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A novel protocol for ultra-trace detection of pesticides: Combined electrochemical reduction of Ellman's reagent with acetylcholinesterase inhibition

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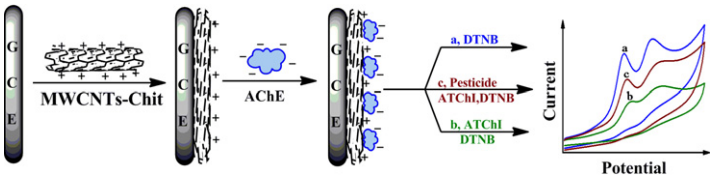
HIGHLIGHTS

- A novel method was proposed for ultra-trace detection of pesticides.
- This method combined electrochemical reduction of DTNB with AChE inhibition.
- The fabricated biosensor determined methyl parathion with high sensitivity.
- The developed biosensor was used in real samples with satisfactory results.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper proposed a novel method for ultra-trace detection of pesticides combining electrochemical reduction of Ellman's reagent with acetylcholinesterase (AChE) inhibition. The amperometric biosensor, fabricated by immobilizing AChE on multi-walled carbon nanotubes-chitosan (MWCNTs-Chi) nanocomposites modified glassy carbon electrode, enjoyed high sensitivity owing to the excellent conductivity and favourable biocompatibility of MWCNTs-Chi nanocomposites. Meanwhile, the sensitivity of the biosensor was further enhanced using the electrochemical reduction signal of DTNB for determination. Under optimum conditions, methyl parathion was detected based on its inhibition effect on AChE activity and the subsequent change in electrochemical reduction response of DTNB. Good relationship was obtained between the reduction current and pesticide concentration in the ranges of 5.0×10^{-7} to 1.0×10^{-12} M with a detection limit of 7.5×10^{-13} M (S/N = 3). Moreover, the proposed protocol was successfully employed for the determination of methyl parathion in water and soil samples.

1. Introduction

Ultra-sensitive detection of pesticide residues has attracted increasing attention in the past couple of decades due to their high toxicity and persistence [1–6]. At present, many techniques such as gas chromatography (GC) [7,8], high performance liquid chromatography (HPLC) [9,10], gas chromatography with mass spectrometry (GC/MS) [11,12] and liquid chromatography with mass spectrometry (LC/MS) [13] have been developed for the determination of pesticides. But some intrinsic disadvantages of either required separation/preconcentration steps and long analysis times, or expensive analysis settings entailing well-trained personnel and inconvenience for in situ applications restrict their further applications. Recently, micro-flow injection system (μFIA) is widely proposed method for pesticide detection due to its automation, efficiency and versatility [14]. The flow rate of μFIA is

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on the order of nL to μL min^{-1}, which drastically reduce waste generation. Nevertheless, this device is usually dedicated to a specific application.

In parallel with typical chromatography, the electrochemical biosensors based on the inhibition of acetylcholinesterase (AChE) activity have been confirmed as one of the most promising alternative for pesticide measurement by virtue of its simplicity, rapidity, high sensitivity and versatility [15–18]. The activity of the enzyme is measured by anodic oxidation of the thiocholine (TCh) produced from the enzymatic hydrolysis of acetylthiocholine by AChE (Eqs. (1) and (2)) [17–19]:

$$\text{acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid} \quad (1)$$

$$\text{2-thiocholine} \xrightarrow{\text{anodic oxidation}} \text{disulfide} + 2H^+ \quad (2)$$

In the presence of pesticides, the enzyme function is inhibited, resulting in a decrease in TCh formation. The inhibition degree of AChE is therefore correlated to the decrease in TCh oxidative current, which depends on the concentration of the pesticide in the sample. This method cannot selectively detect and quantify different pesticides because most organophosphorous and carbamate pesticides can inactivate cholinesterase. However, it is suitable as a screening tool for providing a rapid response and signalling the existence of contaminated samples [20–23]. Unfortunately, the oxidation of TCh, a kind of sulfhydryl compound, occurs at relative high potential (about 0.7 V) which causes high background current and interference from other electroactive compounds with the added problem of electrode surface fouling [24,25]. Accordingly, it still remains a great challenge to develop a rapid, inexpensive but sensitive method for the detection of pesticides in environments.

The most widely used colorimetric technique for the detection of sulfhydryl compounds was first carried out by Ellman in [26]. In the Ellman's test, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) reacted with molecules containing a free thiol group producing highly yellow-coloured 5-thio-2-nitrobenzoic acid (TNBA) (Eq. (3)), which could be detected spectroscopically by UV–vis absorption spectroscopy.

$$\underset{\text{DTNB}}{\text{5, 5-dithiobis(2-nitrobenzoic acid)}} + 2\ \text{sulfhydryl species} \rightarrow \underset{\text{TNBA(yellow)}}{\text{2, 5-thio-2-nitrobenzoic acid}} + \text{disulfide} \quad (3)$$

This method is simple, accurate and relatively economic, and it is adaptable for automated analyzers or plate readers for the rapid processing of large numbers of samples [6,27]. Recent years, several researchers have adapted the traditionally colorimetric Ellman's test into an electrochemical measurement for the determination of sulfhydryl compounds. Nekrassova et al. [28] investigated the electrochemical response of DTNB to increasing additions of thiol species. Experiment results revealed that there was an enhancement in the thiol oxidation current when DTNB was present in the solution. Similarly, free thiol compounds in food flours samples were successfully measured based on the electrochemical adaptation of the Ellman's test [29]. The DTNB reacted with thiol compounds present in food flour extracts, producing electroactive compound TNBA which, in turns, reacted with phenylenediamine. More importantly, Mannino's group [30] found that organic thiols could be detected by electrochemical reduction of DTNB at a bare glassy carbon electrode. The voltammetric protocol, relied on the electrochemical reduction of the nitro groups of DTNB in the presence of thiols, was proposed as a highly selective thiol species detection assay without the need of mediator species.

Motivated by those impressive researches, the present work focused on the development a novel electrochemical detection protocol for pesticides combining electrochemical reduction of Ellman's reagent with AChE inhibition. The amperometric biosensor was fabricated by immobilizing AChE on multi-walled carbon nanotubes-chitosan (MWCNTs-Chi) nanocomposites modified glassy carbon electrode (GCE). The immobilized enzyme retained relatively high bioactivity (about 96.1% relative to free enzyme) and the fabricated biosensor exhibited high sensitivity and fast response to acetylthiocholine iodide due to the inherent merits of MWCNTs-Chi nanocomposites, which not only improved the whole conductivity of the biosensor, but also provided a favourable biocompatible microenvironment for AChE. Moreover, the use of the electrochemical reduction of DTNB as detection signal could avoid the electrode passivation as a result of oxidative electrochemical procedures.

2. Experimental

2.1. Reagents and apparatus

AChE from *Electrophorus electricus* (E.C. 3.1.1.7, 265 U mg^{-1}) and acetylthiocholine iodide (ATChI) were purchased from Sigma (USA). AChE stock solution was prepared with 0.01 M pH 7.4 phosphate buffer solution. Chitosan and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Methyl parathion (*O*,*O*-dimethyl *O*-(4-nitrophenyl) phosphorothioate, MP) was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China), and its stock solutions were prepared in anhydrous ethanol. MWCNTs purchased from Nachen S&T Ltd. (Beijing, China) were treated by refluxing in a 3:1 mixture (v/v) of concentrated H_2SO_4 and concentrated HNO_3 solution for 2 h, followed by a thorough washing with double distilled water until the filtrate became neutral [31,32]. Phosphate buffer solution (PBS) was prepared by mixing stock solutions of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusting the pH with 0.1 M HCl or 0.1 M NaOH. All chemicals were of analytical reagent grade and all the aqueous solutions were prepared with redistilled water.

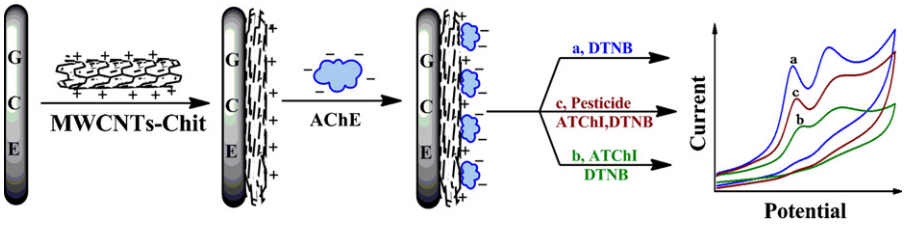
Electrochemical experiments were performed with a CHI660C electrochemical workstation (Shanghai Chenhua Co., China). A conventional three-electrode system was used comprising a bare or modified GCE as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. All measurements were performed at room temperature (25 ± 2 °C).

2.2. Preparation of amperometric biosensor

The GCE was first polished to a mirror-like surface with 0.3 and 0.05 μm alumina slurry, and then sonicated successively in absolute alcohol and doubly distilled water. Finally, the GCE was dried under the stream of high purity nitrogen for further use. In order to prepare the modified electrode, 1.0 mg MWCNTs were dispersed in 1 mL 0.1% chitosan solution with ultrasonication for 1 h to form a homogenous MWCNs-Chi nanocomposites. With a microinjector, 5 μL of the suspension was dropped on freshly prepared GCE surface and dried under an infrared lamp forming MWCNs-Chi nanocomposites modified GCE, denoted as MWCNTs-Chi/GCE. After that, 0.4 U of AChE was coated on the surface of MWCNTs-Chi/GCE and allowed to dry at room temperature. Finally, the biosensor (noted as AChE/MWCNTs-Chi/GCE) was immersed in pH 7.0 PBS to remove the loosely adsorbed AChE and stored at 4 °C when not in use. In parallel, AChE modified GCE (AChE/GCE) was prepared as control.

2.3. Measurement procedure

For electrochemical measurement of pesticides, the AChE/MWCNTs-Chi/GCE was firstly immersed in 0.1 M PBS (pH 7.0) containing different concentrations of standard pesticides



Scheme 1. Schematic diagram of the fabricating processes of the biosensor.

for 15 min, then immediately transferred to an electrochemical cell of 10 mL pH 7.0 PBS (0.1 M) containing 0.70 mM ATChI and 0.50 mM DTNB. After immersion for 120 s, the electrochemical response was recorded by differential pulse voltammetry (DPV). DPV was performed from −0.45 to −0.80 V with the parameters of increment potential, 0.004 V; pulse amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s; pulse period, 0.2 s and quiet time, 2 s. The current change (ΔI) of DTNB was calculated as Eq. (4):

$$\Delta I = I_i - I_0 \quad (4)$$

where I_0 was the initial electrochemical reduction current of DTNB on AChE/MWCNTs-Chi/GCE (without inhibitor) and I_i was the current after the incubation with inhibitor.

2.4. Sample preparation

Water samples were obtained from Naihe River located in the Taian region in Shandong (China). Before use, water samples were filtered with a 0.45 μm filter to eliminate particulate matters and the pH was adjusted to 7.0. These samples were then probed with a known concentration of MP and stored in refrigerator (4 °C) for use.

Soil samples were collected from trial plots of Shandong Agricultural University (China). Before use, soils were homogenized, sieved (2-mm mesh) and air-dried at room temperature. Spiking was performed as follows: Ten grams of homogenized soil was mixed with appropriate amounts of MP-spiked solutions prepared in ethanol. After 24 h, they were extracted with 50 mL of ethanol in an ultrasonic bath for 30 min. Afterwards, the samples were centrifuged at 4000 rpm for 10 min and the supernatant was separated. At last, the soil extracts were diluted with 0.1 M PBS (pH = 7.0) and were assayed as described above.

3. Results and discussion

3.1. Fabricated processes and characterizations of the biosensor

The biosensor was fabricated based on immobilization of AChE on MWCNTs-Chi nanocomposites modified GCE via electrostatic interaction. Under neutral experimental conditions, AChE possesses negative charge because its isoelectric point I_p is 4.5 [33]. Meanwhile chitosan is positively charged due to the protonation of its amino groups [34]. Firstly, positively charged MWCNTs-Chi nanocomposites were cast onto the surface of GCE. And then, a layer of negatively charged AChE was adsorbed on the MWCNTs-Chi film in pH = 7.0 PBS. In the MWCNTs-Chi nanocomposite film, MWCNTs possessed excellent inherent conductivity to enhance the electron transfer rate, and chitosan maintained the high biological activity of the immobilized enzyme due to its excellent biocompatibility, thus a sensitive and stable biosensor was fabricated. The procedure of the construction of AChE/MWCNTs-Chi/GCE was outlined in Scheme 1.

The electrochemical property of different electrodes was characterized by electrochemical impedance spectroscopy (EIS) using $[Fe(CN)_6]^{3-/4-}$ as redox probe. In Fig. S1 (Supporting information), the semicircle diameter of impedance spectroscopy equals the interfacial electron transfer resistance (R_{et}), which controls the electron transfer kinetics of the redox probe at the electrode surface [35]. The R_{et} of bare GCE (curve a) was 240 Ω. When the GCE was modified with MWCNTs-Chi nanocomposites, an almost straight line (curve b) was exhibited, demonstrating a characteristic of a diffusion-controlled electrochemical process [35]. As expected, after AChE was immobilized on the electrode, the penetration of the redox probe was reduced, leading to a higher interface electron resistance (550 Ω, curve c). For proving the advantages of MWCNTs-Chi nanocomposites, the EIS of AChE/GCE was determinated as 1100 Ω (curve d). Compared with that of AChE/GCE, the R_{et} of AChE/MWCNTs-Chi/GCE remarkably decreased. The sharp decrease of R_{et} could be attributed to the excellent conductivity of MWCNTs which promoted electron transfer on electrode interface [36].

3.2. Amperometric response of the biosensor

The amperometric responses of the biosensor were tested by potentiostatic measurements at a potential of +0.7 V (the applied potential was optimized and shown in Fig. S2, Supporting information). Fig. 1A showed a typical current-time plot of AChE/MWCNTs-Chi/GCE biosensor to successive additions of different concentrations of ATChI. With increasing ATChI concentration, the response current of the enzyme electrode increased and reached 95% steady state value within 6 s. As mentioned above, such a fast response was attributed to the ability of MWCNTs-Chi nanocomposites to promote the electron transfer. The linear range spanned the concentration of ATChI from 2 to 700 μM with the correlation coefficient of 0.9991 (Fig. 1B). A detection limit of 1.0 μM was estimated on the basis of an S/N = 3. When the concentration of ATChI was above 700 μM, no significant current improvement was observed, demonstrating the electrode had reached its saturation level. From the above results, 700 μM was selected as optimum concentration for further experiments.

At higher ATChI concentrations the shape of the response was indication of a Michaelis–Menten process (Fig. 1B). The K_M^{app} value for AChE/MWCNTs-Chi/GCE biosensor in this study was calculated to be 0.23 mM according to the electrochemical version of Lineweaver–Burk equation [37]. This value was lower than that for AChE adsorbed on carbon nanotube *N*,*N*-dimethylformamide (DMF) composite film (0.66 mM) [38] and polyethyleneimine modified electrode (1.5 mM) [39], indicating that the immobilized AChE had a great affinity to ATChI. Contrast with free AChE (the K_M^{app} value was 0.221 mM), the immobilized enzyme maintained 96.1% bioactivity, indicating the effective immobilization of AChE. Meanwhile, as a control, the K_M^{app} value of AChE/GCE was calculated to be 17.43 mM, which was 7.58 times of that for AChE/MWCNTs-Chi/GCE. This phenomenon resulted from the contribution of MWCNTs-Chi nanocomposites, which not only improved the conductivity of the biosensor, but also provided a favourable biocompatible microenvironment for AChE.

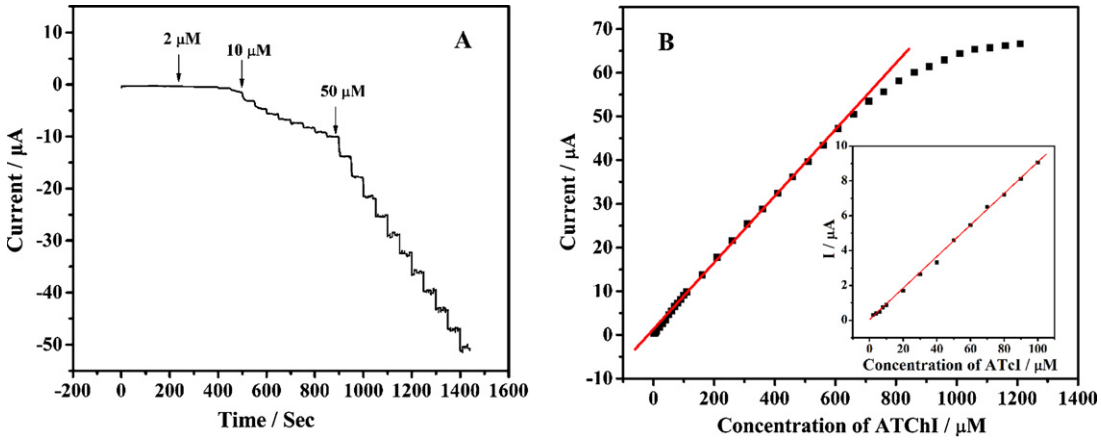


Fig. 1. (A) Typical current–time response of the biosensor. (B) The Lineweaver–Burk plot of response current vs. concentration of ATChI (insert: the amplified calibration curve of the biosensor within 0–100 μM). Applied potential, +0.7 V; supporting electrolyte, 0.1 M pH 7.0 phosphate buffer solution.

3.3. Electrochemical behaviours of DTNB

The proposed protocol is based on the direct electrochemical reduction of DTNB at the fabricated biosensor. Fig. 2A detailed the cyclic voltammetric (CV) response of a solution containing 0.5 mM DTNB towards increasing additions of 0.1 mM ATChI at the fabricated biosensor. In the absence of ATChI, two reductive waves were observed at −0.66 V and −0.95 V in pH 7.0 PBS, which correspond to the reduction of the nitro moieties of DTNB [30]. When a known amount of ATChI was introduced to the solution, the colour of the solution turned immediately yellow, indicating that the coloured TNBA was produced from the rapid reaction between DTNB and TCh. Upon the introduction of ATChI to the solution, the DTNB reagent began to be consumed, thereby the corresponding peak current decreased. With the increasing additions of ATChI, the peak current decreased linearly, as shown in Fig. 2A.

Fig. 2B revealed the difference of electrochemical response of the biosensor in the presence and absence of ATChI. As explained above, DTNB possessed two reductive waves at AChE/MWCNTs-Chi/GCE (Fig. 2B, curve a). A remarkable drop in the peak current emerged upon the introduction of ATChI to the solution resulting from the depletion of DTNB (Fig. 2B, curve b). However, a striking enhancement of response current was found after the biosensor being inhibited by a certain amount of pesticide (Fig. 2B, curve c). This phenomenon resulted from the inhibition of pesticides which deactivated the AChE limiting the production of TCh. Therefore, the depletion of DTNB decreased and the reduction current of DTNB increased relative to the current without an inhibitor [40]. As the reaction ratio of TCh to DTNB formed is 2:1 [30] and the decay of enzyme activity is in proportion with pesticide concentration, the current change of DTNB prior to and after inhibition could be used to assess the concentration of inhibitor. Compare to conventional detection methods based on enzyme inhibition, the new protocol was more sensitive due to the ease reduction of DTNB.

3.4. Methyl parathion detection

Experimental parameters, such as pH, enzyme catalysis reaction time and inhibition time were optimized, and the results were shown in Fig. S3. As can be seen, the optimum conditions were 7.0 for pH, 120 s for enzyme catalysis reaction time and 15 min for inhibition time (Supporting information). In addition, there was no obviously amperometric response for the dissolved oxygen in buffer on the modified electrode, which means that methyl parathion can be determined without interference of oxygen using the proposed method.

Under the above optimal parameters, AChE/MWCNTs-Chi/GCE was used to detect methyl parathion by DPV. It should be noted the potential was stopped at −0.8 V, since lower potential are less attractive for analytical purposes due to the rising risk of

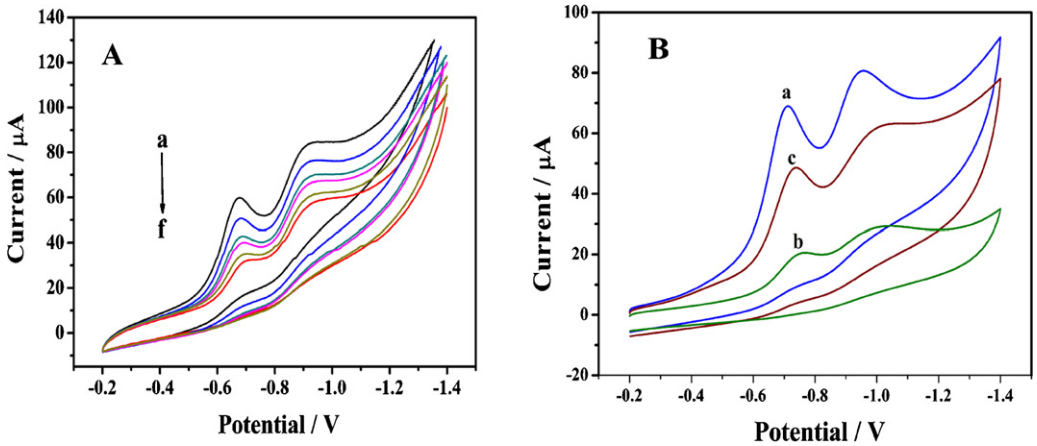


Fig. 2. (A) The CV response of 0.5 mM DTNB towards increasing additions of ATChI (a–f: 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM) at AChE/MWCNTs-Chi/GCE in 0.1 M PBS (pH = 7.0). Scan rate = 100 mV s^{-1}. (B) Comparison of CV response of DTNB (0.5 mM) at AChE/MWCNTs-Chi/GCE (a) in 0.1 M PBS (pH = 7.0), (b) in 0.1 M PBS (pH = 7.0) containing 0.7 mM of ATChI and (c) in 0.1 M PBS (pH = 7.0) containing 0.7 mM of ATChI after the biosensor being inhibited by methyl parathion (100 nM) for 15 min.

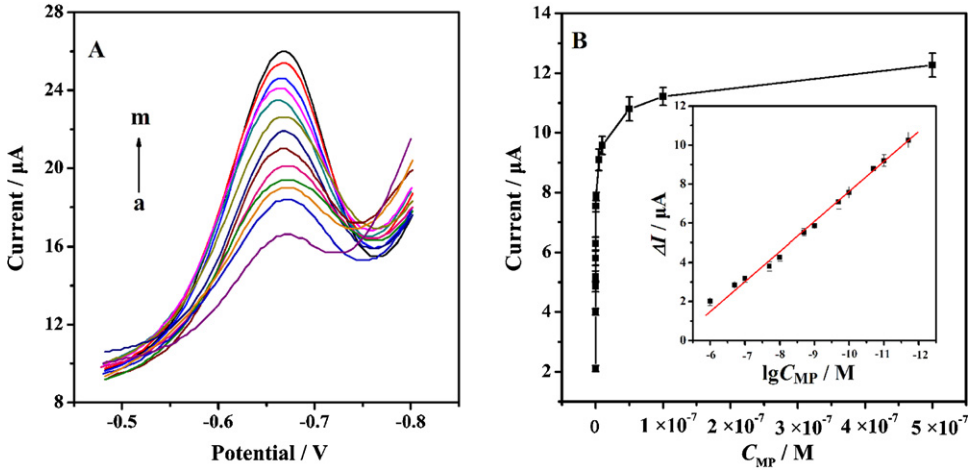


Fig. 3. (A) DPV of the biosensor after inhibition by different concentrations of MP (a–m: 0, 1×10^{-12}, 5×10^{-12}, 1×10^{-11}, 5×10^{-11}, 1×10^{-10}, 5×10^{-10}, 1×10^{-9}, 5×10^{-9}, 1×10^{-8}, 5×10^{-8}, 1×10^{-7}, 5×10^{-7} M). (B) Relationship between I and MP concentration. Inset: ΔI vs. $\log C_{MP}$.

interferences from solvent decomposition and hydrogen evolution. Fig. 3 presented the DPV responses recorded in pH 7.0 PBS containing 0.5 mM DTNB and 0.7 mM ATChI after the biosensor being inhibited by different concentration of MP for 15 min. It was evident that the produced DPV response increased gradually with the increase of the MP concentration. This result could be explained that in the presence of pesticides, the AChE was inhibited and its activity decreased, resulting in a decrease of produced TCh concentration and consequently a decrease of the depletion of DTNB. Good linear relationship between current change of DTNB (ΔI) and $\log C_{MP}$ was obtained from 1.0×10^{-12} M up to 5.0×10^{-7} M with the regression equation of ΔI (μA) = 19.4957 + 1.4897 $\log C_{MP}$ (M) (R = 0.9986) and a detection limit of 7.5×10^{-13} M estimated from signal-to-noise ratio of 3. The detection limit was better than that achieved with a biosensor based on AChE immobilized on polyaniline deposited on vertically assembled CNTs wrapped with single stranded DNA (1.0×10^{-12} M) [41] and significantly lower than those obtained from most other enzyme-based inhibitor electrochemical sensors [42–45]. This result should be attributed to the effective immobilization of AChE, good biocompatibility of the microenvironment, satisfying conductivity of biosensor and the sensitive electrochemical reduction of DTNB.

3.5. Application on real samples

To further demonstrate the practicality of the proposed protocol, the recovery test was carried out by adding different amounts of MP into river water and soil samples. Results were summarized in Table 1. As can be seen, the results were in good agreement with the given concentration with average recoveries from 93.8% to 103.2% (n = 3). These results indicated that this new method could be used for assay of real samples.

Table 1
Recovery studies of MP in river water and soil samples (n = 3).

Sample	Taken (nM)	Found (nM)	Recovery (%)	RSD (%)
River water	0.050	0.0478	95.6	6.1
	1.00	1.032	103.2	4.6
	50.00	48.20	96.4	4.9
Soil samples	0.050	0.0469	93.8	5.4
	1.00	0.973	97.3	4.5
	50.00	51.30	102.6	3.8

3.6. Reproducibility, stability and reactivation

The inter-assay precision was estimated at six different electrodes for the determinations in 0.1 M PBS (pH = 7.0) containing 0.50 mM DTNB and 0.70 mM ATChI after being treated with a certain concentration of MP for 15 min. Similarly, the intra-assay precision of the biosensor was evaluated by assaying one enzyme electrode for six replicate determinations. The relative standard deviation (RSD) values of inter-assay and intra-assay were found to be 4.4% and 5.1%, respectively, indicating acceptable precision and reproducibility. When not in use, the enzyme electrode was stored at 4 °C in 0.01 M pH 7.4 PBS. No obvious decrease in the electrochemistry response was observed in the first week. After a 3-week storage period, the sensor retained 91% of its initial current response, demonstrating the acceptable stability of biosensor.

Enzyme regeneration is one of the key issues in the development of cholinesterase-based biosensors, since it solves the problem of multiple and/or continuous biosensor use. The AChE inhibited irreversibly by organophosphorous pesticides can be reactivated by use of nucleophilic compounds such as pralidoxime iodide [23,46–48]. Reactivation of the enzyme was investigated with pralidoxime iodide as an antidote. The AChE/MWCNTs-Chi/GCE was firstly immersed in pH 7.0 PBS containing 0.1 μM MP for 15 min. Then its electrochemical response to 0.70 mM ATChI was recorded before and after reactivation with 5.0 mM pralidoxime iodide for 15 min. It was observed that inhibited ACh could resume 94.6% original activity after reactivation. Based on this reactivation procedure the proposed biosensor could be used repeatedly with an acceptable reproducibility.

4. Conclusion

In this work, we have demonstrated a sensitive electrochemical sensing strategy for the detection of pesticide residuals by combination electrochemical reduction of DTNB with AChE inhibition mechanism. A remarkably high sensitivity has been achieved through dual-signal amplification offered by the excellent conductivity and favourable biocompatibility of MWCNTs-Chi nanocomposites and the ease reduction of DTNB. Unlike other enzyme-based biosensors, the electrode passivation resulted from oxidative electrochemical procedures could be avoided using the electrochemical reduction of DTNB as detection signal. The proposed protocol with good fabrication reproducibility, acceptable stability, fast response, low detection limit and reusage will pave

the new way for electrochemical detection of other toxic compounds against to AChE.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.11.042.

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