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Acetylcholinesterase biosensor based on chitosan/prussian blue/multiwall carbon nanotubes/hollow gold nanospheres nanocomposite film by one-step electrodeposition

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ABSTRACT

In this paper, chitosan–prussian blue–multiwall carbon nanotubes-hollow gold nanospheres (Chit–PB–MWNTs–HGNs) film was fabricated onto the gold electrode surface by one-step electrodeposition method; and then acetylcholinesterase (AChE) and Nafion were modified onto the film to prepare an AChE biosensor. Incorporating MWNTs and HGNs into Chit–PB hybrid film promoted electron transfer reaction, enhanced the electrochemical response and improved the microarchitecture of the electrode surface. The morphologies and electrochemistry properties of the composite were investigated by using scanning electron microscopy, transmission electron microscopy, cyclic voltammetry and electrochemical impedance spectroscopy, respectively. Parameters affecting the biosensor response such as pH, enzyme loading and inhibition time were optimized. Based on the inhibition of pesticides on the AChE activity, using malathion, chlorpyrifos, monocrotophos and carbofuran as model compounds, this biosensor showed a wide range, low detection limit, good reproducibility and high stability. Moreover, AChE/Chit–PB–MWNTs–HGNs/Au biosensor can also be used for direct analysis of practical samples, which would be a new promising tool for pesticide analysis.

1. Introduction

Pesticides are widely used in modern agriculture due to their high efficiency as insecticides (Xue et al., 2012). Unfortunately, these compounds exhibit high acute toxicity, with the majority being hazardous to both human health and the environment (Chauhan and Pundir, 2012). Therefore, for human health safety and environmental protection purposes, it is necessary to develop sensitive and fast detection technology for pesticide residues. Biosensors can substitute the current analytical methods (liquid chromatography (Ye et al., 2009), gas chromatography (Guan et al., 2010), enzyme-linked immunoabsorbant assays (Rekha et al., 2010)) by simplifying or eliminating sample preparation, thus decreasing the analysis time and cost (Tuoro et al., 2011). Among them, the enzyme-based electrochemical biosensors are particularly attractive because of their high sensitivity, rapid response and miniature size, which have emerged as a promising alternative to rapidly detect pesticides.

In recent years, various nanomaterials have been widely employed to improve the performance of electrodes. Prussian blue (PB), a typical hexacyanoferrate, is a material with increasing interest for applications such as electrochromic devices, and electrochemical sensors. (Pan et al., 2004; Alamini et al., 2011). Several studies showed that PB could oxidize thiocholine, which is the product from the hydrolysis of acetylthiocholine catalyzed by AChE, at low potential (Song et al., 2011; Sun and Wang, 2010).

Chitosan (Chit) has been extensively used for immobilization of enzymes and constructing amperometric biosensors due to its excellent biocompatible, film forming ability, and a susceptibility to chemical modifications (Wang et al., 2009; Salam et al., 2011). Therefore, it is widely used as carrier material for enzyme immobilization (Gong et al., 2009 Ion et al., 2010). Recent study showed that Chit–PB hybrid film possesses excellent biocompatibility and good stability in both neutral and alkalescent solution (Song et al., 2011).

Multiwalled carbon nanotubes (MWNTs) have received increasing scientific and industrial interest due to their great chemical stability, excellent electrical conductivity and extremely high mechanical strength (Du et al., 2010a). When MWNTs were incorporated into Chit–PB hybrid film, and formed Chit–PB–MWNTs composite, which had high catalytic activity, and could form a random mesh on the electrode surface (Che et al., 2011).

In recent years, noble metal nanoparticles have been extensively utilized in biosensing owing to their extraordinary catalytic activities which are strongly dependent on their size, shape and

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the surrounding environment of the particle (Mandal et al., 2004; Marinov et al., 2010; Buiculescu and Chaniotakis, 2012). Particularly, hollow gold nanospheres (HGNs) have attracted a substantial research subject for the fabrication of electrodes. HGNs can promote electron transfer and have good biocompatibility. Fabricating HGNs to electrochemical biosensors can create larger current response and higher-selectivity.

Du et al. have reported the immobilization of AChE on the GCE modified with Chit–MWNTs composite. Using triazophos as a model compound, the detection limit is 0.01 μM (Du et al., 2007b). Song et al. (2011) have reported the AChE biosensor for quantitative determination of carbaryl pesticide based on PB–Chit hybrid film, and the detection limit is about 3 nM. Zhang et al. (2011) have fabricated immunosensor based on HGNs/Chit composite for determination of chloramphenicol. To date, however, the application of Chit–PB–MWNTs–HGNs composite in AChE biosensors has been still very less reported.

In this study, we developed an AChE biosensor based on Chit–PB–MWNTs–HGNs nanocomposite film formed by one-step electrodeposition. Electrodeposition has been reported recently as a method suitable for selective deposition of films with controllable thickness (Deepa et al., 2003). One-step electrochemical co-deposition is a clean and quick method to fabricate the Chit–PB–MWNTs–HGNs composite film. Because of the synergistic effects of Chit, PB, MWNTs and HGNs, the Chit–PB–MWNTs–HGNs film increased electron transfer obviously, improved the microarchitecture and exhibited a high electrocatalytic activity to AChE. Finally, Nafion film was used for immobilizing AChE, which prevented the loss of the enzyme molecules, improved the anti-interference ability of the biosensor and provided a biocompatible microenvironment to maintain enzymatic activity. The prepared biosensor showed a good electrocatalytic ability to oxidate thiocholine and accordingly had a broad linear range, low detection limit, short response time and high sensitivity.

2. Experimental

2.1. Apparatus

Electrochemical measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The working electrod was gold electrode ($d=1$ mm) or modied gold electrode. A saturated calomel electrode (SCE) and platinum wire electrode were used as reference electrode and auxiliary electrodes, respectively. The transmission electron microscopy (TEM) observations were obtained using the JEOL-1200EX TEM (Japan). Scanning electron micrographs (SEM) were studied by JSM-6360LV SEM (Japan).

2.2. Reagents and materials

Acetylcholinesterase (Type C3389, 500 U/mg from electric eel), acetylthiocholine chloride (ATCl), malathion, chlorpyrifos, monocrotophos and carbofuran were purchased from Sigma (USA). MWNTs were obtained from Nanjing XFNano Material Tech Co., China. $HAuCl_4$ and Cobalt (II) chloride hexahydrate ($CoCl_2\cdot 6H_2O$, 99.0%) were obtained from National Chemical Pharmaceutical Co., China. The 0.1 M pH 7.5 phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Other reagents were of analytical grade. All solutions were prepared using double distilled water.

2.3. Fabrication of HGNs

The HGNs were prepared according to the procedure described by Liu et al. (2010). Before the HGNs was synthesized, the Co nanoparticles were firstly prepared. The synthesis of Co nanoparticles was carried out based on the method in Kobayashi et al. (2003). A volume of 50 μL of 0.4 M $CoCl_2$ solution was added into 50 mL of deaerated aqueous solution containing 8 mM $NaBH_4$ and 0.8 mM citric acid to fabricate Co-NPs. High-purity nitrogen was passed into the solution for 20 min to avoid the oxidation of obtained Co-NPs by existed atmospheric oxygen in the solution. After the reaction continued for several minutes, the hydrogen gas was observed to generate gradually. Following with the end of gas evolution, 30 mL of Co-NPs colloidal solutions were extracted and transferred into 18 mL of stirred 1 mM $HAuCl_4$ aqueous solutions. The color of the solution became blackish blue gradually, which showed that a trans-metallation reaction occurred. The obtained HGNs suspension solution was centrifuged at 10,000 rps and the precipitates were redispersed in 5 mL pH 7.5 PBS.

$$3Co+2[AuCl_4]^- = 2Au+3Co^{2+}+8Cl^-$$

2.4. Preparation of the electrodeposition base solution

Chitosan (Chit) solution (0.05%) (w/v) was prepared by dissolving 50 mg Chit powder in 100 mL 1.0% acetic acid solution and stirring it until Chit powder dissolve completely. Then the solution was stored at 4 °C when not in use.

Before the experiment, MWNTs (1 mg/mL) and HGNs (0.5 mg/mL) were dispersed into Chit solution to give a homogeneous dispersion and noted as solution A. Solution B contained 5 mM $FeCl_3$, 5 mM $K_3[Fe(CN)_6]$, 0.2 M KCl, 0.2 M HCl and 0.05% Chit. Prussian Blue was deposited onto the surface of the electrode when $FeCl_3$ (Fe^{3+}) and $K_3[Fe(CN)_6]$ ($Fe(CN)_6^{3-}$) were coexistent in the electrodeposition solution. The process takes place on the anode at a constant potential of 0.4 V versus SCE electrode. The reactions were as presented in Eq. (1) (Abbaspour and Kamyabi, 2005; Ding et al., 2008). Before electrodepositing, the solution A was firstly mixed with solution B under stirring condition (v/v=1:1) to form the electrodeposition base solution.

$$Fe^{3+}\xrightarrow{+e}Fe^{2+}\xrightarrow{Fe(CN)_6^{3-}}KFe[FE(CN)_6] \quad (1)$$

2.5. Preparation of Nafion/AChE/Chit–PB–MWNTs–HGNs/Au biosensor

A gold electrode was polished carefully to a mirror-like surface with 0.3 μm and 0.05 μm Al_2O_3 paste and washed through sonication with ethanol, nitric acid and doubly distilled water for 5 min. After the electrode was dried in air, the clean gold electrode was immersed into the freshly prepared electrodeposition base solution, which was being stirred slightly, and electrodeposited for 300 s with a constant potential of +0.4 V (vs. SCE). After that, the electrode was scanned in the potential range from −0.05 to 0.35 V at a scan rate of 50 mV/s in 0.1 M KCl/ 0.1 M HCl until the cyclic voltammetric (CV) curve was stable. The obtained electrode (Chit–PB–MWNTs–HGNs/Au) was washed thoroughly with ultra-pure water and then dried in air at room temperature. After the water was evaporated, Chit–PB–MWNTs–HGNs/Au was coated with 5.0 μL AChE solution to obtain the AChE/Chit–PB–MWNTs–HGNs/Au. Finally, the AChE/Chit–PB–MWNTs–HGNs/Au electrode was coated with an extra 3.0 μL 0.5% Nafion to maintain the stability of modified electrode. The schematic illustration of the stepwise procedure of the biosensor fabrication was shown in Fig. 1.

2.6. Electrochemical detection of pesticides

The Nafion/AChE/Chit–PB–MWNTs–HGNs/Au biosensor was employed for the determination of pesticides using differential

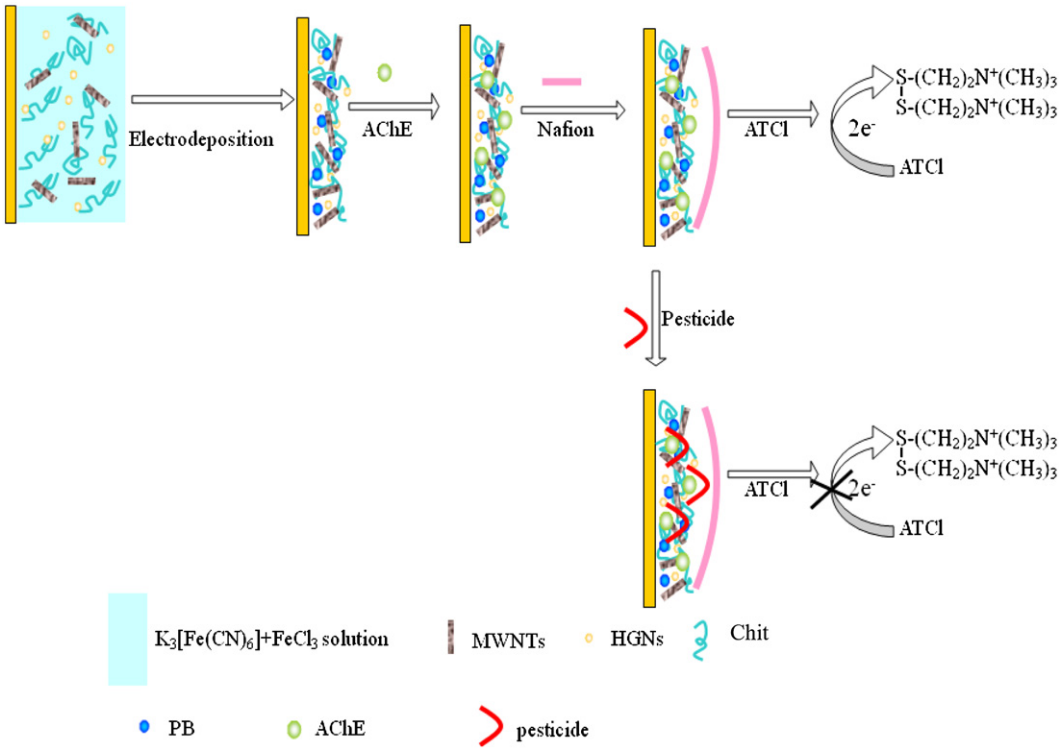

Fig. 1. Schematic illustration of the stepwise AChE biosensor fabrication process and immobilized AChE inhibition in pesticide solution.

pulse voltammetry (DPV) method. The performance of the biosensor was tested by its DPV response in pH 7.5 PBS solution containing 1.0 mM ATCl. Then the electrode was rinsed with water and incubated in an aqueous solution containing the desired concentration of pesticides (malathion, chlorpyrifos, monocrotophos, and carbofuran) for 10 min. Finally, it was transferred into the 1.0 mM ATCl solution for DPV measurements at the same condition. The inhibition rate of pesticides was calculated as follows:

$$\text{Inhibition}(\%) = (I_{P,\text{control}} - I_{P,\text{exp}})/I_{P,\text{control}} \times 100\% \quad (2)$$

where $I_{P,\text{control}}$ was the peak current of ATCl on Nafion/AChE/Chit–PB–MWNTs–HGNs/Au with pesticides inhibition, $I_{P,\text{exp}}$ was the peak current of ATCl on Nafion/AChE/Chit–PB–MWNTs–HGNs/Au with pesticides inhibition. Inhibition (%) was plotted against the concentrations of the pesticides to obtain linear calibration graphs.

2.7. AChE reactivation

After exposure to pesticides, the Nafion/AChE/Chit–PB–MWNTs–HGNs/Au electrode was firstly washed with 0.1 M pH 7.5 PBS, then reactivated by immersing in 5.0 mM pralidoxime iodide for 12 min, and then transferred to 0.1 M pH 7.5 PBS containing 1.0 mM ATCl for DPV analysis of the electrochemical response. The reactivation efficiency was calculated as follows:

$$R(\%) = (I_r/I_{P,\text{control}}) \times 100\% \quad (3)$$

where I_r was the peak current of 1 mM ATCl on Nafion/AChE/Chit–PB–MWNTs–HGNs/Au after 5.0 mM pralidoxime iodide reactivation.

2.8. Preparation and determination of real samples

The real samples were prepared according to the procedure described by Qu et al. (2010). The cabbage, lettuce, leek and pakchoi bought from a local supermarket were washed three times with double-distilled water and then chopped. And then, 5 g of each sample was sprayed with different concentrations of malathion, chlorpyrifos, monocrotophos and carbofuran, respectively. After 24 h of storage at 4 °C, each sample was mixed with the 10 mL mixed solution of acetone/0.1 M pH 7.5 PBS (1/9, v/v), which was obtained through 15 min of ultrasonic treatment. After the suspensions were centrifuged (10 min, 10,000 rpm), the acquired supernatants were detected by DPV directly without extraction or preconcentration. The concentration of pesticides in the samples can be obtained from the calibration curve.

3. Results and discussion

3.1. SEM characterizations of Chit–PB–MWNTs–HGNs composite film and TEM characterization of HGNs

The morphologies of the films were investigated by SEM (Fig. 2A, B and C). As seen from Fig. 2A, the Chit–PB film with lots of small PB particles was formed on the gold surface. After MWNTs added, a large number of filamentous structure was seen in Fig. 2B. When HGNs were incorporated into the Chit–PB–MWNTs film, the structure of the composite had a large change, showing that HGNs was successfully electrodeposited onto the surface of gold electrode (Fig. 2C).

The microstructure of HGNs was also observed by TEM. The contrast between the dark edge and the bright center provided convincing evidence of the hollow structure. Fig. 2D showed that hollow structure of gold spheres obviously possessed a rough outer surface which should be very favorable for the application in biosensor due to its large surface area.

3.2. Electrochemical behavior of the modified electrodes

The stepwise assembly of the biosensor was characterized by EIS, and the results were shown in Fig. 3. As what can be seen, curve **a**

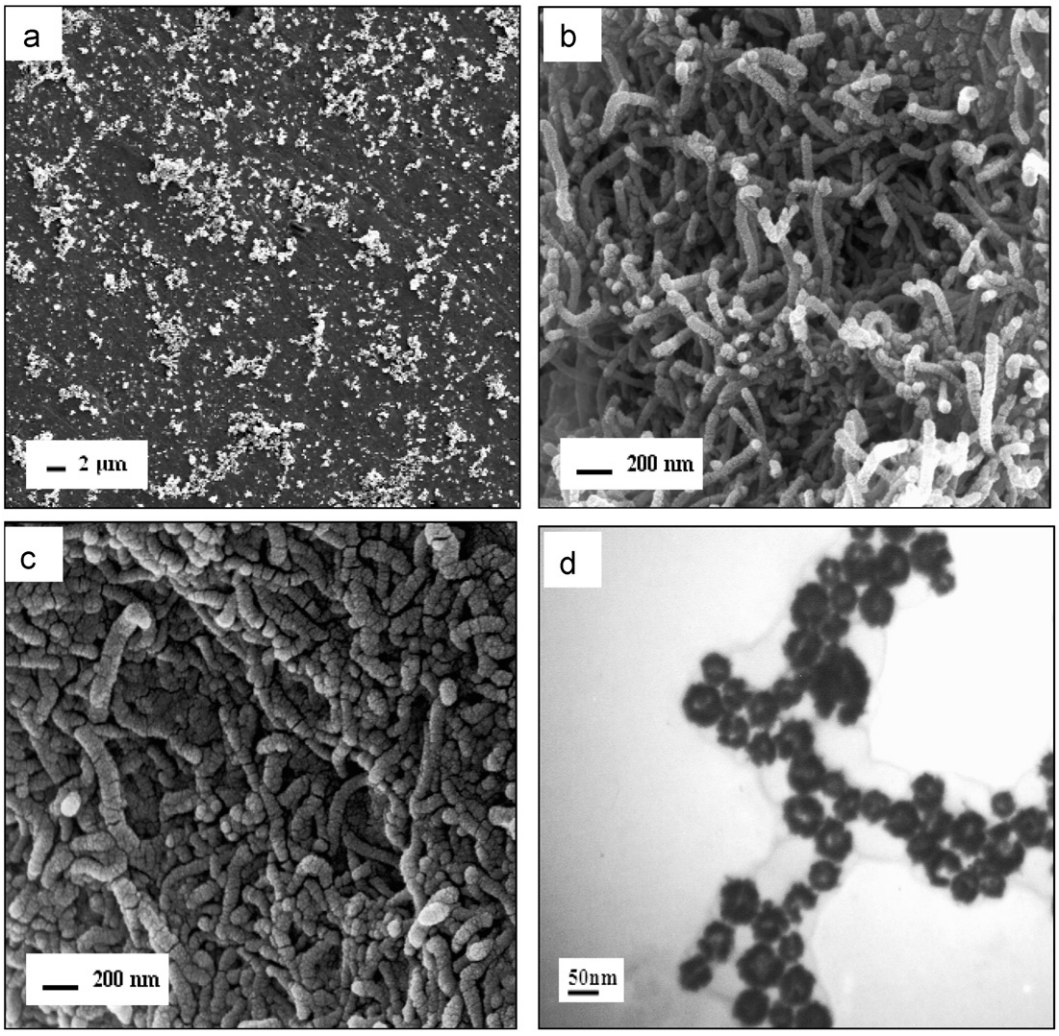

Fig. 2. SEM images of (A) Chit–PB film; (B) Chit–PB–MWNTs film; (C) Chit–PB–MWNTs–HGNs film and (D) TEM image of HGNs.

presented a small semicircle domain implying a low electron transfer resistance (R_{et}) about 85 Ω on the bare gold electrode. After Chit–PB–MWNTs–HGNs composite was deposited onto the electrode surface, the R_{et} decreased dramatically to about 20 Ω (curve **b**). The reason maybe that the excellent conductivities of Chit–PB–MWNTs–HGNs composite have porous network-like structure, and have larger effective surface area than the bare electrode surface. After AChE was modified onto Chit–PB–MWNTs–HGNs/Au electrode, the EIS presented an apparent increase and the R_{et} was about 1255 Ω (curve **c**), which was due to the inhibition effect of the macromolecules for electron transfer. The R_{et} further increased to about 1800 Ω when non-conductive properties of Nafion covered onto the AChE/Chit–PB–MWNTs–HGNs/Au (curve **d**).

The cyclic voltammograms of various electrodes were investigated in pH 7.5 PBS including 0.1 M KCl (see the supporting information, Fig. S1A, B and C). The image showed the CV of bare gold electrode (curve **a**), in which no peak was observed as a result of the lack of redox-active material. After the electrode was electrodeposited in the electrodeposition base solution, the cyclic voltammograms of Chit–PB–MWNTs–HGNs/Au (curve **b**) showed a pair of well-defined redox peaks, indicating that PB had been assembled on the electrode surface through electrochemical co-deposition. The redox peaks decreased drastically when AChE (curve **c**) and Nafion (curve **d**) were modified onto CS–PB–MWNTs–HGNs composite modified gold electrode, respectively.

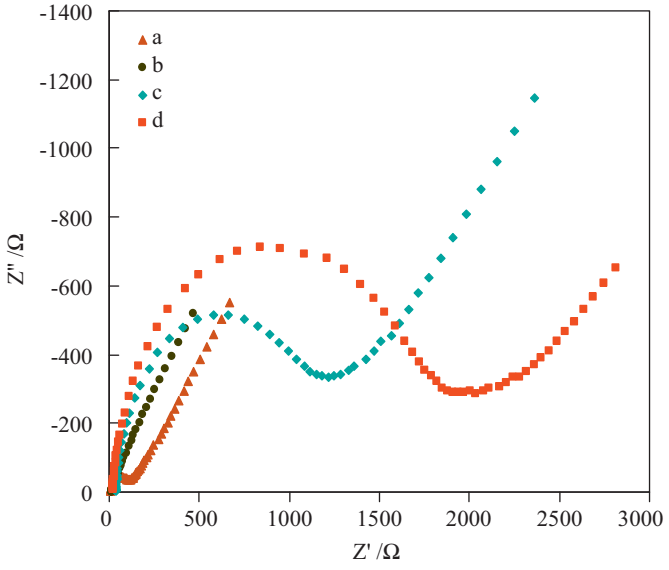

Fig. 3. The EIS of different electrodes in 5.0 mM $[Fe(CN)_6]^{4-/3-}$ and 0.1 M KCl solution of different electrodes in pH 7.5 PBS including 0.1 M KCl: (a) bare Au electrode; (b) Chit–PB–MWNTs–HGNs/Au; (c) AChE/Chit–PB–MWNTs–HGNs/Au and (d) Nafion/AChE/Chit–PB-MWNTs–HGNs/Au.

As shown in Fig. S1B, with incorporating of MWNTs and HGNs into the Chit–PB hybrid film, the peak current of Chit–PB–MWNTs–HGNs/Au was obviously higher than that of Chit–PB/Au, Chit–PB–HGNs/Au and Chit–PB–MWNTs/Au. Moreover, the R_{et} of Chit–PB–MWNTs–HGNs/Au was smaller than that of the electrodes without MWNTs or HGNs nanochains (Fig. S1C). The results indicated that MWNTs and HGNs improved the conductivity of composite, which could enhance the performance of biosensor.

3.3. Optimization parameters of the biosensor performance

The pH plays an important role in achieving good analytical performance. The optimum pH for the AChE enzyme is between 7.0 and 8.0 (Ganesana et al., 2011). The response currents of the enzyme electrode were investigated in a series of PBS (0.1 M, pH from 5 to 9) including 1 mM ATCl (see the Supporting information, Fig. S2A). The maximum value of the response current was at pH 7.5. Thus, a pH 7.5 of PBS was used in the subsequent experiment.

The influence of enzyme amount on the response of biosensor was also investigated (see the Supporting information, Fig. S2B). The results showed that the increasing of the AChE amount, the peak current increased gradually and reached the maximal value at 0.1 U. After that, the amperometric response decreased gradually as the amount of AChE further increased. Therefore, 0.1 U of AChE was chosen as the optimal enzyme amount for preparation of the biosensor.

The effect of inhibition time was investigated with malathion, chlorpyrifos, monocrotophos and carbofuran, respectively. The results showed that the peak current decreased greatly with the increase of inhibition time (see the Supporting information, Fig. S2C). When the immersing time was longer than 10 min, the inhibition curve trended to a stable value, which indicated that the interaction between pesticides and AChE reached equilibrium. Thus, the time of 10 min was selected as the optimum inhibition time.

3.4. Amperometric response to ATCl

Fig. 4 showed a typical current–time plot of AChE/Chit–PB–MWNTs–HGNs/Au biosensor under the optimum experimental conditions in a stirred solution, in which different concentrations of ATCl (0.5 μM, 2.5 μM, 10 μM and 20 μM, respectively) were successively added every 20 s. The oxidation current of ATCl was proportional to the concentration from 54 μM to 254 μM (inset Fig. 4). The equation was I (μA)=0.0051 c (μM)+0.4939 (R=0.9906). The corresponding sensitivities, determined as the slope of the linear calibration curve was 5.1 μA/mM.

The apparent Michaelis–Menten constant K_M^{app} is a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants. It can be obtained from the electrochemical version of Lineweaver–Burk equation (Du et al., 2010a):

$$\frac{1}{I_{SS}} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max} C} \quad (4)$$

where I_{ss} is the steady-state current after addition of substrate, C is the bulk concentration of substrate, and $I_{\max}$ is the maximum current measured under saturated substrate conditions. The K_M^{app} value for AChE/Chit–PB–MWNTs–HGNs/Au biosensor in this study was calculated to be 0.21 mM.

3.5. Pesticides determination

The DPV responses were examined before and after exposure to different concentration pesticides (Fig. 5A). No peak was observed at Nafion/Chit–PB–MWNTs–HGNs/Au (curve **j**) in pH 7.5 PBS containing 1.0 mM ATCl. However, the DPV response of Nafion/AChE/Chit–PB–MWNTs–HGNs/Au showed an obvious peak at 631 mV (curve **a**) in pH 7.5 PBS containing 1.0 mM ATCl. This peak came from the oxidation of thiocholine, hydrolysis product of ATCl, which was catalyzed by immobilized AChE. The DPV responses were associated with the inhibition of AChE activity based on the amount of pesticide added (curve **a**–**i**). The decrease in redox peak current likely occurred as a result of blocking serine hydroxyl groups of AChE by covalently bound of pesticides, which invariably reduced the overall charge of the catalytic active site.

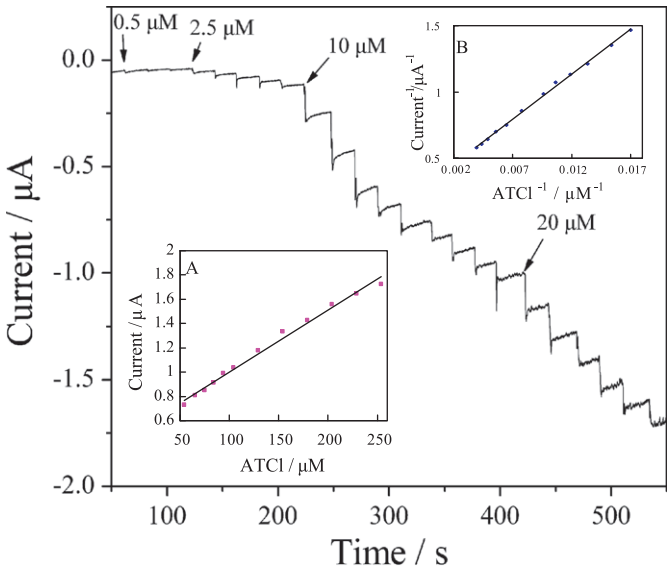

Fig. 4. Typical current-time plot for the sensor on successive addition of ATCl (0.5 μM, 2.5 μM, 10 μM, 20 μM) to pH 7.5 PBS, with stirring, at an applied potential of 0.7 V. Inset A show the calibration curve for ATCl determination; inset B show the Lineweaver–Burk plot of $1/I_{ss}$ vs. $1/C$.

As shown in Fig. 5B, the linear range and detection limit for malathion, chlorpyrifos, monocrotophos and carbofuran were found to be 0.05–75 nM, 0.05–75 nM, 0.1–50 nM, 5–80 nM and 0.05 nM, 0.05 nM, 0.1 M, 2.5 nM, respectively.

The performance of this Nafion/AChE/Chit–PB–MWNTs-HGNs/Au compared with other reported AChE biosensors was summarized in Table S1. It showed that the present Nafion/AChE/Chit–PB–MWNTs–HGNs/Au exhibited a wider range, lower detection limit and higher sensitivity. This indicated that the Chit–PB–MWNTs–HGNs film not only improved the electron transfer rate, but also decreased the detection limit in the quantitative analysis of pesticides. Therefore, Nafion/AChE/Chit–PB–MWNTs–HGNs/Au biosensor would be an excellent platform for the detection of pesticides.

3.6. Precision of measurements

The inter-assay precision was estimated at six different electrodes for the determinations in 1.0 mM ATCl after being treated with 10 nM malathion solution for 10 min. Similarly, the intra-assay precision was estimated at six replicate electrodes. The RSD of intra-assay and inter-assay were found to be 3.3 and 6.7%, respectively, indicating acceptable reproducibility.

3.7. Storage stability

When the enzyme electrode was not in use, it was stored in a refrigerator at 4 °C in dry and hermetic surroundings. The response current of the sensor decreased to 98.5% after 7 days.

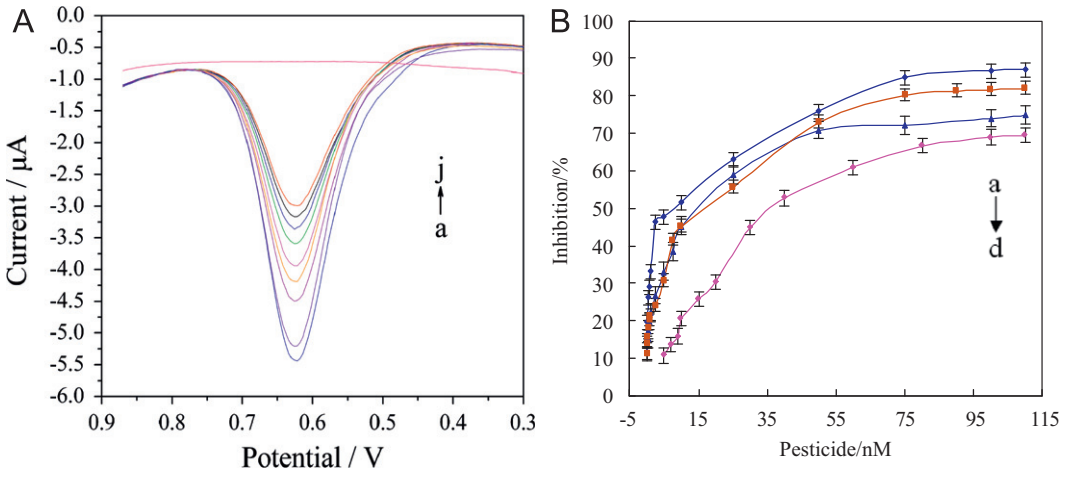

Fig. 5. (A) DPV of Nafion/AChE/Chit–PB–MWNTs–HGNs/Au in pH 7.5 PBS solution containing 1.0 mM ATCl after inhibition with malathion for 10 min. Malathion concentration (a)–(i): 0 nM, 0.05 nM, 0.5 nM, 0.75 nM, 1 nM, 2.5 nM, 5 nM, 10 nM and 25 nM. (B) Relationship between inhibition rate and (a) malathion, (b) chlorpyrifos, (c) monocrotophos and (d) carbofuran concentrations. (Error bars are the standard deviation of three repetitive experiments).

After a 30-day storage period, the sensor retained 90.2% of its initial current response, which was much better than earlier report (Du et al., 2010b).

3.8. Regeneration of acetylcholinesterase

AChE reactivation is a key step for practicable application. According to previous reports (Du et al., 2007a; Yin et al., 2009), the inhibited AChE could be reactivated by oximes, such as pralidoxime iodide and 1,1′-trimethylene bis 4- formylpyridinium bromide.

Here, we selected pralidoxime iodide as activity recovery agent. After inhibited by a certain concentration malathion, the biosensor was immersed in 5.0 mM pralidoxime iodide for 12 min. The results showed that the biosensor could resume higher than 96.2% of its original activity of AChE. The proposed biosensor showed an acceptable reproducibility in repeated use.

3.9. The detection of the real samples

The recovery tests were studied by adding different amounts of pesticides into vegetables samples (cabbage, lettuce, leek and pakchoi), respectively. The recoveries ranged from 94.2% to 105.9% (see the Supporting information, Table S2). The results indicated that this method was highly accurate, precise and reproducible. It can be used for direct analysis of practical samples.

4. Conclusions

The Chit–PB–MWNTs–HGNs composite film had been successfully fabricated by one-step electrochemical co-deposition method. Incorporating MWNTs and HGNs into Chit–PB hybrid film constructed a three-dimensional rough microstructure which increased the electron transfer, improved the microarchitecture and exhibited a high electrocatalytic activity to AChE. Because of the synergistic effects of the Chit, PB, MWNTs and HGNs, the biosensor exhibited excellent performance in the merits of high sensitivity, good stability, good reproducibility, wide linear response range and short response time. Moreover, it can also be used for direct analysis of practical samples, which would be a new promising tool for pesticides analysis.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.10.058.

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