Biosensors and Bioelectronics 26 (2011) 2835–2840

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel immunochromatographic electrochemical biosensor for highly sensitive and selective detection of trichloropyridinol, a biomarker of exposure to chlorpyrifos

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ARTICLE INFO

Article history:
Received 10 August 2010
Received in revised form 20 October 2010
Accepted 9 November 2010
Available online 30 November 2010

Keywords:
Immunochromatographic electrochemical biosensor
Biomarker
Organophosphorus insecticides
Trichloropyridinol
Competitive immunoreaction

ABSTRACT

We present a novel portable immunochromatographic electrochemical biosensor (IEB) for simple, rapid, and sensitive biomonitoring of trichloropyridinol (TCP), a metabolite biomarker of exposure to organophosphorus insecticides. Our new approach takes the advantage of immunochromatographic test strip for a rapid competitive immunoreaction and a disposable screen-printed carbon electrode for a rapid and sensitive electrochemical analysis of captured HRP labeling. Several key experimental parameters (e.g. immunoreaction time, the amount of HRP labeled TCP, concentration of the substrate for electrochemical measurements, and the blocking agents for the nitrocellulose membrane) were optimized to achieve a high sensitivity, selectivity and stability. Under optimal conditions, the IEB has demonstrated a wide linear range (0.1–100 ng/ml) with a detection limit as low as 0.1 ng/ml TCP. Furthermore, the IEB has been successfully applied for biomonitoring of TCP in the rat plasma samples with *in vivo* exposure to organophosphorus insecticides like Chlorpyrifos-oxon (CPF-oxon). The IEB thus opens up new pathways for designing a simple, rapid, clinically accurate, and quantitative tool for TCP detection, as well as holds a great promise for in-field screening of metabolite biomarkers, e.g., TCP, for humans exposed to organophosphorus insecticides.

1. Introduction

Organophosphorus insecticides, like chlorpyrifos (CPF) and chlorpyrifos-methyl (CPFm), were a large class of toxic chemical compounds that have been widely used in the agriculture production and home application (Jiang et al., 2008; Singh and Walker, 2006; Choi et al., 2006). In recent years there has been increasing attention given to the accidents linked to organophosphorus insecticides (Caldas et al., 2008). The major toxic effects of the organophosphorus insecticides are the ability of these insecticides or their active metabolites inhibiting the enzyme activity of the acetylcholinesterase (AChE) in the central or peripheral nervous-system (Wang et al., 2008; Wille et al., 2010; Thompson et al., 2010). For example, the thionophosphate insecticide, like CPF can be metabolized to form 3,5,6-trichloro-2-pyridinol (TCP) and chlorpyrifos-oxon (CPF-oxon) when ingested, inhaled, or absorbed dermally. The CPF-oxon is a super inhibitor to AChE, or it can be metabolized to form TCP (Timchalk et al., 2007; Smith et al., 2010; Kamataki et al., 1976; Chanda et al., 1997; Pond et al., 1998). As the knowledge of the metabolic scheme above, the TCP is the primary metabolite when exposure to CPF (Lee et al., 2010). Thus, it is very important to develop a rapid, portable and sensitive biosensor to biomonitoring the metabolite TCP in-field for the exposure to CPF.

Many analytical methods have been designed and developed to detect the metabolite TCP, such as gas chromatography (Randhawa et al., 2007), gas chromatography-mass spectrometry (Diaz-Cruz and Barcelo, 2006), high performance liquid chromatography (Abu-Qare and Abou-Donia, 2001a,b) and liquid chromatography-positive ion electrospray tandem mass spectrometry (Raina and Sun, 2008). These regular methods, however, generally require expensive equipment and involve complicated and time-consuming sample treatments followed by pre-concentration steps that can be carried out only by trained professionals. Furthermore, the large amount of organic solvent used in the detection process may cause a series of environmental issues. The traditional enzyme-linked immunosorbent assay (ELISA) kit (Strategic Diagnostics Inc., Newark, Delaware) can also be used for the detection of TCP.

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doi:10.1016/j.bios.2010.11.008

However, these traditional immuno-methods need a long time to get quantitative results (Engvall and Perlmann, 1971). Till date, a portable device that can be reliably deployed for the detection of metabolite TCP in a field environment still faces formidable challenges (Timchalk et al., 2007; Lu et al., 2005).

Immunochromatographic electrochemical biosensors (IEBs) have fascinated the great concern for developing a portable, rapid, sensitive and inexpensive immunosensor (Lu et al., 2005; Liu et al., 2007; Lin et al., 2008; Blazkova et al., 2010). With the originally designed IEB, we have successfully detected the Ig G and the prostate-specific antigen (Liu et al., 2007; Lin et al., 2008). Here, we present a novel design of IEB device (Fig. 1A), which combined the electrochemical immunosensor with the immunochromatographic (lateral flow test strip) for rapid and sensitive detection of metabolite TCP in rat plasma with *in vivo* exposure to CPF-oxon. In IEB detection, the enzyme-linked competitive immunoreaction was performed on an immunochromatographic test strip. With the 10 min of immunoreaction and 5 min of substrate reaction (the total detection time of conventional ELISA was commonly more than 60 min), the captured enzyme horseradish peroxidase (HRP) labeled antibodies on the test zone were measured using the screen-printed carbon electrode. Using this new IEB device, we can detect the metabolite TCP as low as 0.1 ng/ml, along with a very broad linear range (0.1–100 ng/ml). Also, the sensor can successfully monitor the metabolite TCP in plasma collected from three rats with *in vivo* exposure to the CPF-oxon. We also measured the *in vivo* plasma using the conventional enzyme-linked immunosorbent assay (ELISA) to compare with the result from the developed IEB.

2. Experimental

2.1. Materials and instruments

Mouse monoclonal anti-TCP antibody was purchased from Strategic Diagnostics Inc. (Newark, Delaware). The TCP derivatized with carboxyl functional group (HTCP) was synthesized according to previous literature (Zou et al., 2010). SuperBlock T20 TBS Blocking Buffer (SPB) was purchased from Thermo Scientific Inc. 1× Phosphate Buffered Saline With 1% Casein (Casein) was the production of Bio-Rad (Hercules, CA). The analytical reagent grade chemical used, such as TCP, phosphate buffer saline (PBS, 0.01 M), bovine serum albumin (BSA), N-hydroxy-succinimide (NHS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), horseradish peroxidase (HRP), Tween-20, and N, N′-dimethylformamide (DMF, 99.8%) o-Phenylenediamine (OPD) were purchased from Sigma–Aldrich (St. Louis, MO). Nitrocellulose membrane, absorbent pad, sample pad, and conjugation pads as well as backing cards were purchased from Millipore (Temecula, CA).

All of the electrochemical experiments were performed on an electrochemical analyzer CHI 1232A (CH Instruments, Austin, TX, USA) 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, USA)

The ELISAs were carried out on the Coring® 96 well High binding EIA/RIA plate (Product # 3590). The Absorbance measurement was performed with a TECAN's Monochromator Microplate Reader connected with a Personal Computer.

2.2. Preparation of HTCP-HRP

The conjugation HTCP-HRP was synthesized according to the previous literature with a slight modification (Qian et al., 2009; Zhang et al., 2008). All of the HTCP, NHS, EDC were diluted into DMF. The HRP was diluted in 0.1 M $NaHCO_3$ (pH 8.1). 1 ml of 0.1 M HTCP was incubated 1 h at Room Temperature (R.T.) with 0.5 ml of 0.2 M NHS and 0.5 ml of 0.2 M EDC with shaking. 20 μl EDC/NHS preactivated HTCP was slowly dropped into the 1 ml of 2 mg/ml HRP. The mixture was allowed to react for 3 h at R.T, and finally the conjugation was purified under the PD-10 desalting column used the PBS as the elution buffer. Centrifuge the desalted solution at 7000 rpm for 15 min with the 3.5 K filter to make the solution concentrated to 500 μl.

2.3. Rat plasma collection

The details of collecting *in vivo* rat plasma were described as previous report (Lee et al., 2010; Busby-Hjerpe et al., 2009). Several adult male Sprague-Dawley rats were purchased from Charles River Laboratories Inc (Raleigh, NC). Rats were dosed by intraperitoneal (IP) injection with 0.5 or 1 mg/kg CPF-oxon in 1 mL/kg corn oil. Rats were humanely euthanized 1 h post dosing by CO_2 asphyxiation, and blood was collected by intracardiac puncture using sodium heparin as the anticoagulant. Rats without CPF-oxon dosed were used as control. Blood was centrifuged for 10 min at 1600 RCF× *g*, and plasma was separated from the packed red blood cell fraction. All procedures involving animals were in accordance with protocols established in the National Research Council 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.

2.4. Design of IEB device

As shown in Fig. 1A, the IEB consists of three components: a cover with a cutter (a), a salver of test strip (b) and a salver of electrode (c). The components (b) and (c) have chambers for test strip (d) and electrode (e) respectively. The competitive immunoreactions were performed in the test strip. The IEB was combined the test strip with the electrochemical detector. The cutter in the cover (a) was designed to cut the test zone from test strip (d) after the completion of competitive immunoreactions. Under the test zone, there was a substrate reaction cell in the salver of test strip (b) designed to combine the test strip with the electrochemical detector. When the substrate reaction completed, the electrochemical measurements were conducted to obtain the quantitative information. There are five zones in the test strip which is housed in the chamber of (b): glass fiber sample loading pad, glass fiber enzyme-linked HTCP conjugation pad, nitrocellulose membrane, test zone immobilized with the antibody on the nitrocellulose membrane and the absorbance pad. The electrochemical detector and SPE were embedded in the chamber of component (c).

2.5. Preparation of the immunochromatographic test strip

An enzyme-linked TCP competitive assay were conducted based on a fluid flow on an immunochromatographic test strip. The preparation of the immunochromatographic test strip was described as follows: The sample loading pad and the conjugation pad were both made of glass fiber. A desired volume of diluted TCP-HRP conjugate solution was dispensed on the conjugation pad with the dispenser XYZ-3050 BioJet Quanti 3000, and stored at 4 °C. The test zone of the strip was prepared by dispensing a desired volume of 1 mg/mL mouse monoclonal TCP antibody solution with the dispenser onto a nitrocellulose membrane. After overnight of drying at 4 °C, the nitrocellulose membrane was blocked with blocking agents including SPB, Casein and 3% BSA. And the membrane was stored at 4 °C. Both the sample loading pad and the absorbent pad were stored at room temperature without any treatments. All of the above parts were assembled on a plastic adhesive backing card using the Batch

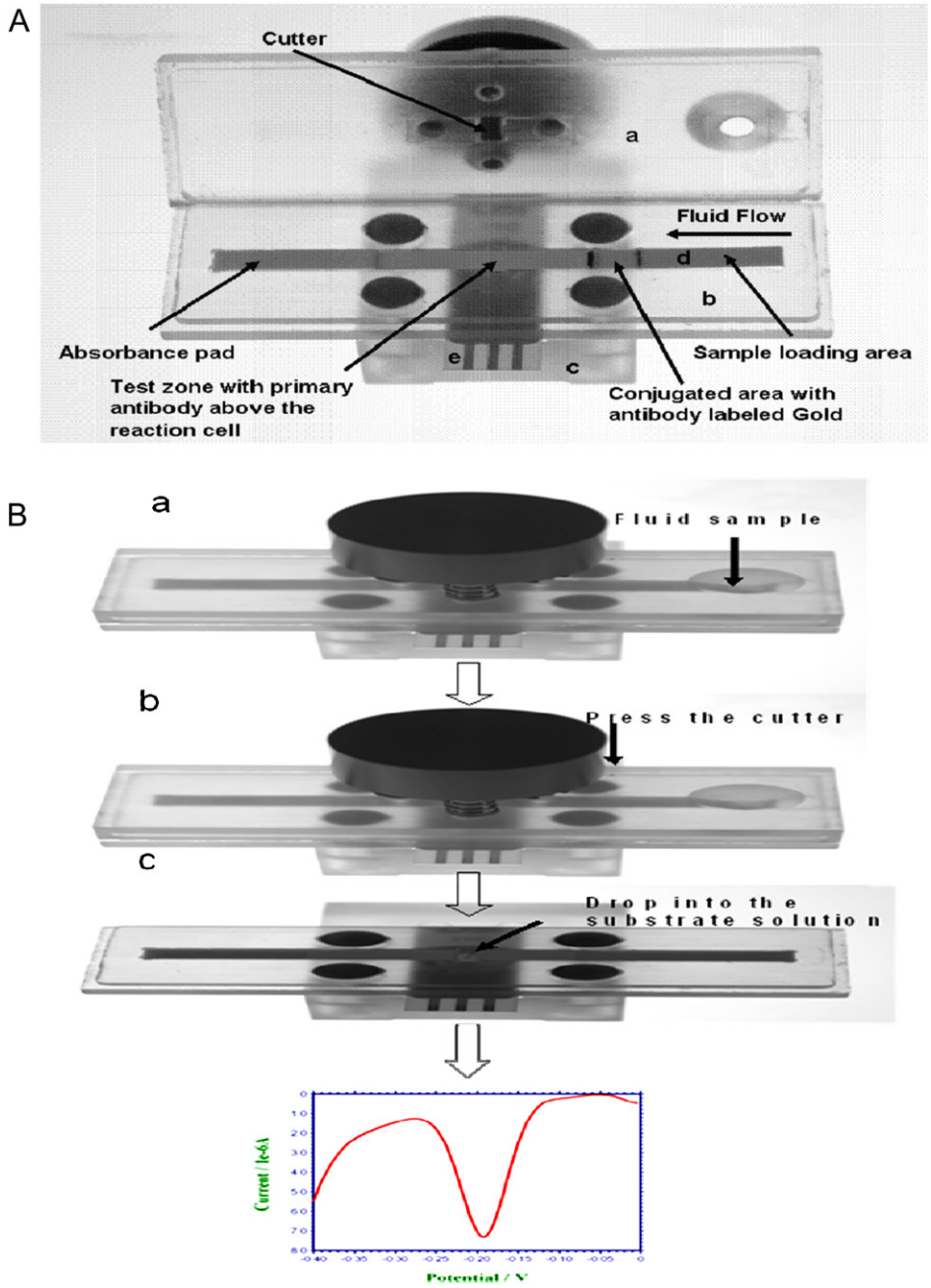

Fig. 1. A: The schematic diagram of an IEB. a: The cover with a cutter. b: The salver of the test strip. c: The salver of the electrode. d: A test strip. e: A SPE; B: Measurement principle of IEB. a: 50 μl of liquid sample dropped into the sample loading zone of the glass fiber. b: Cut the captured HTCP-HRP in the test zone into the reaction cell of sensor. c: The substrate solution of desired concentration of OPD and 2 mM of H2O2 was dropped into the reaction cell. d: The SWV response.

Laminating System LM5000. Finally, the TCP test strips with a 4 mm width were cut using the Guillotine Cutting System CM 4000 and assembled in the strip cassettes.

2.6. Lateral flow immunoreaction

Fig. 1B demonstrated a competitive immunoreactions measurement principle on the IEB. The competitive immunoreaction was performed on the test strip, where the HTCP-HRP conjugation physically absorbed on the glass fiber conjugate pad, and the anti-TCP mouse antibody was immobilized on the test zone. 50 μl of sample solution was casted onto the glass fiber sample loading pad and allowed the solution to flow through the whole test strip (Fig. 1B(a)). After a desired immunoreaction time (e.g. 10 min), when the competitive immunoreactions of TCP and HTCP-HRP binding to anti-TCP antibody on the test zone were completed, the cutter inside the cover of the device was pressed down to cut the test zone with captured HTCP-HRP to inserted into the reaction cell (Fig. 1B(b)). Before the electrochemical measurements, a 50 μl of substrate solution consisted of desired concentration of OPD and 2 mM of H_2O_2 in PBS (pH 4) was added into the reaction cell (Fig. 1B(c)). After s 5 min reaction time for HRP with the substrate solution, the Square Wave Voltammetric (SWV) responses were recorded for quantitative study (Fig. 1B(d)).

2.7. Electrochemical detection

The electrochemical experiments were performed with a portable electrochemical analyzer CHI 1232A (CH Instruments, Austin, TX, USA) connected to a personal computer. The SWV

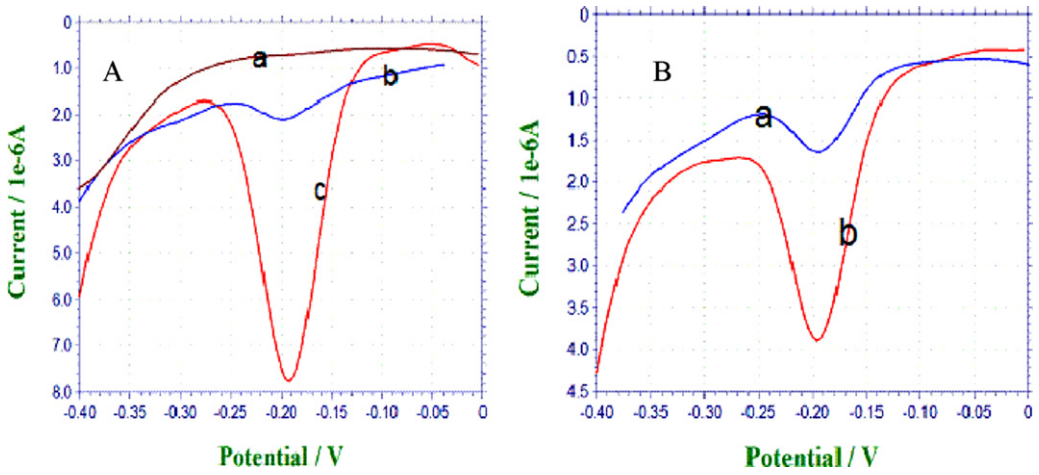

Fig. 2. A: Square Wave Voltammetry of a) 0.1 M PBS (pH 4.0), b) (a) + 2.0 mM H_2O_2 + 10 mM o-phenylenediamine and c) (b) + 2 mg/mL HRP at a screen printed carbon electrode; B: The typical SWVs of the TCP assay with the enzyme-linked IEB in the presence (curve a) and absence (curve b) of TCP.

experiments were carried out under the following conditions: the scanning potential is from 0 to −0.4 V, with increments of 4 mV, amplitude of 25 mV, and a frequency of 15 Hz. Baseline corrections were carried out using CHI software.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The one-side competitive ELISA experiments were carried out with the coring® high-binding microplates. The concentrations of analyte (TCP) and the competitor (HTCP-HRP) were as same as the one used in IEB detection. The responses were recorded using a Tecan microplates reader.

3. Results and discussion

3.1. Biosensor detection principle

In this paper, the HRP linked to HTCP, which can be captured by antibody on the test zone, was chosen as labeling material. During the detection, the HTCP-HRP (competitor) competitive binding with liquid sample (analyte, TCP within PBS buffer or Rat plasma) to the anti-TCP antibody immobilized on the test zone of the strip (see SI for details). First, a liquid sample solution (50 μl) was dropped into the sample loading zone. The liquid can flow laterally to the other end of the test strip according to the capillary action driven by the absorbent pad. The competitor HTCP-HRP pre-loaded in the conjugated zone of glass fiber flowed laterally together with the liquid sample when the liquid reached the zone. The competitive immunoreaction of HTCP-HRP and TCP occurred when they appeared in the test zone. The excess analytes and competitors contained in fluid fraction to continue flowing into the absorbent pad at the end of the strip. For the control experiment, the competitors can be fully binded to the antibody when the liquid sample is in the absence of TCP. The cutter in the cover of the device was pressed down to cut the test zone of the strip after the completion of competitive immuoreaction (10 min). The piece of test zone with the captured HTCP-HRP dropped into the reaction cell of IEB device, and ready for electrochemical detection.

The labeling material, HRP, can catalyze the oxidation of the OPD in the present of H_2O_2 (Ju et al., 1999; Chen et al., 2005; Lin and Ju, 2005). The production, 2,2′-diaminoazobenzene, showed a sensitive voltammetric response. As shown in Fig. 2A, there was no obvious peak observed in the scanned voltage range in PBS buffer solution (pH ~4.0). When 10 mM OPD and 2 mM H_2O_2 were added into the PBS buffer solution, the voltammograms changed slightly, with a small peak appears near the potential of −0.19 V, indicating that the spontaneous oxidation of OPD by H_2O_2 without the HRP catalysis was very slow. However, when the HRP was added into the solution, the curve displayed a much higher peak at −0.19 V. Fig. 2B demonstrated the typical SWVs of the TCP assay with the enzyme-linked IEB in the presence or absence of TCP in the liquid sample. Therefore, the voltammetric response was inversely proportional to the concentration of the TCP residues in the sample, which can be used for quantitation of TCP.

3.2. Optimization of the experimental parameters

A set of parameters such as immunoreaction time, concentration of OPD, the amount of HTCP-HRP pre-loaded on the conjugation zone and the blocking agents were examined in order to achieve an optimal experimental system. In this study, the ratio of signals (SWV responses recorded in the absence and presence of TCP in sample) was chosen as the criteria to evaluate the IEB system. And the concentration of antigen (TCP) was 1 μg/ml. The effect of the immunoreaction time was tested using the standard TCP sample. Several immunoreaction times including 5, 8, 10, 15, 30 min were chosen for this study. As shown in Fig. 3a, the ratio increases with the immunoreaction times up to 10 min, then begins to level off for longer periods. It means that the competitive immunoreactions on test zone were completed for the 10 min immunoreactions, and the competitive reaction would be stable when the immunoreactions over10 min. The 10 min was thus chosen for following experiments. In order to obtain the best competitive purpose, the ratio of the competitor (HTCP-HRP) pre-loaded on the conjugation zone and the antibody on the test zone was very important. Under the desired amount of the antibody, we studied the influence of the concentration of the competitor. As shown in the Fig. 3b, the highest ratio of the signal can be obtained with 3 μl of 2 μg/ml HTCP-HRP on the conjugation zone, indicates that the optimal amount of HTCP-HRP was most suitable to the competitive immunoreaction. The influence of the concentration of OPD was also examined using 2 mM H_2O_2 PBS buffer (Fig. 3c). The highest ratio of the signal can be obtained at 10 mM OPD, indicates that totally amount of OPD is related to the amount of the HRP. Furthermore, the Fig. 4d demonstrated that the ratio of the signal can get the highest when the SPB was used to block the nitrocellulose membrane. It means that the SPB can greatly decrease the non-specific affinity of the other proteins onto the membrane. So, according to the experimental results, as shown in Fig. 4a–d, the optimal conditions for the detection of

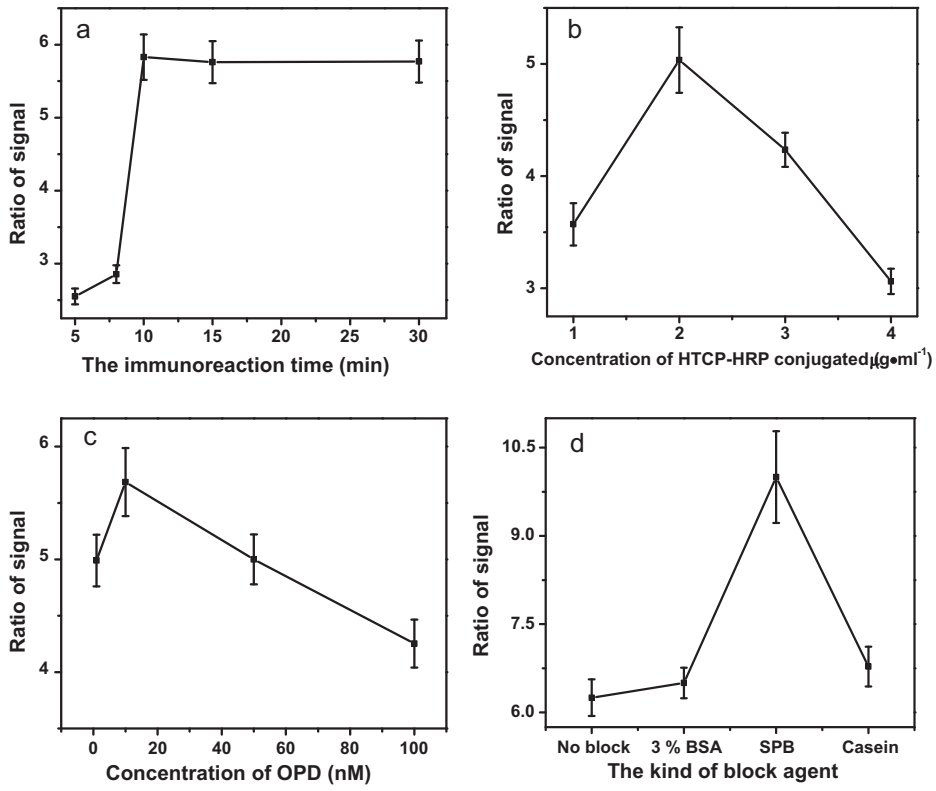

Fig. 3. Optimization of experimental parameters: immunoreaction time ((a) 5, 8, 10, 15, 30 min), concentration of HTCP-HRP pre-loading on the glass fiber ((b), 1, 2, 3, 4 μg ml^{-1}), concentration of OPD ((c), 1, 10, 50, 100 mM) and the kind of block agent ((d), no block, BSA, SPB and Casein).

TCP with the IEB can be described as follow: immunoreactions time was 10 min, the concentration of OPD was 10 mM, the concentration of HTCP-HRP was 2 μg/ml and the blocking agent was SPB.

3.3. Analytical characterization

Under the optimal conditions, the analytical performance of the IEB for the assay of TCP was examined with the desired concentration (0, 0.1, 1, 10, 20, 50, 100, 1000 ng/ml) of standard TCP in PBS buffer. As shown in Fig. 4a, well-defined peaks were observed following a 10 min reaction period. The peak current decreases proportionally with the concentration of TCP from 0.1 ng/ml to 100 ng/ml to yield a highly linear calibration plot (Fig. 4b, correlation coefficient is 0.94). The detection limit is about 0.1 ng/ml according to the real detectable concentration of the assay (based on 90% of C/C_0).

3.4. Evaluation of TCP in rat plasma with in vivo exposure to CPF-oxon

To study the clinical application of the enzyme-linked IEB, the optimized IEB were evaluated by the rat plasma with *in vivo* exposure to CPF-oxon. The plasma samples were collected from adult male rats that have been exposed to the CPF-oxon directly, under the dose of 0, 0.5, 1 mg/kg respectively. In order to reduce the effect of matrix, the plasma was 5-fold diluted with PBS buffer (pH 7.4). The rat plasma sample without CPF-oxon exposure was considered as control. The results shown in Fig. 5 (red color) demon-

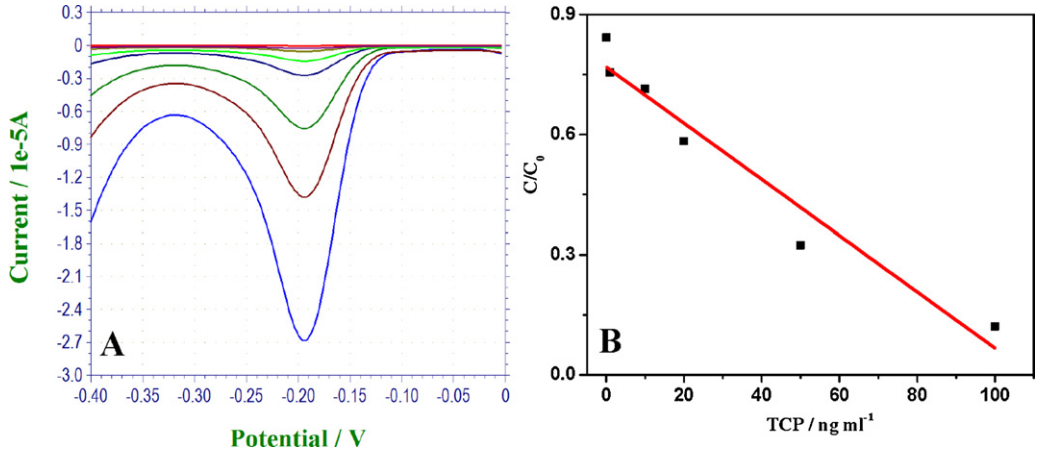

Fig. 4. A: Typical SWV responses of the IEB with increasing TCP concentrations, from top to bottom: 1000, 100, 50, 20, 10, 1, 0.1, 0 ng/ml, respectively; B: The resulting calibration curve from A (signals expressed as C/C_0, where C and C_0 are the voltammetric signal obtained with the TCP analyte and the blank sample. The inset is the calibration curve of the linear range (from 0.1 to 100 ng/ml).

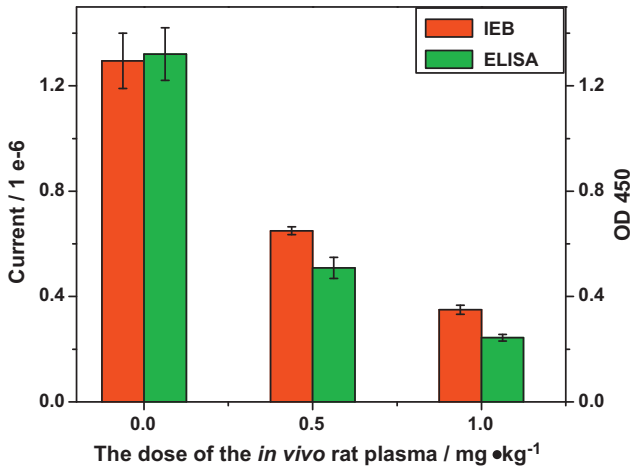

Fig. 5. Detection of TCP from the *in vivo* plasma samples collected from rats which were exposure to desired amount of CPF-oxon. Red color: monitoring the TCP using the IEB, Green color: monitoring the TCP using the ELISA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

strated that our novel IEB device can detect the TCP residues in the rat plasma after the rats were exposed to the CPF-oxon, with the relative standard deviations (R.S.D) of 9.6%, 5% and 5.1% respectively. We also measured the metabolite TCP in the rat plasma samples using the tradition ELISA method to compare with the results using IEB. As shown in Fig. 5 (green color), the ratio of responses for detecting TCP in rat plasma with *in vivo* exposure to different dose of CPF-oxon by using two sensing methods were highly consistent. Compared with traditional ELISA, the developed IEB is more portable and sensitive, which can be employed for fast quantitative analysis of TCP and in-field screening of organophosphorus insecticides exposure (based on 90% of C/C_0).

4. Conclusion

We have successfully demonstrated a highly portable, rapid, sensitive and cost effective detection method for detection of TCP based-on the enzyme-linked IEB. Our new approach takes advantages of the test strip for fast immunoreaction and separation, the combination of enzyme-linked immunoassay with electrochemical techniques to achieve a high sensitivity and selectivity, minimal space and power requirements, along with the low-cost instrumentation for on-site measurements. The IEB device developed in this study provides the capability of accurate biomonitoring of the metabolite TCP in the rat plasma which has been *in vivo* exposed to the CPF. The novel IEB device also opens up a new pathway for the design of hand-held device for many other clinical applications.

Acknowledgments

This work was conducted at Pacific Northwest National Laboratory (PNNL) and supported partially by Grant R01 OH008173-01 from the Centers for Disease Control/National Institute for Occupational Safety and Health, and Grant Number U01 NS058161-01 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke. This work was also supported partially by Key Project in the National Science & technology Pillar Program (2009BADB9B03). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. The characterization work was performed at the Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research located at PNNL. PNNL is operated for DOE by Battelle under Contract DE-AC05-76L01830. L.W. would like to acknowledge the fellowship from the China Scholarship Council and the fellowship from PNNL.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.11.008.

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