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ORIGINAL PAPER

A novel and highly sensitive acetyl-cholinesterase biosensor modified with hollow gold nanospheres

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Abstract In this work, a highly sensitive acetylcholinesterase (AChE) inhibition-based amperometric biosensor has been developed. Firstly, a glassy carbon electrode (GCE) was modified with chitosan (Chits). Then, hollow gold nanospheres (HGNs) were absorbed onto the surface of chitosan based on the strong affinity through electrostatic adsorption. After that, L-cysteine (L-cys) was assembled on HGNs through Au–S bond. The hollow gold nanospheres were prepared by using Co nanoparticles as sacrificial templates and characterized by scanning electron microscopy, transmission electron microscopy and ultraviolet spectra, respectively. Finally, AChE was immobilized with covalent binding via –COOH groups of L-cysteine onto the modified GCE. The AChE biosensor fabrication process was characterized by cyclic voltammetry and electrochemical impedance spectroscopy methods with the use of ferricyanide as an electrochemical redox indicator. Under optimum conditions, the inhibition rates of pesticides were proportional to their concentrations in the range of 0.1–150 and 0.1–200 μg L^{-1} for chlorpyrifos and carbofuran, respectively, the detection limits were 0.06 μg L^{-1} for chlorpyrifos and 0.08 μg L^{-1} for carbofuran. Moreover, the biosensor exhibited a good stability and reproducibility and was suitable for trace detection of pesticide residues in vegetables and fruits.

Xia Sun and Chen Zhai are equally contributed to this manuscript.

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Keywords Biosensor · Pesticides · Hollow gold nanospheres · Acetylcholinesterase

Introduction

In recent years, the extensive utilization of organophosphorus and carbamate pesticides for insect control in the field of agriculture has raised serious public concern regarding the health, environment and food safety [1]. Therefore, rapid determination and reliable quantification of trace level of pesticides have become increasingly important for public security and health protection [2]. During the past, pesticides have been effectively monitored by various chromatographic techniques such as liquid chromatography [3], gas chromatography [4] and high performance liquid chromatography (HPLC) [5]. Although these chromatographic techniques are rationally selective and have low limits of detection, these methods require extensive sample preparation, specialized analytical equipment, and technical expertise, which are all unsuitable for rapid, immediate and large-scale sample analysis [6, 7]. So it is of great significance to develop fast, reliable and field-deployable methods for determination of trace amounts of organophosphorus and carbamate pesticides.

Biosensors can be used to monitor pesticides residue and they overcome the shortcomings of the conventional chromatographic methods for pesticides determination [8]. Amperometric enzyme-based biosensors have been considered to be one of the most suitable for biochemical analysis due to their sensitivity, rapid response, miniature size and reproducible results. Among them, electrochemical acetylcholinesterase (AChE) biosensors have shown satisfactory results for pesticides analysis based on their inhibition on AChE, in which the enzymatic activity is

employed as an indicator of quantitative measurement of pesticides [9, 10].

When AChE is immobilized on the working electrode surface, its interactions with the substrate acetylthiocholine chloride (ATCl) produce the electroactive product of thiocholine, and the inhibition on the enzyme system can be monitored by measuring the oxidation current of thiocholine (Fig. 1a) [11].

The immobilization of AChE onto the electrode surface represents a crucial step in the development of the biosensor [12]. In recent years, the use of nanomaterials in AChE biosensors has resulted into their improved analytic performance, such as ZnS nanomaterials [13], multiwall carbon nanotubes (MWNTs) [14], graphene (GR) [15], gold nanoparticles (AuNPs) [16], hollow gold nanoparticles (HGNs) [17], CdS nanoparticles [2], and iron oxide nanoparticles (Fe_3O_4 NPs) [18]. These nanomaterials are considered to promising materials in catalysis owing to their satisfying biocompatibility and good conductivity [19]. Among these nanomaterials, HGNs have received increasing interest in the preparation of biosensors due to their satisfying properties. Hollow gold nanospheres have several advantages over the solid counterparts such as high specific surface, low density and reduction of costs [20–22].

HGNs can promote electron transfer; therefore, fabricating HGNs onto electrochemical biosensors can get larger current response and higher-selectivity. In addition, because of its good biocompatibility, HGNs are favorable to immobilize bioactive substances onto the electrode surface [23]. To date, however, the application of hollow metal nanospheres in AChE biosensors has been still very less reported.

Chitosan (Chits) as membrane material contains large groups of $–NH_2$ and –OH which is preferable to maintain the high biological activity of the immobilized biomolecules. In addition, chitosan also has the properties of nontoxicity, low cost, high mechanical strength, good filmforming ability and biocompatibility [24, 25]. Therefore, it is widely used as modification material for preparing biosensor.

In this work, the HGNs were simply fabricated based on the displacement reaction between the Co nanoparticles and $HAuCl_4$. We investigated the behaviors of a novel AChE based on the AChE/L-cys/HGNs/Chits/GCE. The bare glassy carbon electrode was first modified with chitosan. Then, HGNs were absorbed on the surface of chitosan based on the strong affinity through electrostatic adsorption. After that, L-cysteine (L-cys) was assembled on HGNs through Au–S bond. Finally, AChE was immobilized onto the GCE via -COOH groups of L-cys. Typical pesticides such as chlorpyrifos and carbofuran were selected to verify this method. Compared with other kinds of electrochemical AChE biosensor, this method was simple, stable and more sensitive for pesticides determination with much lower detection limit.

Experimental

Apparatus

Electrochemical measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The working electrode was glassy carbon electrode (GCE) ($d = 3$ mm) or modified GCE. A saturated calomel electrode (SCE) and platinum electrode were used as reference and auxiliary electrodes, respectively. UV spectra were recorded using an ultraviolet spectrophotometer UV-2550 (Shimadzu Co., Japan). The transmission electron microscopy (TEM) observations were

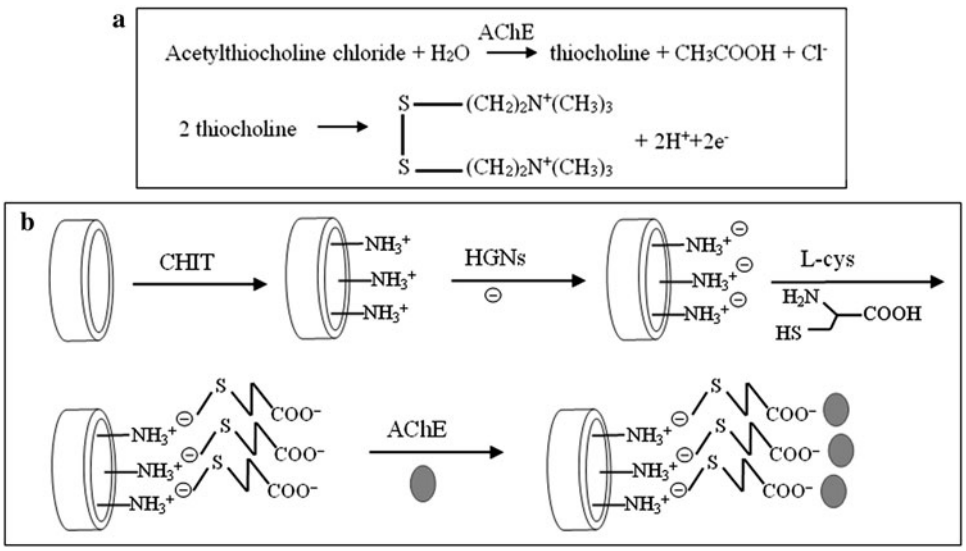

Fig. 1 **a** The principle of immobilized acetylcholinesterase of electrochemical response. **b** The procedure of preparation of AChE/L-cys/HGNs/Chits/GCE

obtained using the JEOL-1200EX TEM (Japan). Scanning electron micrographs (SEM) was studied by JSM-6360LV SEM (Japan).

Reagents and materials

Acetylcholinesterase (Type C3389, 500 U mg^{-1} from electric eel), acetylthiocholine chloride (ATCl), pralidoxime iodide were purchased from Sigma (St. Louis, USA). Chlorpyrifos, carbofuran and L-cysteine were obtained from Solarbio Co. (Beijing, China). $HAuCl_4$ and Cobalt (II) chloride hexahydrate ($CoCl_2{\cdot}6H_2O$, 99.0 %) were obtained from National Chemical Pharmaceutical Co., China. 0.1 M pH 7.5 phosphate buffer solutions were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Sodium borohydride ($NaBH_4$), CHIT (95 % deacetylation), $[Fe(CN)_6]^{4-/3-}$, ethanol and other reagents were of analytical grade. All solutions were prepared using double distilled water.

Fabrication of hollow gold nanospheres (HGNs)

The HGNs were prepared according to the procedure described by Liu et al. [17] with a slight modification. For the synthesis of HGNs, the Co nanoparticles were first prepared. The synthesis of Co nanoparticles was carried out based on the method outlined in Kobayashi et al. [26]. 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 prepare Co nanoparticles. To avoid the oxidation of obtained Co nanoparticles by existing atmospheric oxygen in the solution, high-purity nitrogen was passed into the solution for 20 min to create a nitrogen atmosphere, which was maintained during the reaction. After the reaction continued for several minutes, gas generation could be observed obviously. Following the end of gas evolution, 30 mL of Co nanoparticle colloidal solutions were extracted and transferred to 18 mL of stirred 1 mM $HAuCl_4$ aqueous solutions to achieve the preparation of the HGNs. The obtained suspension solution was centrifuged at 10,000 rps and the precipitates were redispersed in 5 mL pH 7.5 phosphate buffer solutions.

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

Preparation of sensor

A GCE was polished carefully to a mirrorlike surface with 0.3 and 0.05 μm Al_2O_3 paste and washed sonication with ethanol, nitric acid and doubly distilled water. Before modification, the electrode was applied a potential scanned from −0.6 to 1.0 V in 0.5 mol L^{-1} H_2SO_4 for 300 s until a steady-state curve was obtained. 5 μL 0.5 wt % chitosan solution was coated onto the pretreated GCE and dried in the air (Chits/GCE). Then the electrode was immersed in HGNs solution for 12 h under the nitrogen atmosphere. Having been rinsed thoroughly with distilled water to remove some weakly adsorbed species, the resulting HGNs/Chits/GCE electrode was immersed in 1 mM L-cysteine for 3 h to prepare L-cysteine monolayer modified HGNs/Chits/GCE. The obtained L-cys/HGNs/Chits/GCE was washed thoroughly with double distilled water. Then 5.0 μL AChE solution (200 mU) was dropped and dried in air at room temperature for another 2 h. After washing carefully with pH 7.5 phosphate buffer solutions to remove nonspecifically adsorbed enzyme, the AChE/L-cys/HGNs/Chits/GCE was obtained. As shown in Fig. 1b. The obtained biosensor was stored at 4 °C when not in use.

Measurement procedure

For detection of pesticides, the obtained AChE/L-cys/HGNs/Chits/GCE was first immersed in pH 7.5 phosphate buffer solutions containing different concentrations of standard pesticides for 10 min, and then transferred to the electrochemical cell of pH 7.5 phosphate buffer solutions containing 0.5 mM ATCl to study the electrochemical response by cyclic voltammetry (CV). The inhibition of pesticides was calculated as follows:

$$\text{Inhibition } (\%) = \left(i_{\text{P,control}} - i_{\text{P,exp}}\right)/i_{\text{P,control}} \times 100\ \%$$

where $i_{\text{P,control}}$ and $i_{\text{P,exp}}$ were the peak currents of 0.5 mM ATCl on AChE/L-cys/HGNs/Chits/GCE before and after exposure to pesticides, respectively.

Enzyme reactivation

After the AChE/L-cys/HGNs/Chits/GCE was inhibited by pesticides, it was washed with phosphate buffer solutions and reactivated with 4.0 mM pralidoxime iodide for 12 min, then transferred to electrochemical cell of pH 7.5 phosphate buffer solutions containing 0.5 mM ATCl to study the electrochemical response by CV. The reactivation efficiency (R %) was estimated as follows:

$$R\ (\%) = \left(i_r - i_{\text{P,exp}}\right)/\left(i_{\text{P,control}} - i_{\text{P,exp}}\right) \times 100\ \%$$

where, i_r was the peak current of 0.5 mM ATCl on AChE/L-cys/HGNs/Chits/GCE after 5.0 mM pralidoxime iodide reactivation.

Preparation and determination of real samples

Fresh cabbage, lettuce, leek and pakchoi bought from a local supermarket, were pulped after removing the rotten leaves and dirt. Then 10 mL mixed solution of acetone and 0.1 M pH 7.5 phosphate buffer solutions (1/9, v/v) was added into 5 g of each sample, and then a 3 min blending

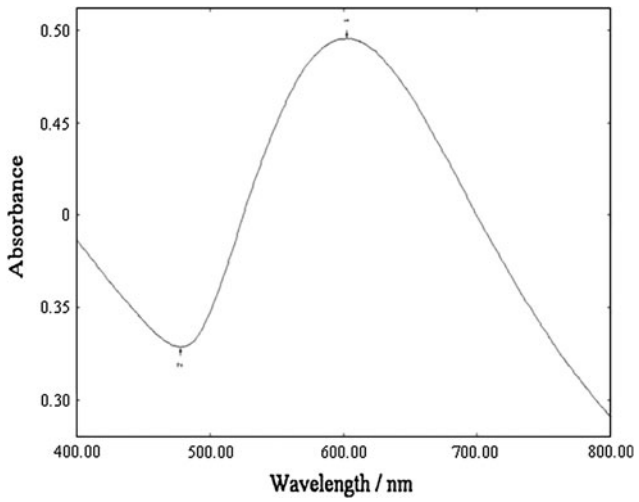

Fig. 2 UV–vis spectra of HGNs

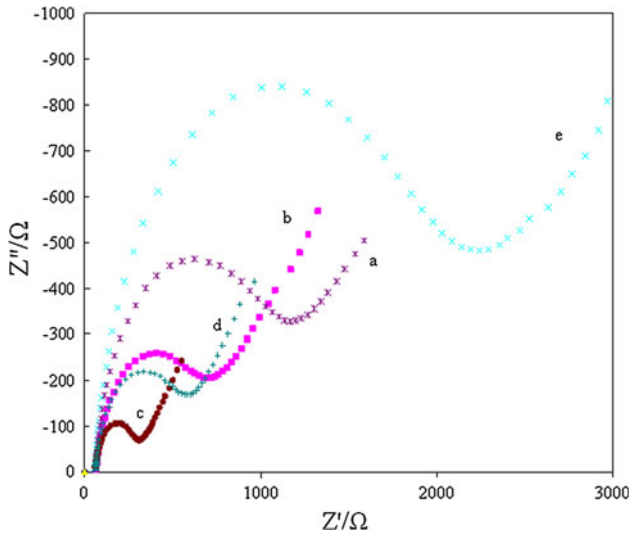

Fig. 4 Electrochemical impedance spectra of *a* bare GCE, *b* Chits/GCE, *c* HGNs/Chits/GCE, *d* L-cys/HGNs/Chits/GCE, *e* AChE/L-cys/HGNs/Chits/GCE in pH 7.5 phosphate buffer solutions containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl

and a 5 min supersonics were finished in sequence. After that, the suspensions were centrifuged (10 min, 10,000 rpm), the acquired supernatants were detected by CV directly without extraction or preconcentration. The content of pesticides in the samples can be achieved from the calibration curve.

Results and discussion

Characterization of HGNs

The UV–visible absorption spectra of the gold solid colloids have a λ_{max} at about 520 nm, which is the characteristic absorbance peak of gold solid colloids. Figure 2 shows the UV–visible absorption spectra of aqueous solution of HGNs. The UV–vis spectrum of HGNs shows a strong peak at 600 nm, the characteristic absorption peak of HGNs due to their surface plasma resonance [27]. The results indicated that we have fabricated hollow gold nanoparticles.

The morphology of HGNs was investigated by TEM. Figure 3a exhibits a TEM image of HGNs. There is a strong contrast difference in all of the spheres with a bright center surrounded by a much darker edge, that is, a hollow structure. The average outer diameter of the hollow nanospheres is about 50 nm. In the Fig. 3a obviously shows hollow structure of gold spheres owned a rough outer

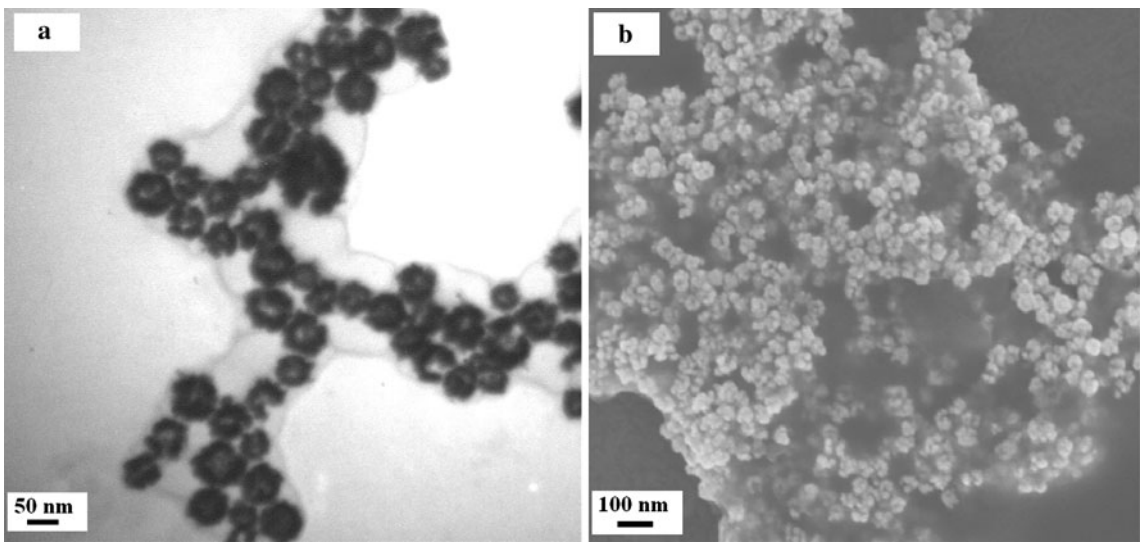

Fig. 3 TEM (**a**) and SEM (**b**) images of HGNs

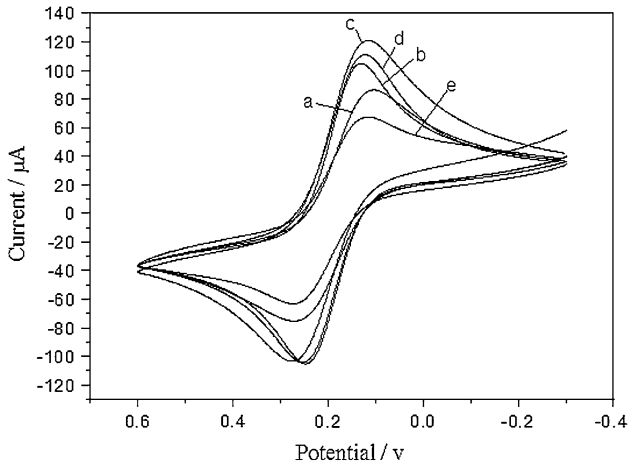

Fig. 5 Cyclic voltammograms of *a* bare GCE, *b* Chits/GCE, *c* HGNs/Chits/GCE, *d* L-cys/HGNs/Chits/GCE, *e* AChE/L-cys/HGNs/Chits/GCE in pH 7.5 phosphate buffer solutions containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl

surface which should be very favorable for the application in biosensor due to its large surface area. Figure 3b is a typical SEM image of HGNs, showing the as-synthesized product of large-scale uniform spherical materials.

Electrochemical reactivity of different electrodes

EIS study was carried out to characterize the modified electrode surface. In a typical Nyquist plot, the semicircle portion corresponds to the charge-transfer resistance (R_{ct}) at higher frequency range, while a linear part at lower frequency range representing the diffusion limited process [15]. In this report, EIS study of the modified electrodes was carried out in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl with a frequency range of 0.01 Hz–100 kHz. As shown in Fig. 4 curve, the R_{et} value of the bare GCE was about 1,100 Ω (curve a); by contrast, the R_{et} of Chits/GCE (curve b) was a little smaller, which was about 710 Ω (curve b). This was mainly due to the electrostatic attraction between positively charged Chits and the negatively charged $[Fe(CN)_6]^{3-/4-}$. After modification with HGNs, the EIS of the resulting layer showed semicircle domain with an R_{ct} value of about 260 Ω (curve c) suggesting that HGNs layer greatly improved the conductivity of the modified electrode. When L-cys was bound to the HGNs via Au–S bond, the R_{et} value of L-cys/HGNs/Chits/GCE (curve d) increased to about 620 Ω. This phenomenon was due to the exclusion between the negatively charged COO^- and $[Fe(CN)_6]^{3-/4-}$. The R_{ct} value increased for the enzyme-modified electrode up to about 2,200 Ω as shown in curve e. This increase in R_{ct} is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer. These results were supported by the cyclic voltammogram study (Fig. 4).

The CV in $Fe(CN)_6^{3-/4-}$ solution is also a valuable and convenient tool to monitor the barrier of the modified

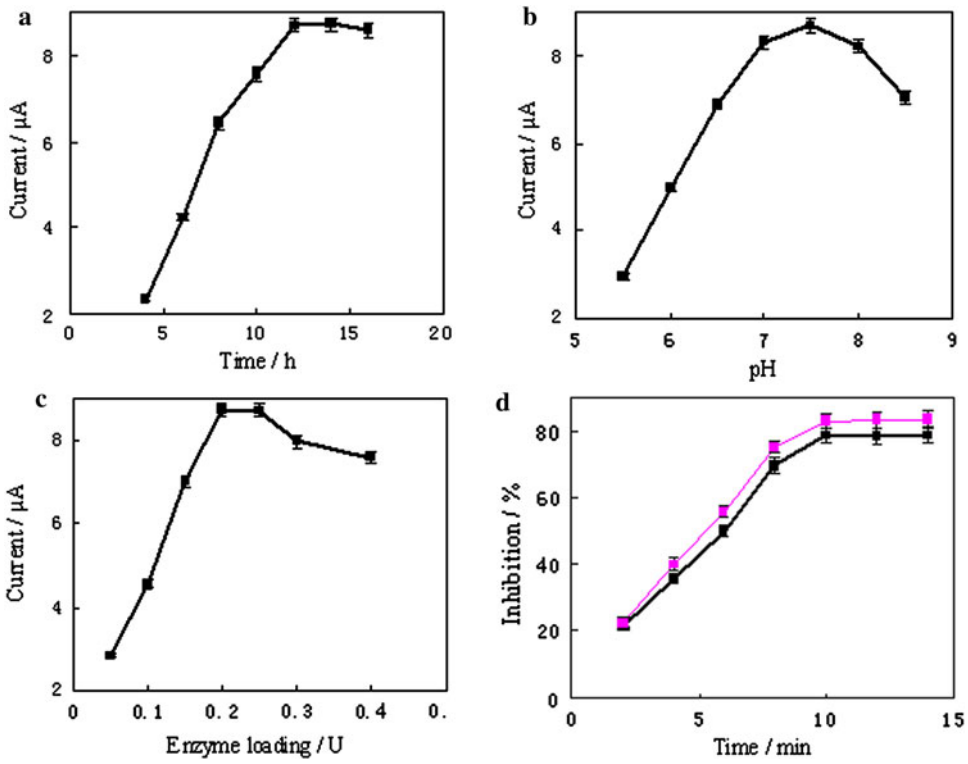

Fig. 6 **a** Relationship between peak current and immersion time in HGNs solution. **b** Relationship between peak current and pH 5.5–8.5. **c** Relationship between peak current and the unit of immobilized AChE. **d** Relationship between inhibition rate and inhibition time. (*Error bars* are the standard deviation of three repetitive experiments)

electrode [28]. Figure 5 displays the CV behaviors of different layers of the modified electrode in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl solution. When the electrode was dropped in Chits (curve b), the peak currents increased compared with those of the bare GCE (curve a). After being modified with HGNs, the peak currents of HGNs/Chits/GCE increased again (curve c). On the contrary, the peak currents decreased more and more when L-cys (curve d) and AChE (curve e) were in turn immobilized on the surface of the electrode. All these changes were consistent with the conclusion of EIS.

Optimization parameters of the biosensor performance

Effect of immersion time in HGNs solution on response of AChE/L-cys/HGNs/Chits/GCE

The Chits/GCE was immersed in the HGNs solution for different time (4–16 h at an interval of 2 h), then continue to immobilized L-cys and AChE. It was found that shorter immersion time (less than 12 h) resulted in thin and unstable HGNs films which are not good for immobilization of proteins. When the immersing time was longer than 12 h, the curve tended to a stable value. In order to make the prepared biosensor stable and sensitive response, 12 h was optimized for the immersion time in HGNs solution (Fig. 6a).

Effect of pH on response of ATCl on the AChE/L-cys/HGNs/Chits/GCE

The pH value is one of the parameters which affects the performance of the AChE biosensor since the activity of the immobilized AChE is pH dependent. Therefore, we further investigated the effect of pH. Figure 6b shows the response current of the enzyme electrode in a series of phosphate buffer solutions with the pH from 5.5 to 8.5 including 0.5 mM ATCl, the response current showed the maximum value at pH 7.5. Certain enzymes have ionic groups on their active site and these ionic groups must be in a suitable form to function. Variations in the pH of the medium result in change in the ionic form of the active site and changes the activity of the enzyme and hence the reaction rate [29]. The enzyme lost activity irreversibly at the lower or higher pH values [30]. Thus, pH 7.5 was used in the detection solution.

Effect of enzyme amount on response of ATCl on the AChE/L-cys/HGNs/Chits/GCE

The amount of AChE immobilized onto the electrode surface is crucial for pesticides determination. Figure 6c shows the influence of enzyme amount on the response of

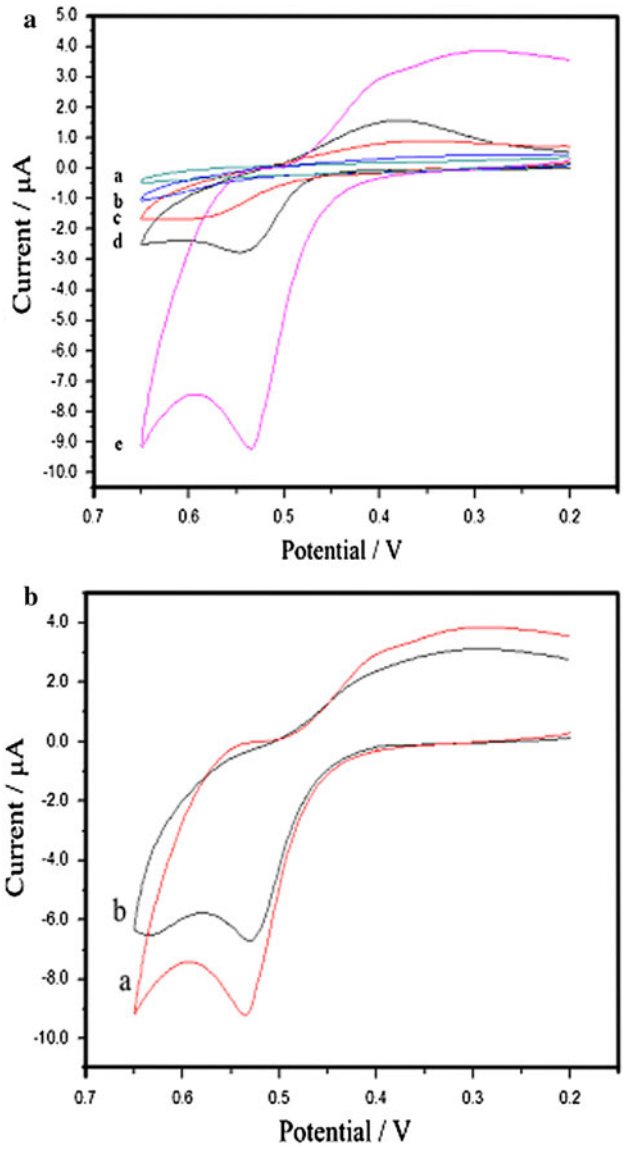

Fig. 7 **a** Cyclic voltammograms: *a* bare GCE and *b* AChE/L-cys/HGNs/Chits/GCE in pH 7.5 phosphate buffer solutions, *c* L-cys/HGNs/Chits/GCE, *d* AChE/L-cys/Chits/GCE and *e* AChE/L-cys/HGNs/Chits/GCE and in pH 7.5 phosphate buffer solutions containing 0.5 mM ATCl. **b** Cyclic voltammograms of AChE/L-cys/HGNs/Chits/GCE in pH 7.5 phosphate buffer solutions containing 0.5 mM ATCl before *a* and after *b* inhibition with 15 μg L^{-1} chlorpyrifos for 10 min. Scan rate: 50 mV/s

biosensor. Amounts of AChE between 0.05 and 0.4 U were immobilized onto the L-cys/HGNs/Chits/GCE, the oxidation current increased obviously from 0.05 to 0.2 U and reached the maximum at 0.2 U. However, when the amount of AChE increased further the current response decreased, which may be ascribed to the over-thickness of enzyme layer hindering the transmission of electrons to the electrode surface. Therefore, 0.2 U of AChE was chosen as the optimal enzyme amount for preparation of the biosensor.

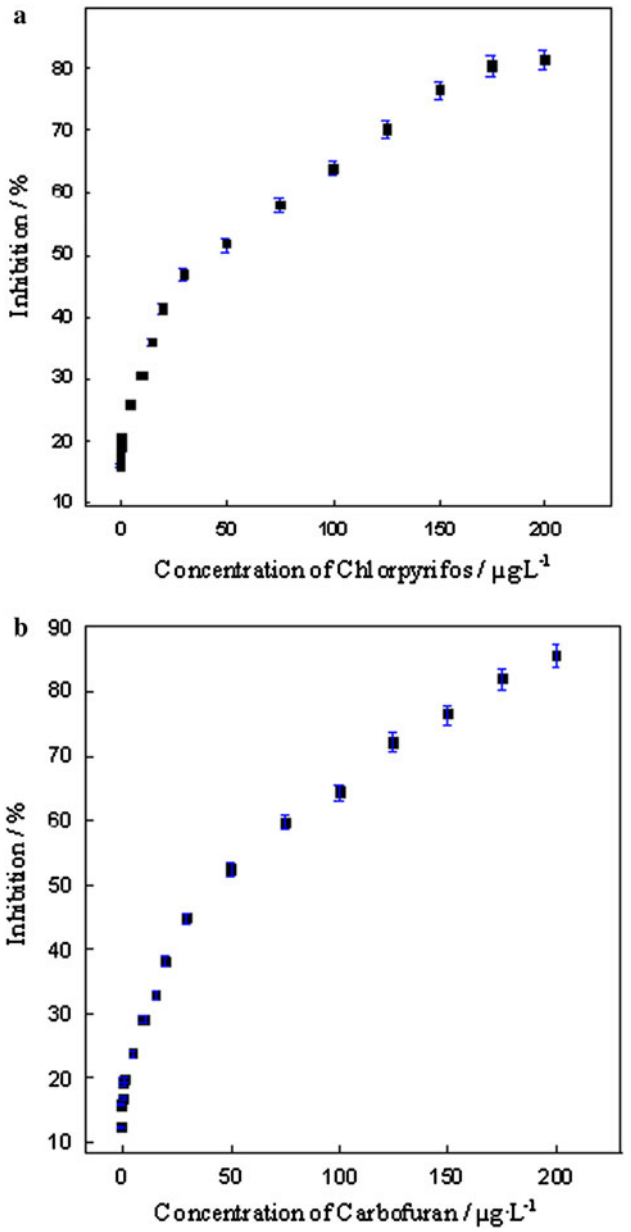

Fig. 8 Relationship between inhibition rate and chlorpyrifos (**a**) and carbofuran (**b**) concentrations. *Error bars* are the standard deviation of three repetitive experiments

Effect of incubation time on response of AChE/L-cys/HGNs/Chits/GCE

The incubation time is the reaction time of the enzyme with the inhibitor. The effect of inhibition time was investigated with chlorpyrifos and carbofuran, respectively. As seen from Fig. 6d, chlorpyrifos and carbofuran displayed increasing inhibition to AChE with immersing time. When the immersing time was longer than 10 min the curve trended to a stable value. However, the maximum values of inhibition of chlorpyrifos and carbofuran were not 100 %, which was likely to attribute to the binding sites between pesticides and enzymes could reach saturation.

Electrochemical behavior of AChE/L-cys/HGNs/Chits/GCE

The cyclic voltammograms of AChE/L-cys/HGNs/Chits/GCE were examined, as shown in Fig. 7a. No peak was observed at bare GCE (curve a), AChE/L-cys/HGNs/Chits/GCE (curve b) in pH 7.5 phosphate buffer solutions. However, when 0.5 mM ATCl was added into phosphate buffer solutions, the cyclic voltammogram of AChE/L-cys/HGNs/Chits/GCE showed an irreversible oxidation peak of 8.7 μA at 535 mV (curve e), while no detectable signal was observed at L-cys/HGNs/Chits/GCE (curve c). Obviously this peak came from the oxidation of thiocholine, hydrolysis product of ATCl, which was catalyzed by immobilized AChE. Furthermore, this oxidation peak current (553 mV) at AChE/L-cys/Chits/GCE without HGNs (curve d) is much lower than that at AChE/L-cys/HGNs/Chits/GCE (curve e). This phenomenon was attributed to the presence of HGNs, which played an important role in facilitating the electron transfer between the enzyme and the electrode surface. This result indicated that the HGNs can greatly improve electron transfer and decrease the over-potential of thiocholine oxidation better than other biosensors [9, 15]. The produced current by thiocholine on AChE/L-cys/HGNs/Chits/GCE reflected enzymatic activity, which could be used for pesticides analysis involved in the inhibition action [31]. Thus, the AChE/L-cys/HGNs/Chits/GCE was used in following experiments of chlorpyrifos and carbofuran determination.

Determination of pesticides

Upon construction AChE/L-cys/HGNs/Chits/GCE was immersed in 15 μg L^{-1} standard solution of chlorpyrifos for 10 min, the produced current decreased drastically (Fig. 7b). This was because chlorpyrifos as one of the pesticides exhibited fairly high acute toxicity and involved in the irreversible inhibition action on AChE, thus reduced the enzymatic activity to its substrate. Due to the notable change in cyclic voltammetric signal of the AChE/L-cys/HGNs/Chits/GCE, a simple method for determination of pesticides was established.

Inhibition effects were investigated by measuring the biosensor response towards 0.5 mM ATCl after being inhibited by different concentrations of chlorpyrifos and carbofuran. The calibration plots of inhibition percentage versus chlorpyrifos and carbofuran concentration are shown in Fig. 8. Linear relationships between inhibition percentage and the concentration of chlorpyrifos and carbofuran were obtained and shown in Table 1.

Table 1 Linear relationships between inhibition percentage and the concentration of chlorpyrifos and carbofuran

Pesticides	Regression equation	Linear range/ μg L^{-1}	Detection limit/ μg L^{-1}	Correlation coefficients
Chlorpyrifos	$y = 1.0329x + 17.429$;	0.1–30;	0.06	0.9922;
	$y = 0.2411x + 40.021$	30–150		0.9988
Carbofuran	$y = 1.113x + 14.047$;	0.1–30;	0.08	0.9943;
	$y = 0.2307x + 41.218$	30–200		0.9937

Table 2 Comparison of the response of the fabricated biosensor for detection of chlorpyrifos and carbofuran with other biosensors based on immobilized AChE

Pesticide	Electrode	Linear range/μg L^{-1}	Detection limit/μg L^{-1}	Reference
Chlorpyrifos	AChE/L-cys/HGNs/Chits/GCE	0.1–30; 30–150	0.06	This work
	AChE-[BMIM][BF_4][a]-MWCNT/CP[b]	3.5–350	1.4	[32]
	AChE/CPBA[c]/GR-AuNPs/GCE	0.5–10; 10–100	0.1	[14]
Carbofuran	AChE/L-cys/HGNs/Chits/GCE	0.1–30; 30–200	0.08	This work
	AChE/PAMAM[d]-Au/CNTs/GCE	1–20	0.89	[33]
	AChE-ChOx[e]/Fe_3O_4/Au SPCE[f]	50–1,000	10	[34]

[a] 1-Butyl-3-methylimidazolium tetrafluoroborate,

[b] Carbon paste electrode

[c] 3-Carboxyphenylboronic acid

[d] Dendrimers polyamidoamine

[e] Choline oxidase

[f] Screen printed electrode

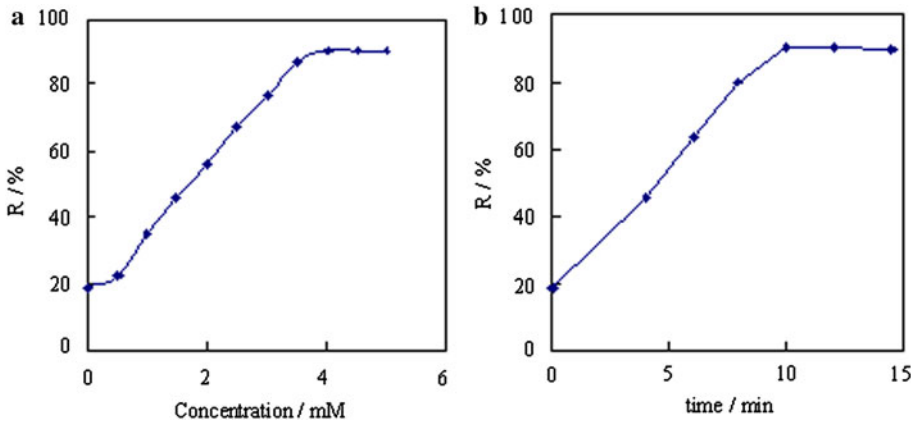

Fig. 9 Effects of incubation concentration (**a**) and incubation time (**b**) of pralidoxime iodide on reactivation efficiency. (*Error bars* are the standard deviation of three repetitive experiments)

The characteristics of chlorpyrifos and carbofuran determination are comparable with those reported for other AChE biosensors. As can be seen in Table 2, the AChE/L-cys/HGNs/Chits/GCE biosensor offered a reasonable linear range for chlorpyrifos and carbofuran detection and the detection limit was lower than some of those reported literatures [15, 32–34]. This indicated that the HGNs can decrease the detection limit in the quantitative analysis of pesticides. Therefore, the developed biosensor will be an excellent platform for the detection of pesticides.

Storage stability and reproducibility of biosensor

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 98.2 % after 10 days. After a 40-day storage period, the sensor retained 85.4 % of its initial current response, the modified electrode lost 52.7 % of its initial current response, when stored at 4 °C for 60 days, which is much better than earlier electrodes [35], indicating acceptable stability of biosensor.

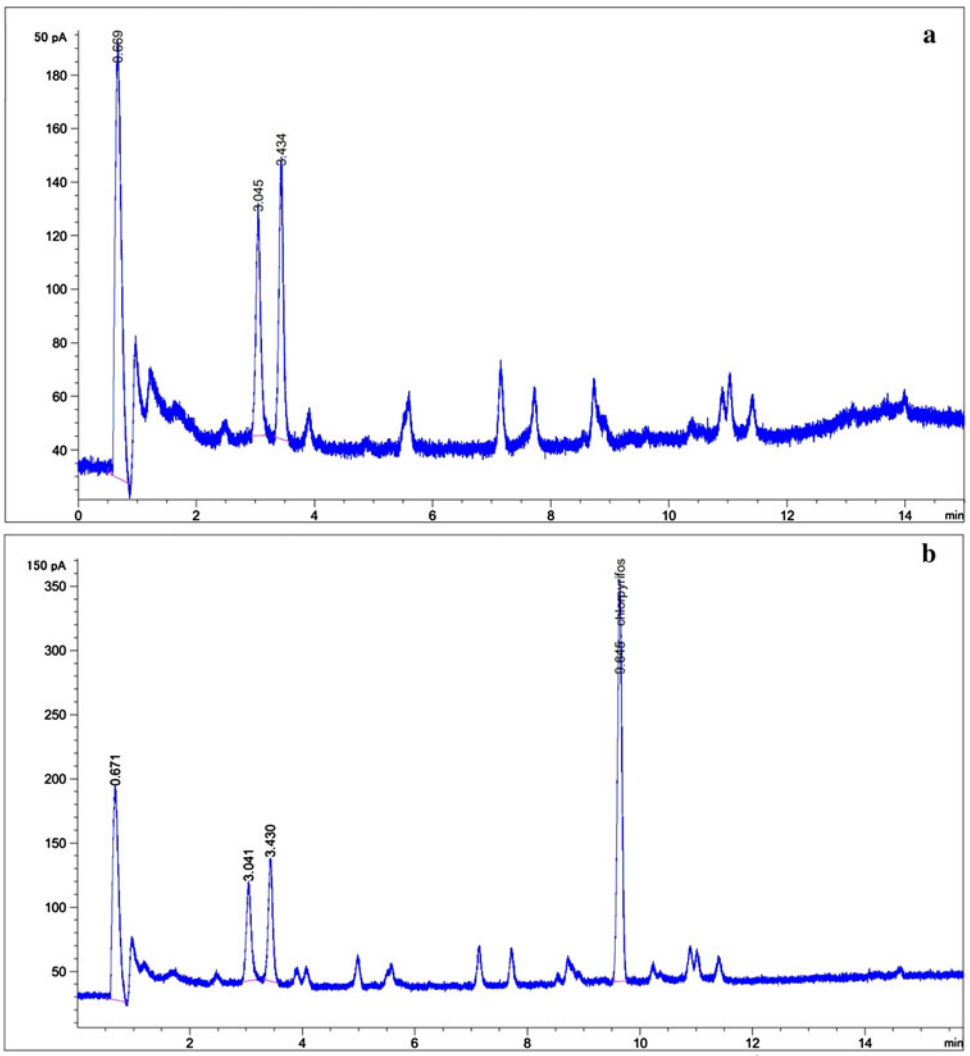

Fig. 10 The GC chromatograms of pakchoi sample: blank (**a**) and added with chlorpyrifos (**b**)

Table 3 Comparisons of AChE/L-cys/HGNs/Chits/GCE biosensor and gas chromatograph

Detection method	Pesticide	Detection time	Detection limit (μg L^{-1})	Recovery (%)
Gas chromatograph	Chlorpyrifos	4 h	10	94.8–103.9
	Carbofuran		50	91.2–106.6
AChE/L-cys/HGNs/Chits/GCE biosensor	Chlorpyrifos	30 min	0.06	93.7–106.2
	Carbofuran		0.08	95.5–105.8

The fabrication reproducibility of biosensor was estimated for the determination of 30 μg L^{-1} chlorpyrifos and carbofuran, respectively, at five different electrodes for 10 min. The coefficient of variation was found to be 3.5 and 2.9 %, respectively, which is acceptable.

Regeneration of acetylcholinesterase

A crucial problem in practical detection of pesticides is the activity of the immobilized enzyme of the biosensor will have been inhibited after use, which limits the reuse of biosensor. In order to settle this problem, pralidoxime iodide has been used to recover the activity of the inhibited AChE [36, 37]. The AChE/L-cys/HGNs/Chits/GCE was firstly immersed in pH 7.5 PBS containing 10 μg L^{-1} chlorpyrifos for 10 min. Then its electrochemical response to 0.5 mM ATCl was recorded before and after reactivation with pralidoxime iodide solution. Figure 9a shows the reactivation degree of the biosensor incubation with pralidoxime iodide 1–7 nM. After immersing in 5.0 mM pralidoxime iodide, the activity of regenerated AChE could reach to 93.1 % of its original value, and which it was

constant (up to 6 nM). This value was higher than that of 90 % for AChE-PAn-PPy-MWCNTs/GCE [38], 91.3 % for AchE-CdS-G-CHIT/GCE [2]. The reactivation efficiency increased with the increase of reactivation time, until it reached to a constant value after 12 min (Fig. 9b). Based on this, the proposed biosensor showed an acceptable reproducibility in repeated use.

The detection of the real samples

AChE/L-cys/HGNs/Chits/GCE biosensor was used in the detection of actual sample, firstly the blank tests of cabbage, lettuce, leek and pakchoi were done, whose inhibition ratios were 5.7, 6.4, 8.9, 7.5 %, respectively, none of them attained the detection limit, which showed that the contents of pesticide residues in these vegetables bought from that supermarket were less than the detection limit. To further demonstrate the practicality of this method, the recovery tests were studied by adding different amounts of chlorpyrifos and carbofuran into vegetables samples, respectively. The recoveries ranged from 93.7 to 106.2 %.

The blank tests and recovery tests of fresh cabbage, lettuce, leek and pakchoi bought from market were done by gas chromatograph (GC), according to the detection method in Liu et al. [39]. Figure 10a shows the background spectra of pakchoi, Fig. 10b shows the spectra of pakchoi added with 200 μg L^{-1} chlorpyrifos. Table 3 shows the comparisons of detection time, detection limit and recovery between GC and AChE/L-cys/HGNs/Chits/GCE biosensor; from these it is obvious that AChE/L-cys/HGNs/Chits/GCE biosensor has the advantages of short detection time and low detection limit, and their recoveries were both higher than 90 %. The results indicated that this method was highly accurate, precise and reproducible. It can be used for direct analysis of practical samples.

Conclusion

A highly sensitive amperometric biosensor based on the HGNs modified electrode was constructed for fast determination of chlorpyrifos and carbofuran pesticides. The prepared HGNs show a special morphology which could upgrade the AChE immobilization and promote electron transfer. Under the optimal conditions, the AChE/L-cys/HGNs/Chits/GCE exhibited a good electrochemical response in terms of high sensitivity, wide linear range (0.1–150 and 0.1–200 μg L^{-1} for chlorpyrifos and carbofuran, respectively) and low detection limit (0.06 and 0.08 μg L^{-1} for chlorpyrifos and carbofuran, respectively), which was further applied to determine organophosphorus and carbamate pesticides. The developed biosensor provides a new promising tool for pesticides analysis.

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