analysis.

Inspired by the fate of OP−AChE formed with structurally precise phosphoserine esters, we attempted to use two kinds of

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commercially available antibodies, Ab_1 and Ab_2, to bind the phosphoserine and AChE moieties of the OP−AChE, respectively, achieving a specific recognition of the meaningful adducts. It is well established that ChE (i.e., AChE) is made up of subunits structurally containing inter- and intramolecular disulfide bonds[36−38] that can be cleaved by DDT serving as a reducing agent.[37,38] Accordingly, DDT was employed to reductively unfold OP−AChE to expose its phosphoserine moieties for more accessible immunorecognition. Magnetic separation was then combined with electrochemical SWV analysis using QDs as signal amplification tags to develop an electrochemical immunoassay method. Figure 1 schematically illustrates the process of electrochemical immunoassays for OP−AChE in a sandwich detection format, which was performed with centrifuge tubes and disposable SPEs. OP−AChE is first captured by MP−Ab_1 through binding the phosphoserine moieties to be magnetically separated from the sample matrixes. QD−Ab_2 is then introduced to selectively recognize the captured OP−AChE. The resultant immunocomplex is subsequently treated by acid to release cadmium ions from the bound QDs for SWV measurements on the SPEs. The yielded SWV signals are proportional to the OP−AChE concentrations in the samples. Figure 2 presents the typical TEM images of as-formed immunocomplex and QDs originally used (top right). One can note that amine-terminated MPs are amorphous, and the QD tags are uniform in size and short-rod shaped with dimensions of about 4 nm × 12 nm. Upon the immunorecognition of targeting OP−AChE, QDs are attached onto the MP surface forming the sandwich immunocomplex, as clearly shown in the high-magnitude TEM image (bottom right of Figure 2).

In this protocol, using amorphous MPs can achieve a higher surface-to-volume ratio for loading a larger amount of antibodies. The magnetic separation of OP−AChE can allow for the enrichment of the analytes from the samples to facilitate the electrochemical SWV detections conducted on the SPEs, which might largely avoid the formidable interferences from the complex sample media. Moreover, SWV with mercury film is recognized as an ultrasensitive technique for determining heavy metals due to its unique capability of preconcentrating target species in combination with pulse measurements that yields a high signal-to-background ratio. Additionally, QDs were coated with a thin layer of functionalized polymer providing terminal amine groups for antibody conjugation and spacing between the antibody and QD, which greatly increases the flexibility of antibodies on the surface of the QDs. Using QD tags for the SWV analysis may further benefit from the amplified response signal, achieving enhanced detection sensitivity of the immunoassays.

Evaluation of Antibody Immunoaffinities to OP−AChE. The manufacturer's instructions state that antiphosphoserine polyclonal antibodies are specific to free and conjugated phosphoserine as well as phosphoserine in modified cellular proteins without the cross-reaction with nonphosphorylated serine. However, it is not clear whether both Ab_1 and Ab_2 can specifically recognize the phosphorylated AChE that was reductively unfolded by DDT.

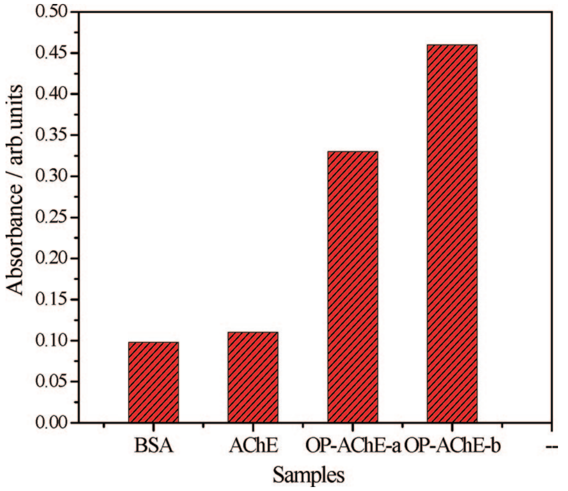

Figure 3. Typical ELISA responses to 10 mg/mL BSA, 300 ng/mL AChE, and 300 ng/mL (a) non-DDT-unfolded and (b) DDT-unfolded OP−AChE using HRP-labeled Ab_1 and pure Ab_2.

We first studied the immunoaffinity of Ab_1 and Ab_2 to OP−AChE by the classic ELISA method using HRP-conjugated Ab_1 and pure Ab_2. DDT-unfolded OP−AChE was employed as the test target, with non-DDT-unfolded OP–AChE, nonphosphorylated AChE, and BSA as the protein controls. Figure 3 displays the ELISA results. Obviously, the response to non-DDT-unfolded OP−AChE (a) is smaller than that to DDT-unfolded ones (b). Moreover, the responses obtained from the control experiments using AChE and high-concentration BSA are >2.1 times lower than that from OP−AChE. The ELISA data demonstrate that Ab_1 and Ab_2 could bring about good immunoaffinity and specificity to the phosphorylated AChE treated by DDT.

Furthermore, the immunoaffinities of MP−Ab_1 and QD−Ab_2 to OP−AChE were investigated by electrochemical measurements. Here, non-DDT-unfolded OP−AChE, nonphosphorylated pure AChE, BSA, and human plasma were simultaneously examined as the challenging protein rivals and sample background. The typical electrochemical responses are shown in Figure 4. Note that the current response for DDT-unfolded OP−AChE (curve a) is higher than that of non-DDT-unfolded ones (curve b), indicating the effective role of DDT in reductively unfolding the phosphorylated enzymes for maximizing exposure of their phosphoserine moieties. In contrast, negligibly low signals were observed in the control experiments for AChE (curve c), plasma (curve d), and BSA (curve e). Signals from the control experiments are presumably ascribed to the nonspecific adsorptions of QD−Ab_2 to the magnetic particles and centrifuge tubes used. Such a phenomenon may make it necessary to block both of the MP−Ab_1 conjugates and QD−Ab_2 labels as well as the plastic centrifuge tubes, which is discussed afterward. The signal differences between OP−AChE and these controls indicate that the possibly coexisting AChE and plasma background can have no significant interference with the magnetic electrochemical immunoassays for OP−AChE. The synergistic use of MP−Ab_1 conjugates and QD−Ab_2 labels can achieve desirable specific immunorecognition to OP−AChE.

Minimization of Nonspecific Adsorption of the Immunoassay. Nonspecific adsorption is one of the important issues to be addressed in the development of magnetic immunoassays with high selectivity and sensitivity. In the current study, we found

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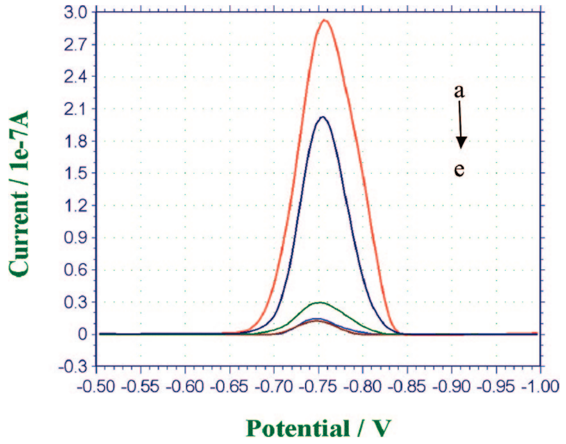

Figure 4. Comparison of SWV responses of the immunoassays to 300 ng/mL (a) DDT-unfolded and (b) non-DDT-unfolded OP−AChE, and (c) 300 ng/mL AChE, (d) plasma, and (e) 10 mg/mL BSA, all of which were prepared in Tris-HCl buffer (pH 7.4) to be detected separately under the experimental conditions including 45-min magnetic capture with MP−Ab_1 conjugates (1.0 mg/mL) and 30-min selective recognition with QD−Ab_2 labels (1/120 dilution, v/v).

Table 1. Comparison of Minimization Effectiveness of Nonspecific Adsorption for Immunoassays Using Different Blocking Agents[a]

	blocking agents (current, A)		
samples	none	3% BSA	3% BSA−1% PEG
OP-AChE	6.4×10^{-7}	3.4×10^{-7}	2.9×10^{-7}
AChE	4.3×10^{-7}	5.1×10^{-8}	3.0×10^{-8}
plasma	2.9×10^{-7}	3.3×10^{-8}	1.7×10^{-8}

[a] Each of the current responses is the average of triplicate measurements of the samples of 300 ng/mL OP−AChE, 300 ng/mL AChE, and plasma under the optimized experimental conditions as described in Figure 4, except for use of different blocking agents.

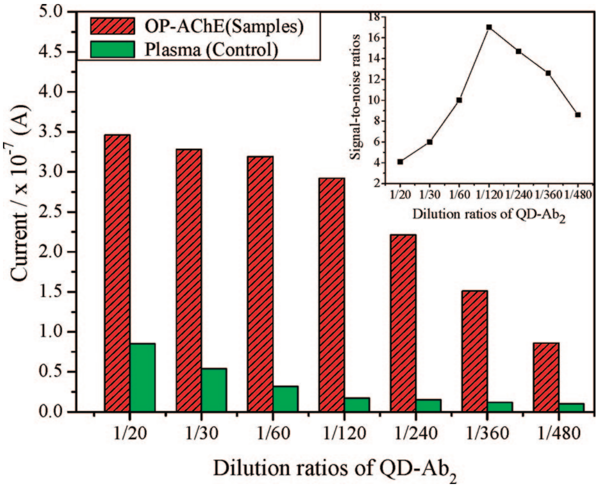

Figure 5. Effects of the dosage of QD−Ab_2 labels in dilutions on the SWV responses of the immunoassays where the detections for 300 ng/mL OP−AChE and plasma (control) were performed in parallel under the conditions as described in Figure 4, except for using Qdot−Ab_2 labels of different concentration dilutions.

that the immunoassays for OP−AChE could have serious nonspecific adsorptions, especially for probing the analytes in human plasma. To minimize such a nonspecific adsorption, we tried to separately introduce 3% BSA and the BSA−PEG mixture as the blocking agents to pretreat MP−Ab_1 conjugates and QD−Ab_2 labels prior to immunoassays. The minimization effectiveness of two kinds of blocking agents was evaluated with the same OP−AChE concentration together with nonphosphorylated AChE and plasma as the controls. The test results are summarized in Table 1. When no blocking step was carried out for two antibody conjugates, the current responses showed no significant difference among OP−AChE, AChE, and the plasma samples. However, when both antibody conjugates were separately blocked by 3% BSA, nonspecific adsorptions could be largely depressed. Herein, the current response for OP−AChE changed to 3.4×10^{-7} A, while the current responses for AChE and plasma were sharply down to 5.1×10^{-8} and 3.3×10^{-8} A, respectively. Moreover, nonspecific adsorptions could be further depressed when the BSA−PEG agent was used to block MP−Ab_1 conjugates and QD−Ab_2 labels. As is noted from Table 1, negligible current signals were observed for the protein rival (3.0×10^{-8} A) and plasma background (1.7×10^{-8} A), in contrast to the adduct sample (2.9×10^{-7} A). The above results imply that using the BSA−PEG blocking agent can achieve desirable minimization of nonspecific adsorptions in the immunoassays for OP−AChE. In addition, a pretreatment of the plastic centrifuge tubes with the BSA−PEG agent could be helpful for avoiding any nonspecific adsorption of QD tags onto the tube walls (data not shown).

Such an excellent limitation of nonspecific adsorptions may be attributed to the synergetic blocking effects of BSA and PEG that were simultaneously applied for MP−Ab_1 conjugates and QD−Ab_2 labels. BSA, a kind of small inert molecule of proteins, has been well recognized and commonly applied as a blocking agent in varying immunoassays. PEG as a water-soluble and nonimmunogenic polymer can possess the unique capability of depressing some nonspecific protein adsorptions and cell adhesions.[39,40] Many surfaces are accordingly derivatized with hydrophilic PEG coatings to improve their biocompatibility.[41,42] PEG layers in water, with rapidly moving hydrated anchored chains and a large excluded volume, tend to repel protein molecules approaching the meaningful surfaces.[40,42] Additionally, PEG is also widely used as a reaction rate accelerator and detection sensitivity promoter in immunoassays.[43,44] The mechanism regarding the steric exclusion effects is described elsewhere.[45] Therefore, the minimization of nonspecific adsorptions by using the BSA−PEG blocking agent could additionally endow the immunoassays with better analysis performances for quantifying OP−AChE in terms of detection rate, sensitivity, and selectivity.

Optimization of Major Parameters for Electrochemical Immunoassays. The dependence of current signals of immunoassays on the amount of QD−Ab_2 labels was examined using different dilutions of the stock QD−Ab_2 suspension. The experimental results are described in Figure 5, in which the current responses to the plasma are shown as the control. As can be seen

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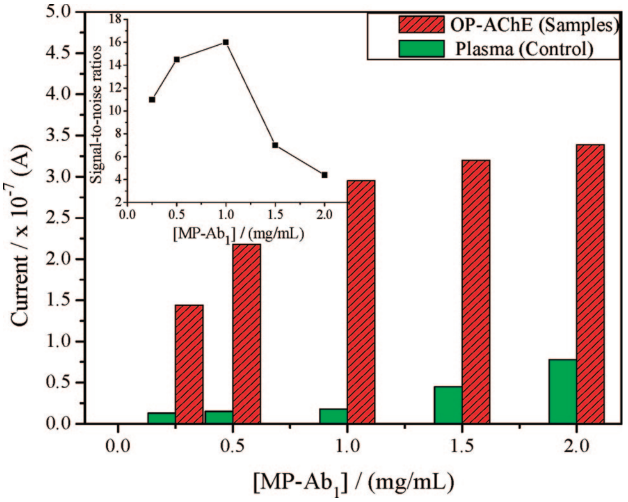

Figure 6. Effects of the amount of MP−Ab$_1$ conjugates on the SWV responses of the immunoassays where the detections for 300 ng/mL OP−AChE and plasma (control) were conducted in parallel under the conditions as described in Figure 4, except for using MP−Ab$_1$ conjugates of different concentrations.

from Figure 5, electrochemical SWV responses to the samples and the control decrease with greater dilution ratios of QD tags. In this regard, an excess of QD−Ab$_2$ labels might cause an increasing nonspecific adsorption, indicating the need to obtain a maximal response while using a minimum amount of QD−Ab$_2$ labels. Accordingly, the optimal amount of QD−Ab$_2$ labels in the reactant solution was determined to be a 1/120 dilution, at which the biggest signal-to-noise ratio was observed as shown in the inset of Figure 5.

Figure 6 displays the SWV response currents that could be affected by the amount of MP−Ab$_1$ conjugates used in which human plasma was determined in parallel as the control for different concentrations of MP−Ab$_1$ conjugates. The current responses for the same OP−AChE concentration can increase with MP−Ab$_1$ particle concentrations rising from 0.25 to 2.0 mg/mL, and the SWV responses to the plasma (noise) perform in the same manner. Obviously, high MP−Ab$_1$ concentrations may result in high noise responses to the plasma. As is shown in the inset of Figure 6, however, their signal-to-noise ratios may peak at 1.0 mg/mL MP particle concentration, after which the ratios may start to decrease. Therefore, 1.0 mg/mL MP−Ab$_1$ was selected for the experiments by compromising between high sample responses with low background interference from the plasma.

Reaction times for both magnetic capture and QD recognition of OP−AChE are considered to be two vital factors largely affecting the detection performances of the proposed immunoassays. On the one hand, we have checked the effects of the adduct capture time on magnetic immunoassays using MP−Ab$_1$ conjugates and a high-concentration OP−AChE (Figure 7, curve a). It was found that a longer reaction time could bring about a larger current response, which might result from a more complete capture of OP−AChE from the sample. From Figure 7 (curve a), we can see that the current responses can increase with increasing capture time until 45 min, after which the responses reach a plateau. Accordingly, the time for the capturing immunoreaction between MP−Ab$_1$ conjugates and OP−AChE has been chosen as 45 min. It should be pointed out that the reaction time needed for magnetic capture of phosphorylated AChE may additionally depend on the analyte concentration and the physiochemical property of the sample media. For example, the high viscosity of a sample may lead to a high transferring resistance for the reactants, resulting in a relatively long capture time for targeting adducts by the magnetic conjugates. On the other hand, we have investigated the reaction time for the recognition of OP−AChE by QD−Ab$_2$ labels with the data manifested in Figure 7 (curve b). One can observe that the SWV responses may show no increase after a 30-min reaction. That is, the immunoreaction time of 30 min may be enough for the recognition between the analytes and QD−Ab$_2$ labels, which is thereby recommended as the secondary recognition time of OP−AChE in the immunoassays.

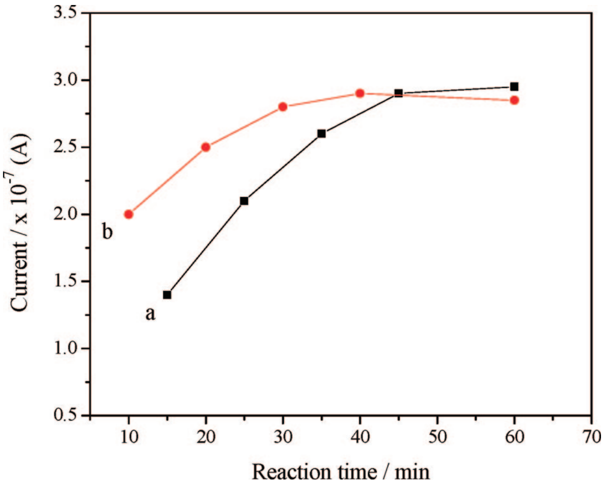

Figure 7. Reaction time dependences of SWV responses of immunoassays on (a) magnetic capture and (b) selective recognition using MP−Ab$_1$ conjugates and QD−Ab$_2$ labels, respectively, under the experimental conditions as described in Figure 4, except for different reaction times of magnetic capture and selective recognition. Each point refers to the average of triplicate experiments.

Analytical Characteristics of SWV Immunoassays. To further verify the feasibility of the application of the developed immunoassay, OP−AChE with different plasma concentrations was probed under optimal experimental conditions. Figure 8A displays typical characteristics of electrochemical SWV responses for increasing OP−AChE concentrations in plasma (curves a−j, from 0.01 to 600 ng/mL). Well-defined voltammetric peaks of cadmium were observed at −0.76 V, and the peak current intensities increase with increasing OP−AChE concentrations. Figure 8B exhibits the resulting calibration curve between current versus OP−AChE plotted on a semilog scale. As shown in Figure 8B, linear responses are obtained over the concentration range of 0.3−300 ng/mL OP−AChE with a detection limit of 0.15 ng/mL, as estimated by the "S/N = 3" rule. The top inset of Figure 8B manifests a large difference between the "as-received" SWV responses to the 0.15 and 0 ng/mL (control) of OP−AChE. A trivially low current signal was observed in the control. Such a noise-depressed behavior is ascribed to the use of the BSA−PEG blocking agent as mentioned above. Moreover, a series of measurements of 300 ng/mL OP−AChE in plasma could yield reproducible electrochemical SWV responses with a relative standard deviation of 11.5%, indicating that the sensitive and selective response of the magnetic immunoassays can be accompanied with favorable detection reproducibility.

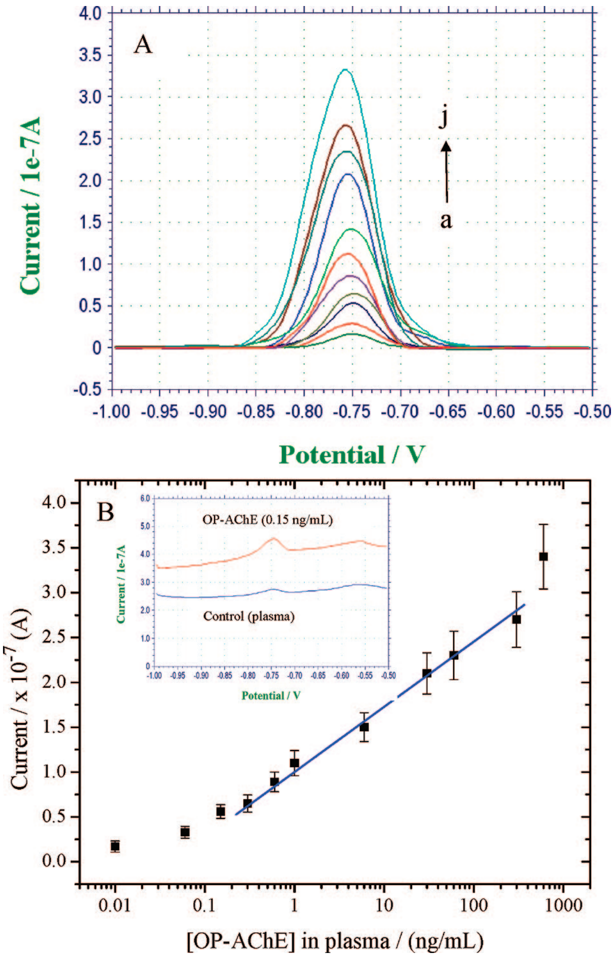

Figure 8. (A) Typical electrochemical SWV responses of the immunoassays for OP−AChE with increasing plasma concentrations under the optimized conditions, where curves a−j correspond to 0.01, 0.06, 0.15, 0.3, 0.6, 1.0, 6, 30, 60, 300, and 600 ng/mL OP−AChE in plasma, respectively. (B) The resulting calibration curve plotted on a semilog scale where the inset shows comparably the SWV responses to the detection limit of 0.15 and 0 ng/mL (control plasma) of OP−AChE.

This work aims at the biomonitoring of low-dose OP exposures, which are defined as <15% inhibition of plasma cholinesterase at which the victims will not show acute symptoms but may have a harmful biological effect. Since the average AChE level in human plasma is ~8.0 ng/mL,[46] the OP−AChE level in human plasma is estimated to be ~1.2 ng/mL for the victims of low-dose OP exposures. Accordingly, the developed immunoassay can have enough sensitivity for biomonitoring of low-dose exposure to OPs, and it holds great promise for analyzing real samples in a clinic.

CONCLUSIONS

The current evaluation methods of potential exposures to nerve agents and pesticides are generally established by quantifying free OPs or their metabolites or cholinesterase activities, which may in a way have some intrinsic disadvantages. In this paper, a new electrochemical immunoassay method has been described that assesses the formation of phosphorylated AChE. This immunoassay protocol incorporates magnetic separation with QD-based electrochemical SWV analysis. It possesses some merits over the common methods for biological monitoring of OP exposure. First, two different antibodies with high immunoaffinities to the phosphoserine moieties and antigenic AChE, respectively, were employed in a synergetic way, achieving specific recognition of the enzyme adducts toward selective quantification. Second, the enzyme adducts were reductively unfolded by DDT to maximize the exposure of their phosphoserine moieties, resulting in enhanced immunorecognitions. Third, using magnetic separation for capturing OP−AChE can largely avoid the interferences from the complex sample matrixes, facilitating the direct analysis of phosphorylated AChE in plasma without complex purification steps. Fourth, metal ion-based SWV measurements using QDs as signal-amplifying tags could ultrasensitively probe the enzyme adducts with a plasma concentration as low as 0.15 ng/mL, which is comparable to that of MS analysis for phosphylated butyrylcholinesterase in human serum.[16] Fifth, introducing the BSA−PEG blocking agent with protein-repelling behavior can significantly minimize nonspecific adsorptions in the immunoassays, making a selective and sensitive immunoassay for OP−AChE. Finally, the utilities of the new immunoassay may include centrifuge tubes, a biomagnetic processing platform, and disposable SPEs, which are truly portable, low cost, and especially convenient for on-site biomonitoring and point-of-care diagnosis of OP exposures if additionally coupled with a miniaturized electrochemical analyzer. Overall, this new electrochemical immunoassay is demonstrated to be a simple, selective, sensitive, and field-deployable alternative tool for biomonitoring and diagnosis of OP exposures to nerve agents and pesticides. This kind of detection format may pave the way toward developing immunoassays for other formidable biomarkers, such as phosphated or nitrated proteins, with low exposure levels in complex biological systems.

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