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Enzyme-linked immunosorbent assay for detection of organophosphorylated butyrylcholinesterase: A biomarker of exposure to organophosphate agents

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ABSTRACT

A sandwich enzyme-linked immunosorbent assay (sELISA) has been developed for detection of organophosphorylated butyrylcholinesterase (OP-BChE), a potential biomarker for human exposure to organophosphate insecticides and nerve agents. A pair of antibodies specific to OP-BChE adduct were identified through systematic screening of several anti BChE antibodies (anti-BChE) and anti-phosphoserine antibodies (anti-P_{ser}) from different sources. The selected anti-BChE (set as capture antibody) antibodies recognize both phosphorylated and nonphosphorylated BChE. These antibodies can therefore be used to capture both BChE and OP-BChE from the sample matrices. The anti-P_{ser} (set as detecting antibody) was used to recognize the OP moiety of OP-BChE adducts. With the combination of the selected antibody pair, several key parameters (such as the concentration of anti-BChE and anti-P_{ser}, and the blocking agent) were optimized to enhance the sensitivity and selectivity of the sELISA. Under the optimal conditions, the sELISA has shown a wide linear range from 0.03 nM to 30 nM, with a detection limit of 0.03 nM. Furthermore, the sELISA was successfully applied to detect OP-BChE using *in vitro* biological samples such as rat plasma spiked with OP-BChE with excellent adduct recovery ($z > 99\%$). These results demonstrate that this novel approach holds great promise to develop an ELISA kit and offers a simple and cost-effective tool for screening/evaluating exposure to organophosphate insecticides and nerve agents.

1. Introduction

The organophosphates (OPs) are a large class of chemical compound which are widely used as pesticides in modern agricultural or chemical weapon nerve agents [1–4]. The high residues of OPs in the environment such as air, soil, water and food, and the terrorist attacks with nerve agent gas in public [5–10], are major public health and national security concerns. The primary toxicity of these OPs arises as a result of covalently binding to some important proteins [11,12] such as cholinesterase (ChE) including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [3,9,13]. The covalently binding of OPs to ChE leads to an inhibition of enzyme activity including both serum ChE (BuChE) and erythrocyte ChE [14,15]. Owing to the high toxicity of the OPs, it is highly desirable to design a reliable method for rapid, specific and sensitive detection of OP exposure in order to develop timely medical countermeasures for both current and future chemical warfare agent threats and for use in biomonitoring agricultural workers or the general public who are exposed to low levels of these agents [16–18].

There are four types of biomarkers used for biomonitoring OP exposure: oragnophosphorylated proteins such as OP-ChE adducts; OP metabolites in blood and urine; decreased enzyme (ChE) activity; and free unbound OP in blood. However, ultra-sensitive technology is mostly required for the detection of extremely low levels of free OPs in blood due to the high affinity of OPs for ChE, resulting in OP-ChE adducts [19]. The level of OP metabolites in urine/blood was considered as a suitable biomarker of exposure, but in some cases, the results confounded by the fact that these metabolites may not be derived exclusively from exposure to OPs. Although the enzymatic activity assay (e.g. Ellman assay) [20,21] has served as a simple and rapid biomonitoring approach, it has some disadvantages, for example, it needs a baseline enzyme levels to measure meaningful changes which usually lead to inaccurate clinical results because of the variation in enzyme levels between individuals. However, it is well established that OPs covalently bind to the serine of ChE at the catalytic site to product stable inhibition enzyme once absorbed into the body [1]. Hence, a rapid and

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accurate detection of OP-ChE adducts has received a considerable interest as a new biomarker for biomonitoring OP exposure.

In recent years, gas or liquid chromatography coupled with mass spectrometry (GC/LC–MS) [22–24] has been utilized to monitor OP adducts. However, the GC/LC–MS needs to be operated in a stable lab-based environment, which is unsuitable for the point-of-care testing (POCT) under the emergency conditions such as terrorist attacks. Also, the GC/LC–MS have some intrinsic disadvantages such as the low sensitivity, high cost, and the measurements can only be carried out by the well-trained technicians. In order to meet these challenges, the enzyme-linked immunosorbent assay (ELISA) has received more attention as a simple, cost-effectively and sensitively tool for biomonitoring OP adducts [25,26].

There are now some immunoassays available which are designed to monitor OP-AChE adducts in biological samples [18,27,28]. However, it is extremely challenge to sensitively detect low levels of AChE (~3 nM of AChE) in the erythrocyte [29,30]. In contrast, the average plasma BChE concentration is substantially greater, up to 80 nM [31,32]. Thus, plasma BChE has more advantages as a potential biomarker for biomonitoring of exposure to low level of OPs [33]. Also, the BChE can hydrolyze or scavenge a broad range of toxic esters such as OPs [14,34], and the OP-BChE displays a long-lasting stability in human circulation [33,35]. Therefore, compared with OP-AChE, there are advantages to monitor OP-BChE adducts as an early warning for low-level OP exposures.

In this paper, we present the first study of the enzyme-linked immunosorbent assay (ELISA) development for biomonitoring and early warning of low-dose OP exposure based on the new potential biomarker, OP-BChE adducts, in biological samples. To develop such ELISA with high sensitivity and selectivity, the first key step was selection of suitable antibodies for this assay [36]. OP-specific antibodies, e.g. antiphosphoserine, and BChE-specific antibodies from different sources were screened, and a pair of antibodies specific to OP-BChE was identified. Using the selected pair of antibodies, the ELISA developed in this study shows a high sensitivity with a detection limit of 0.03 nM OP-BChE, along with a wide linear range (0.03–30 nM). Furthermore, the ELISA was successfully applied to detect OP-BChE in *in vitro* biological samples such as rat plasma spiked with OP-BChE. The recovery of the measurements is from 81% to 116%. Thus, the novel ELISA holds a great promise to develop ELISA kit to offer a simple and cost-effective tool (such as lateral flow test strip) for screening/evaluating exposure to OP pesticides and nerve agents.

2. Experimental

2.1. Apparatus and reagents

The absorbance measurement was performed with a TECAN's Monochromator Microplate Reader connected with a Personal Computer. For the absorbance measurement, the high binging of Corning® 96 Well EIA/RIA Plate (Product# 3590) were used. The absorbance was measurement at 450 nm after the immune reaction was terminated by 2 M H_2SO_4. For dialysis process the Slide-A-Lyzer® 3.5 K Dialysis Cassettes (Thermo Scientific) was used. All of the antibodies used in this study were purchased from several companies, such as Abcam Inc. (Cambridge, MA), Sigma–Aldrich, Qiagen in USA, Abnova Logistic Center (USA) and Santa Cruz Biotechnology Inc (Santa Cruz, CA). Human BChE, butyrylthiocholine (BTcH), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), bovine serum albumin (BSA), Tween-20,3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma–Aldrich. SuperBlock T20 (TBS) blocking buffer and bicinchoninic acid assay (BCA) kit were purchased from Thermo scientific (Rockford, IL). Paraoxon was bought from Chem Service, Inc. (West Chester, PA).

2.2. Preparation of the OP-BChE adducts

BChE was firstly dissolved in 1.0 mL of distilled water. 50 μL of 75 mM paraoxon prepared in acetone was then slowly added to BChE solution. And the mixture allowed to stay at room temperature for 72 h. After reaction, the mixture was further dialyzed against distilled water for 72 h using 3.5 K dialysis cassettes. The enzyme activity of the dialyzed production was determined by Ellman assay [20,37]. Routinely no enzyme activity was detected when the covalent modification of BChE by paraoxon is completed. The dialyzed product was defined as the standard OP-BChE adducts. Also, the concentration of BChE and OP-BChE adducts were determined by BCA method. The BChE solution without paraoxon addition was used as a control.

2.3. Unfolding the BChE and OP-BChE by urea

The three dimensional structure of BChE [38,39] demonstrates that the catalytic site including a serine is protected within the bottom of a deep gorge [40]. In order to efficiently recognize the OP moiety of OP-BChE by a specific antibody, it is necessary to unfolding the OP-BChE. It has been well known that urea can softly denature proteins [41–43]. In this study, all the samples (BChE, OP-BChE) were denatured by using 8 M urea diluted in water. In order to reduce the matrix effect from urea, the denatured samples were diluted with TBST buffer before the measurements.

2.4. Collection of rat plasma

The details of collecting rat plasma were described in previous report [44,45]. Several adult male Sprague-Dawley rats were purchased from Charles River Laboratories Inc. (Raleigh, NC). Rats were housed in solid bottom cages with hardwood chips under standard laboratory conditions. Water and feed (PMI 5002, Certified Rodent Diet) were provided ad libitum. Rats were humanely euthanized using CO_2, and blood was collected via cardiac puncture. Sodium heparin was used as an anticoagulant, and blood was centrifuged at $1600 \times g$ for 10 min to separate plasma from the packed red blood cell fraction. All procedures involving animals were in accordance with protocols established in the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

2.5. Screening of antibodies by using ELISA and/or Western blot

We collected several polyclonal and monoclonal antiphosphoserine (anti-P_{ser}) from different commercial sources. The directly one-side ELISA and Western blot were used to select the antibody which captured the OP moiety of OP-BChE adducts. There were three types of anti-BChE which were generated from (a) the full length of native human BChE (e.g. monoclonal ab 17246 from ABcam), (b) epitope-specific antibodies that are raised against the sequence near the N-terminus of BChE of human origin (polyclonal ab N1-15 from Snta Cruz)) and (c) an antibody against the peptide domain that contains 120 amino acids residues include the active site (polyclonal ab 1560 from Sigma–Aldrich) to recognize the different location of BChE with one-side ELISA. In the screening experiments of ELISAs, all of the concentrations of samples were set as 30 nM, which were diluted in TBST buffer. And in the western blot, the OP-BChE and BChE were set as 1 mg lane^{-1} in the gel.

2.6. Searching an optimal antibody-pair for performing sandwich-ELISA

With the selected anti-P_{ser}-HRP and the three anti-BChEs, there were three combinations of capture (anti-BChE)/detecting (anti-P_{ser}) antibody. We identified the best pair from these three combinations which can be used to detect the paraoxon-BChE adducts based-on the ELISA. The anti-BChE diluted in Tris buffer (TBS_1, 150 mM NaCl, pH 7.4) was coated on the EIA plate (4 °C, overnight). Then the plate was block with the superblock agent (the superblock with 3% BSA and the 0.01% Tween-20). After that, 50 μL 30 nM of BChE or OP-BChE, which has be diluted in Tris buffer (TBS_2, 3% BSA, 0.01% Tween-20, pH 7.4), was added into the wells of the plate, and the plate was allowed to incubate for 1 h at 37 °C. 50 μL of 80 ng mL^{-1} anti-P_{ser}-HRP was used to detect the OP moiety of BChE-OP adducts. Then 50 μL of TMB was added into wells. Then, 25 μL of 2 M H_2SO_4 was used to terminate the substrate reaction after 5 min. The absorbance measurements were performed with a TECAN's Monochromator Microplate Reader at 450 nm and the plates were washed for 3–5 times with washing buffer after each incubation step.

2.7. Evaluation of the accuracy of sELISA—test on system with biological samples

The accuracy of the developed sELISA was evaluated using rat plasma spiked (*in vitro*) with known concentrations of denatured OP-BChE and BChE. In order to reduce the matrix effect, the spiked rat plasma was diluted 10-fold with TBS_2. And the final concentrations of the OP-BChE and BChE spiked in the rat plasma were 0.3, 3, 7.5, 15 and 30 nM. Also, a calibration curve was established, and the recovery (%) was used to evaluate the accuracy of the ELISA.

3. Results and discussion

3.1. The confirmation of the formation of OP-BChE adducts

The paraoxon was used as a model agent to covalently bind to BChE. Ellman assay was used to monitor the reaction and to confirm whether the OP-BChE adducts was successfully prepared. We firstly prepared the assay solution by mixing BTcH and Ellman assay solution (DTNB) at a ratio of 1:1 (v/v). Then, 2 μL of OP-BChE and control BChE were added into the pre-mixed assay solution, respectively. After 30 min of reaction, the color of assay solution mixed with control BChE or OP-BChE was measured (the color of BChE was yellow and the OP-BChE was colorless). The results of the enzyme activity assay demonstrated that the OP-BChE adducts were successfully prepared (data not shown). The concentrations of OP-BChE and the BChE, which determined by BCA method, were 660 μg mL^{-1} and 706 μg mL^{-1}, respectively. And the sample was used when at least 6 days passed after the preparation of OP-BChE was completed.

3.2. Systematic screening of antibodies and selection of a pair of antibodies

The identification of antibodies that are specific to the OP moiety of organophosphate-BChE adducts is extremely challenging. Especially, there was a side reaction, named "aging", which can change the structure of organophosphorylated butyrylcholinesterase. The process was investigated by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry [46,47]. According to the literature [46], the aging process of OP-BChE faster than OP-AChE, and the half-time of aging of OP-AChE ranges from several minutes to several days. And we found that the structure of phosphoserine was similar with the OP compound which combined at active site of ChE [1]. So, six anti-phosphoserine antibodies (anti-P_{ser}) were selected from several biological companies and experiments were conducted to evaluate their capability to recognize the OP moiety of OP-BChE. The one-side ELISA and the Western-blot were performed to test the affinity of the anti-P_{ser} to OP-BChE. Fig. 1 demonstrated the affinity of the best anti-P_{ser} from our collections to OP-BChE and BChE with Western blot and one-side ELISA. As shown in Fig. 1, the antibody can selectively differentiate OP-BChE adducts from BChE.

The anti-phosphoserine antibody can be served as either a capture antibody or a detecting antibody for sELISA. If it is used as a capture antibody, the method, however, may have a limitation resulting from the fact that the anti-P_{ser} can also capture other types of phosphorylated proteins which exist abundantly in the sample matrix [48,49], subsequently greatly decreasing the sensitivity of the method. Therefore, we used the antiphosphoserine as a detecting antibody, and anti-BChE as capture antibody. As such, BChE and OP-BChE will first be captured minimizing the matrix effect and thereby improving sensitivity. A suitable anti-BChE is needed for specific capture of BChE. Fig. 2 displays the result of one-side ELISA for screening specific anti-BChE, where all of the anti-BChE antibodies recognized the BChE and OP-BChE equally. Thus, there were three possible combinations of the antibody pairs to be used to detect the OP-BChE. As shown in Fig. 3, we observed that the best result was from the combination of ab 17246/ab 9334. It may be ascribed to the fact that the ab 17246 was the anti-full length of BChE, which means it can recognize most epitopes along the enzyme molecule, except those including the blocked active serine site. For the pair of ab 1560/ab 9334, the capture efficiency is very low because the ab 1560 may block the active serine site of BChE he color of BChE was yellow and the OP-BChE was colorless. For the combination of ab N15/ab 9334, there was a large non-specific affinity of the sELISA. So, this combination of antibody pair

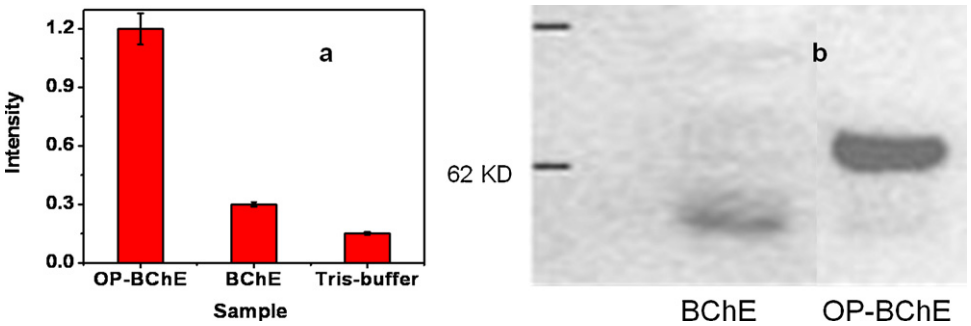

Fig. 1. The affinity testing of anti-P_{ser} to OP moiety of BChE-OP. (a) Tested by one-side ELISA, the concentrations of OP-BChE and BChE were set as 30 nM; (b) tested by Western blot, the OP-BChE and BChE were set as 1 mg $lane^{-1}$ in the gel.

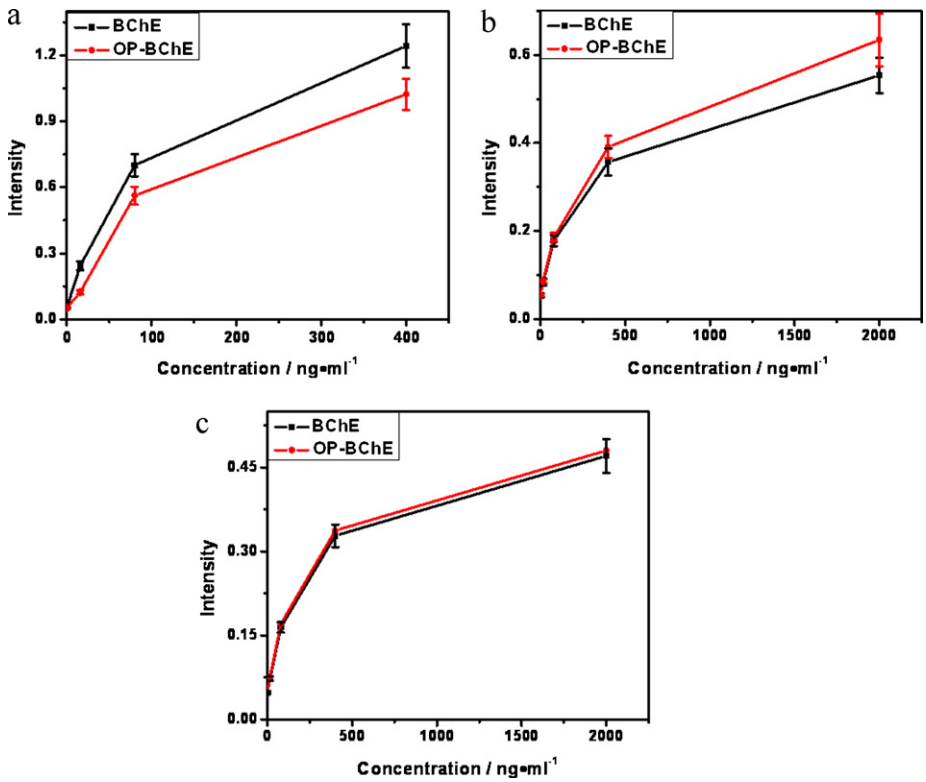

Fig. 2. The affinity testing of three anti-BChE antibodies to BChE and OP-BChE by one side ELISA, all the concentrations of samples were set as 30 nM, which were diluted in TBST buffer. (a) ab 17246; (b) ab N15; (c) ab 1560.

cannot differentiate OP-BChE adducts from BChE. Based on these tests, we therefore selected the pair of ab 17246/ab 9334 in our study to detect the OP-BChE adducts.

3.3. Optimization of the ELISA

Some parameters, which may affect the analytical performance of the assay, were examined and include concentrations of anti-BChE and anti-phosphoserine, and blocking agents. Fig. 4 shows the results of these optimization experiments. In this study, the ratio of signal from OP-BChE to BChE was considered as the main criteria for evaluating the ELISA assay. The concentration of antigen (OP-BChE and BChE) used in the experiments is 30 nM and different concentrations of anti-BChE, ranging from 0.2 to 10 $\mu g\,mL^{-1}$ were coated on the plate. According to the Fig. 4a, the largest difference between the signals from OP-BChE to BChE was obtained with 5 $\mu g\,mL^{-1}$ of the anti-BChE, which indicates the lowest nonspecific adsorption. Also, the different concentrations of anti-P_{ser} ranging from 12.5 to 250 $ng\,mL^{-1}$ were also tested. Fig. 4b demonstrated that the largest difference between the signals from OP-BChE to BChE was obtained with 83.3 $ng\,mL^{-1}$ of anti-P_{ser}. Furthermore, in order to minimize nonspecific adsorption, we performed the measurements using different blocking agents. As shown in the Fig. 4c, the super blocking (SPB) agent resulted in the highest signal difference. While, BSA blocking produced the lowest signal, which may be attributed to the fact that that the BSA not only blocked the empty spaces within the plate but also blocked some active site of antibody. In summary, we have achieved a best optimization for the sELISA to detect OP-BChE adducts when a combination of the following conditions were used: the concentration of anti-BChE is 5 $\mu g\,mL^{-1}$, the concentration of anti-P_{ser} is 83.3 $ng\,mL^{-1}$, and the blocking agent is SPB.

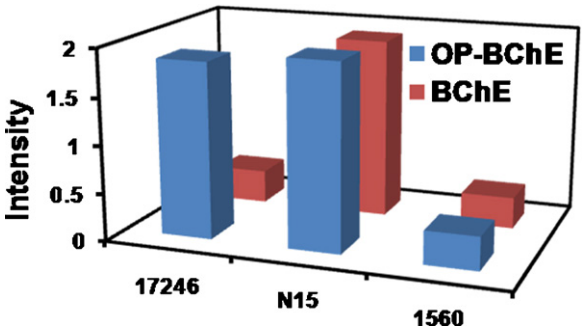

Fig. 3. The response of three pairs of capture antibody and detect antibody to OP-BChE adducts by ELISA. The *x*-axis was the kind of capture antibodies. The detecting antibody was anti-P_{ser}-HRP, and the concentrations of samples were used as 30 nM in TBST buffer.

3.4. Analytical ELISA for OP-BChE

With the optimal conditions, the analytical performance of the ELISA for detection of OP-BChE was examined with different concentrations of standard OP-BChE (0.03, 0.3, 3, 15, 30, 60 nM) in TBS_2 and the results are shown in Fig. 5. The results indicate that ELISA can be employed to differentiate the OP-BChE from BChE, with the detection limit as low as 0.03 nM. As shown in the Fig. 5, the ELISA also demonstrated a broad linear range from 0.03 to 30 nM (R^2 = 0.998), and the relative standard deviations (RSD) for 6 measurements of three concentrations of standard OP-BChE (0.03, 0.3, 3, 15, 30 nM) are 3.4%, 4.5%, 6.2%, 2.5%, 4.9%, respectively. This clearly indicates a good reproducibility of this novel ELISA approach.

3.5. Evaluation of the ELISA with spiked rat plasma

To study the feasibility of clinical application of the ELISA, the method was examined *in vitro* using rat plasma that was spiked

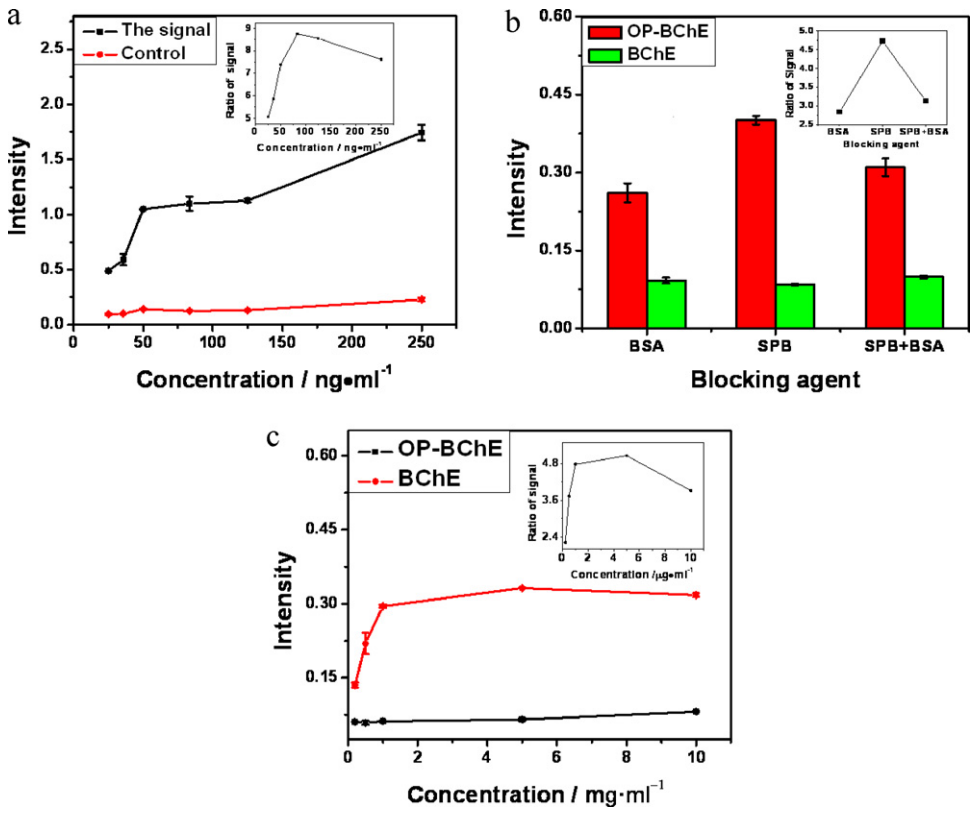

Fig. 4. Optimization of the Sandwich ELISA system. (a) Concentrations of capture antibody (0.2, 0.5, 1, 5, 10 μg mL^{-1}); (b) concentration of detecting antibody (25, 35.7, 50, 83.3, 125, 250 ng mL^{-1}); (c) influence of blocking agents. All of the insets in (a), (b) and (c) are ratio of the signal from OP-BChE and BChE, resepectively.

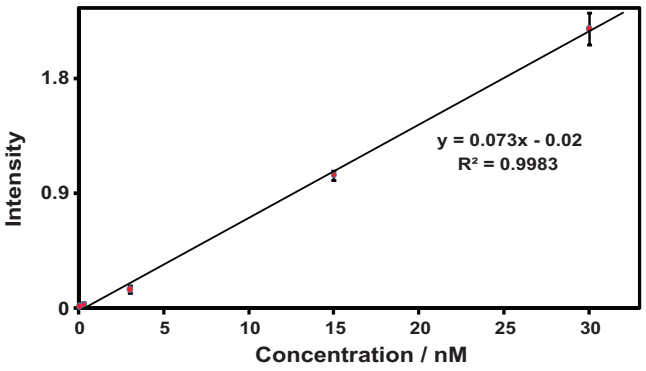

Fig. 5. The calibration curve for detection OP-BChE during the linear range (0.03, 0.3, 3, 15, 30 nM) with the optimized ELISA.

Table 1
Recovery of OP-BChE spiked into plasma samples obtained by the ELISA.

Spiked concentration (nM)	Final concentraton (nM)	Found concentraion (nM)	Recovery (%)
300	30	34.08 ± 2.03	114 ± 6.8
150	15	17.31 ± 0.81	115 ± 5.4
75	7.5	7.91 ± 1.21	105 ± 16.1
30	3	2.97 ± 0.24	99 ± 8.1
3	0.3	0.36 ± 0.13	121 ± 43.9

with known concentrations of OP-BChE. As shown in Fig. 6 and Table 1, the ELISA was successfully applied for the detection of OP-BChE from spiked rat plasma. The recoveries of the spiked BChE-OP in plasma were 99% to 121%. And extremely low level of BChE (0.3 nM) in the plasma sample can also be detected with recov-

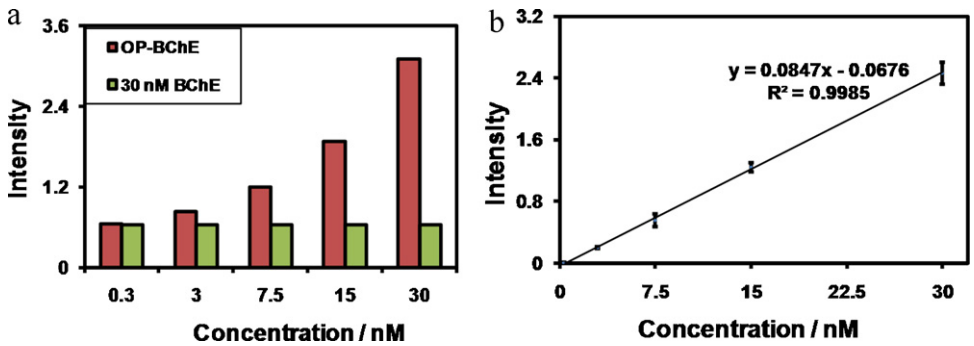

Fig. 6. Evaluation of the ELISA with *in vitro* rat plasma samples. (a) Detect the known concentration of OP-BChE and BChE spiked into rat plasma; (b) resulting calibration curve from (a).

ery of 121% by this approach. The results clearly demonstrate that this ELISA holds a great potential to quickly identify the low dose exposure of OP agents.

4. Conclusions

A pair of antibodies have been identified and further applied for the design of ELISA for sensitive and selective detection of OP-BChE adducts in biological fluids. The novel approach can be employed as sensitive biomarker for exposure to low level of OPs. This ELISA shows the capability for the detection of OP-BChE adducts in plasma samples with *in vitro* exposure to OPs. This novel method shows great promise for developing ELISA kit and offers a simple and cost-effective tool for screening/evaluating exposure to OP agents.

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