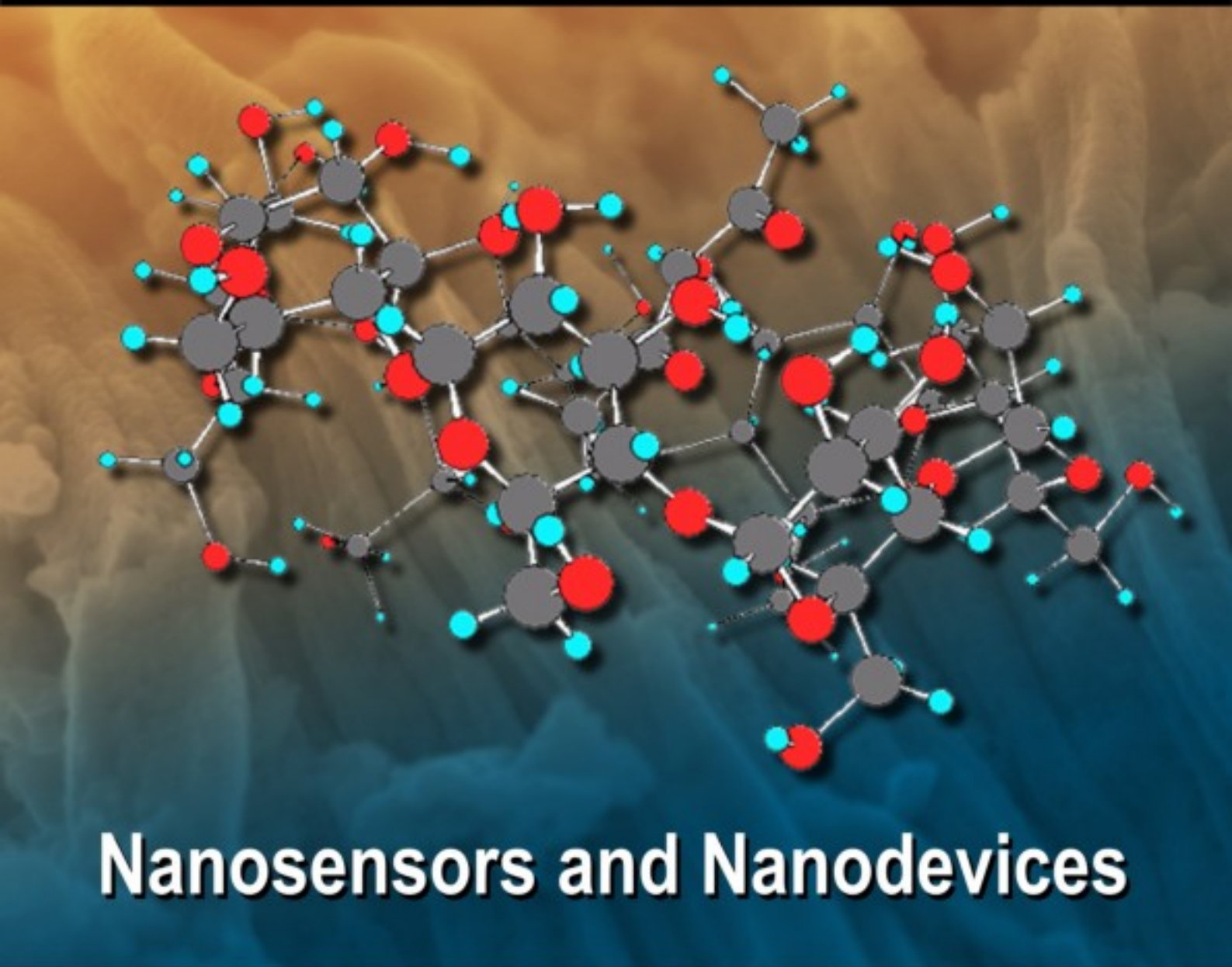
ISSN 1726-5479

SENSORS & TRANSDUCERS

vol. 146
11/12



Nanosensors and Nanodevices

International Frequency Sensor Association Publishing

IFSA

Sensors & Transducers

Volume 146, Issue 11
November 2012

www.sensorsportal.com

ISSN 1726-5479

Sensors & Transducers Journal (ISSN 1726-5479) is a peer review international journal published monthly online by International Frequency Sensor Association (IFSA).
Available in electronic and on CD.

Sensors & Transducers Journal

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Issue 11
November 2012

www.sensorsportal.com

ISSN 1726-5479

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Sensors & Transducers Journal, Vol. 146, Issue 11, November 2012, pp. 96-109

www.sensorsportal.com

Sensors & Transducers

ISSN 1726-5479

http://www.sensorsportal.com

A Novel Amperometric Immunosensor Based on {MWCNTs-COOH-CHIT}$_2$/GNPs for Detection of Chlorpyrifos

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Received: 16 September 2012 /Accepted: 23 November 2012 /Published: 30 November 2012

Abstract: In this study, a novel label-free amperometric immunosensor based on gold nanoparticles and dual-layer carboxylated multiwall carbon nanotubes-chitosan ({MWCNTs-COOH-CHIT}$_2$/GNPs) for the determination of chlorpyrifos residues was exploited. To construct the immunosensor, MWCNTs-COOH was first dispersed with CHIT to obtain a homogeneous solution and then it was dropped on the surface of glassy carbon electrode (GCE) which was first modified by the electrodeposition of GNPs. And then the modified GCE was immersed in the poly (diallyldimethylammonium chloride) (PDDA) which was positively charged. Next, the negatively charged MWCNTs-COOH-CHIT was assembled to the interface through the electrostatic force. The stepwise assembly process of electroactive species on electrode surface and the performance of the immunosensor was characterized by means of cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and scanning electron microscopy (SEM), respectively. {MWCNTs-COOH-CHIT}$_2$ was acted as an electron promoter and had excellent immobilization effects for antibody, thus, it improved the detection sensitivity and stability of the immunosensor. Under optimal experimental conditions, the proposed immunosensor had a wide linear range from 0.1 to 50 ng/mL and from 50ng/mL to 1mg/mL, and with a detection limit of 0.069 ng/mL. The proposed immunosensor exhibited high reproducibility, specificity, stability and regeneration performance, which provided a new promising tool for the detection of chlorpyrifos residues in fruits and vegetables.

Keywords: Amperometric immunosensor, Gold nanoparticles, MWCNTs-COOH-CHIT, Chlorpyrifos.

1. Introduction

Chlorpyrifos (O,O-diethyl-O- (3, 5, 6-trichloro-2-pyridyl)-phosphorothioate), a broad spectrum OP insecticide, is one of the most widely used organophosphate insecticides on food and fiber crops and in the treatment of termites [1, 2]. However, it is highly toxic and its accumulation in living organisms can be a cause of serious diseases [3-5]. Thus, the analysis of foods to assess the presence of pesticide residues is crucial importance for ensuring food safety and quality. Traditional analytical methods for chlorpyrifos detection involving gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS) are very sensitive, reliable and standardized techniques [6-8], but have disadvantages such as extensive time consumption, expensive instrumentation and complicated pretreatment procedure, which limit their application for real-time detection [9-13].

Immunosensors are biosensors that are provided with the selectivity in view of immunological interactions and also being proposed and proved to be efficient analytical devices for the monitoring of organic pollutants in food and the environment because of their simple fabrication, easy operation, rapid response and high sensitivity [14-16]. The immobilization of biomolecules on the electrode is the most significant factor in improving the performance of the immunosensors. With the development of nanotechnology, various kinds of nanomaterials have been widely applied in immunosensor fabrication, such as metal nanoparticles [17], multiwall carbon nanotubes (MWCNTs) [18, 19], and so on. Gold nanoparticles (GNPs) have been widely used in the biosensors for detection of pesticide residues due to their high electron-transfer ability and large specific surface area [20, 21]. Chitosan (CHIT) is a biological cationic macromolecule with primary amines. It has been widely used for dispersion because of its good biocompatibility and film-forming ability [22-24]. In addition, MWCNTs have received increasing interest due to their great chemical stability, large aspect ratio, excellent electrical conductivity, and extremely high mechanical strength and stiffness and demonstrated to be an excellent material for the development of electrochemical sensors [25-27]. However, MWCNTs with their high van der Waals force, surface area, and high aspect ratio inevitably cause self-aggregation bringing about a poor connection to the active material particles. Thus, the functionalization of MWCNTs is an actively discussed topic in contemporary nanotube literature because the modification of the properties of MWCNTs is believed to open the road toward real nanotechnology applications [28, 29]. A simple route for functionalization of MWCNTs had been achieved by an oxidation process, which involves their treatment in a mixture of concentrated HNO_3 and H_2SO_4; after this treatment, the end and side walls of MWCNTs are decorated mainly with carboxylic acid groups which is negatively charged [30, 31]. Therefore, MWCNTs-COOH-CHIT can be fixed onto the electrode surface that has been modified by GNPs because amino group has intensive reaction with GNPs, and it also can provide a good biocompatible interface for the immobilization of biomolecules.

To the best of our knowledge, little research on the use of chlorpyrifos immunosensors based on $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ for determination of chlorpyrifos has been reported. In this study, the carboxylated MWCNTs were employed and we aimed to improve the stability and maintain the activity of antibodies to develop a simple, stable, sensitive, and low-cost amperometric immunosensor based on $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE. The poly (diallyldimethylammonium chloride) (PDDA), positively-charged, was used to assemble the second layer of MWCNTs-CHIT (negatively charged) [31] to the interface through the electrostatic force. In this way, we can get a stable, rough and compact surface. $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ not only had good conductivity, but also provided more carboxyl groups to adsorb antibodies, which could improve the detection stability of the immunosensor. As a result, the obtained immunsensor exhibited excellent characteristics including high sensitivity, low detection limit and long-term stability. The preparation, characterization, optimal conditions, and preliminary analysis of real samples for the determination of chlorpyrifos were investigated in detail.

2. Materials and Methods

2.1. Apparatus

Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and different pulse voltammetry (DPV) measurements were performed with CHI660D electrochemical workstation (Shanghai Chenhua Co., China). A conventional three-electrode system was employed with a saturated calomel electrode (SCE) as the reference electrode, a platinum electrode as the auxiliary electrode, and a glassy carbon electrode (GCE) (d=3 mm) or modified GCE as the working electrode. GNPs, MWCNTs-COOH-CHIT and $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ were observed by a scanning electron microscope (SEM, S-3000N, Hitachi, Japan).

2.2. Reagents

Anti-chlorpyrifos monoclonal antibody, chlorpyrifos, N-(3-dimethyl-aminopropyl)-N'-ethyl-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were all purchased from Sigma (St. Louis, USA). Gold chloride ($HAuCl_4$) was obtained from Shanghai Sinopharm Chemical Reagent Co. Ltd. (China). Poly (diallydimethylammonium chloride) (PDDA) (35 %, w/w in water) was from Sigma-Aldrich. Carboxylated MWCNTs (MWCNTs-COOH) was obtained from Nanotech Port Co. (Shenzhen, China). Bovine serum albumin (BSA) was from BioDev-Tech. Co. Ltd. 0.01 M Phosphate buffer solution (PBS, pH 7.4, high-pressure sterilization) was used for dissolving the anti-chlorpyrifos monoclonal antibody. A PBS (0.1 M, pH 7.0) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl was used as the detection solution. CHIT (95 % deacetylation), ethanol and other reagents were of analytical grade and distilled water was used throughout the experiments.

2.3. Preparation of the Immunosensor

150 mg chitosan flakes were weighed and dissolved in aqueous solution of 30 mL 1.0 % acetic aced. Then 30 mg MWCNTs-COOH was put into 9 mL absolute ethyl alcohol, and the solution was sonicated. Subsequently, the above 0.5 wt% CHIT was added into the acid-treated MWCNTs-COOH and the miscible liquid should be ultrasonically dispersed for 8 h to give a stable black suspension (MWCNTs-COOH- CHIT composite) [32]. The prepared composite was stored at 4°C and was treated by ultrasonication for 10 min before use.

The bare GCE (d=3 mm) was polished carefully with 0.3 and 0.05 μm alumina powder, and then cleaned through sonication in 6.0 M HNO_3, absolute ethanol and distilled water for 5 min respectively. Before modification, the bare GCE was scanned in 0.5 M H_2SO_4 between -1 and 1 V with a scan rate of 0.1 V/s until a steady-state curve was obtained. After that, the electrode was rinsed with distilled water, and dried in air.

Firstly, the cleaned electrode was treated with electrodeposition performed with current-time curve scanning at potential of -0.2 V for 200 s in 3 mM $HAuCl_4$ (denoted as GNPs/GCE). Then the homogeneous MWCNTs-COOH-CHIT composite was dropped to the modified interface (denoted as MWCNTs-COOH-CHIT/GNPs/GCE). After that, the electrode was immersed in 10g/L PDDA which was electropositive and then the second layer of MWCNTs-COOH-CHIT was coated on the electrode surface through the electrostatic force (denoted as $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE). The modified GCE was flushed by distilled water to remove the unstable components, yielding a stable interface. The electrode was then placed in a solution of EDC/NHS for 90 min at room temperature to form the NHS ester compound, washed with water, and dried with nitrogen [33]. Subsequently, the electrode was immersed in 10 μg/mL anti-chlorpyrifos antibody solution at 4 °C for about 12 h, thus the

surface modification was completed by forming a covalent amide bond between the surface-bound MWCNTs-NHS ester and the antibody. Finally, the multilayer modified electrode was incubated with 0.25 % BSA (denoted as BSA/anti-chlorpyrifos/{MWCNTs-COOH-CHIT}$_2$/GNPs/GCE) at room temperature for 2 h in order to block nonspecific binding sites. The resulted immunosensor was stored above the 0.1 M PBS at 4 °C when not in use. The fabrication process of immunosensor was shown in Fig. 1.

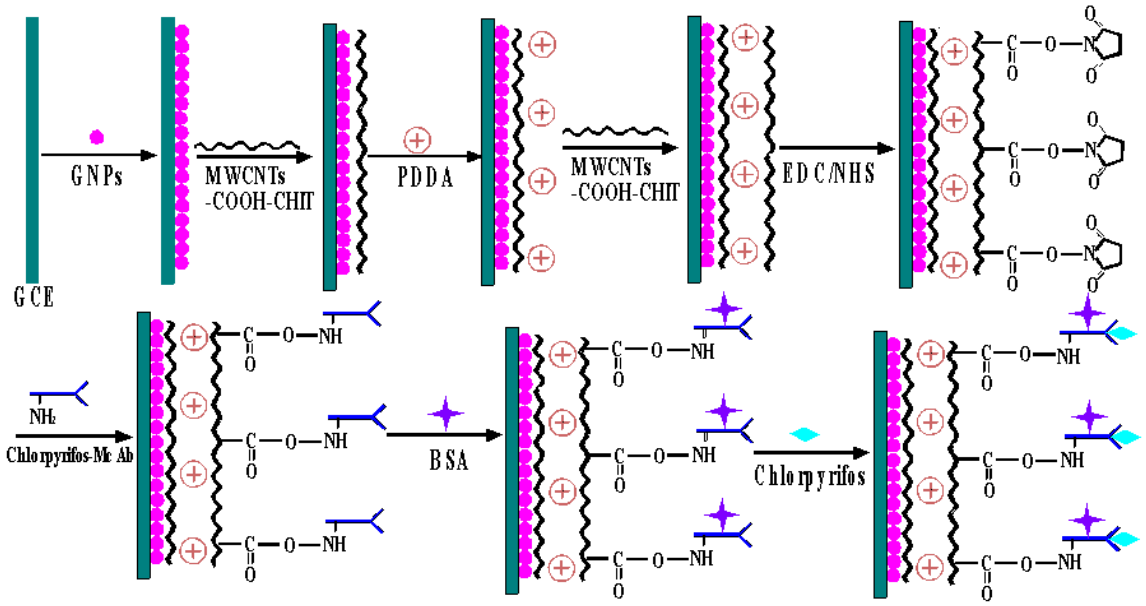


Fig. 1. Schematic illustration of the stepwise immunosensor fabrication process.

2.4. Electrochemical Measurements

The scanning electron micrographs of GNPs and MWCNTs-COOH-CHIT film were observed with SEM. The electrochemical characteristics of the modified electrode were investigated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) which were performed in 10 mL of 0.1 M PBS (pH 7.0) containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1 mixture as a redox probe) and 0.1 M KCl at room temperature. The cyclic voltammograms were recorded with a scan rate of 50 mV/s from -0.2 to 0.6 V (vs. SCE) in the above mentioned detection solution (pH 7.0). The impedance spectrum was measured at a potential of 0.2 V in the frequency range from 0.1 to 10^5 Hz with voltage amplitude of 5 mV. The chlorpyrifos detection was based on the inhibition (%), the relative change in current response, which was calculated as follows:

$$\%\Delta I = (I_0 - I_1)/I_0 \times 100\ \%\ , \tag{1}$$

where I_0 is the peak current of the DPV after blocking nonspecific binging sites by BSA, and I_1 is the peak current of the DPV after immunoreaction to the chlorpyrifos.

2.5. Preparation and Determination of Real Samples

The cabbage, carrot, green peppers and lettuce purchased from a supermarket were cleaned several times with double-distilled water. Then, different concentrations of chlorpyrifos solution were sprinkled on their surfaces. After sealing with plastic wrap at 4 °C for 24 h, 10 g of each sample was weight, chopped and meshed. Afterwards, the mixed solution of 1 mL acetone and 9 mL 0.1 M phosphate buffer (pH 7.5)

was added to each sample. And then the suspensions, treated under ultrasound for 20 min, were centrifuged (10 min, 10000 rpm). At last, the acquired supernatants were detected by DPV directly without any extraction or preconcentration.

3. Results

3.1. SEM Characterization of the Different Modified GCE Interfaces

Fig. 2 displayed the morphologies and microstructures of the modified electrode surface at each immobilization step by the SEM observation. As shown in Fig. 2a, GNPs were uniformly electrodeposited on the electrode without obvious aggregation. The images of Fig. 2b and Fig. 2c exhibited the morphologies of one and two layers of well-dispersed MWCNTs-COOH-CHIT nanocomposite films coated on the electrode, respectively. It was found that the MWCNTs was in the form of small bundles or single tubes with about 50 nm in diameter which was fatter than the unmodified MWCNTs-COOH, indicating it was tightly wrapped by CHIT. Compared with Fig. 2b, $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ (Fig. 2c) was more uniformly, tightly and roughly. The three-dimensional structure of the MWCNTs-COOH-CHIT nanocomposite film could provide a favorable microenvironment for immobilization of antibodies, which was essential for the electrochemical immunosensor construction. In addition, the nanocomposite film could provide a conductive pathway of electron-transfer.

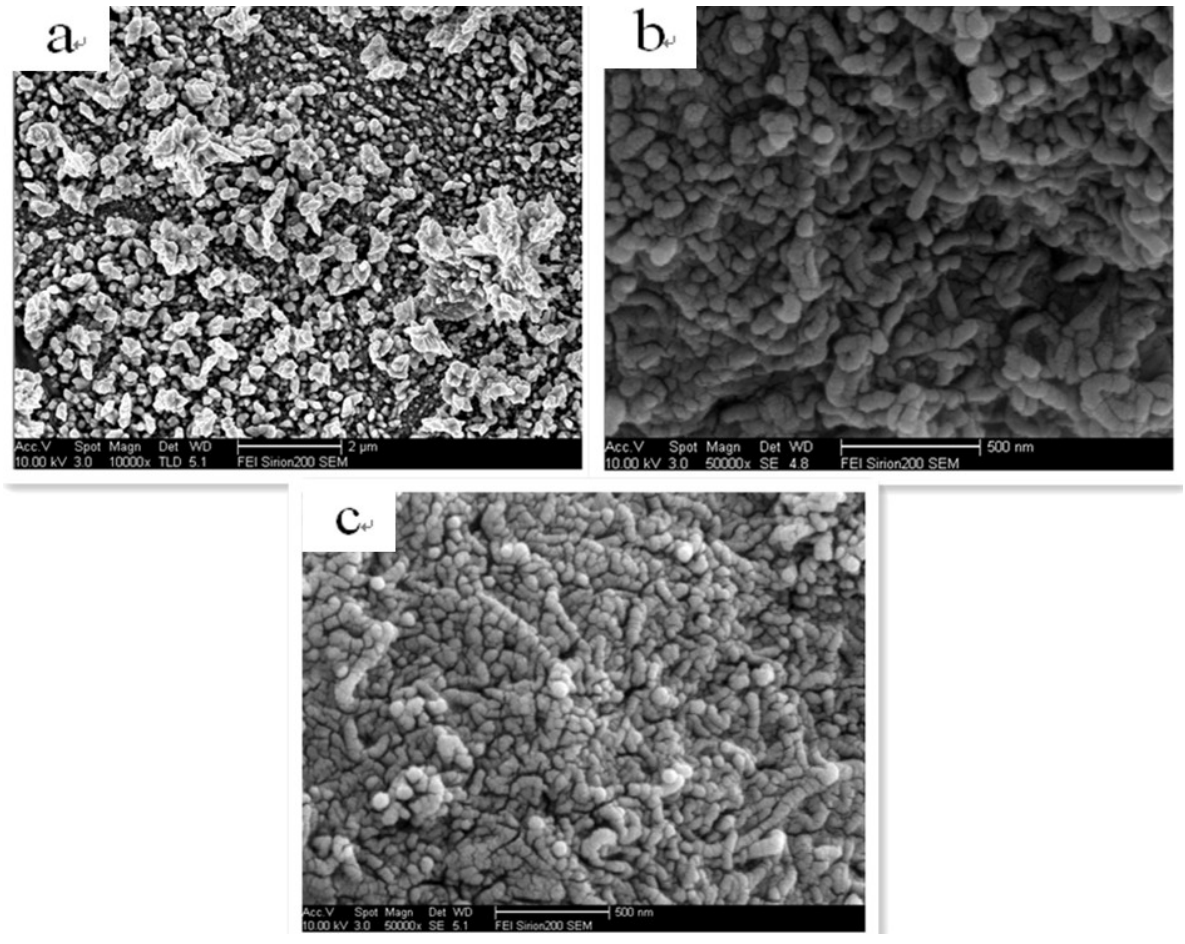


Fig. 2. SEM images of (a) GNPs film; (b) MWCNTs-COOH-CHIT film; (c) $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ film.

3.2. Cyclic Voltammetry Characterization

Fig. 3 showed the CV of different electrodes in the presence of 0.1 M PBS (pH 7.0) and 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 50 mV/s. As shown in Fig. 3, the immobilization of GNPs on the bare GCE (Fig. 3a) leaded to an obvious increase in peak current of the redox probe (Fig. 3b), confirming the fact that GNPs functioned as an electron-conducting tunnel. Similarly, after coated with MWCNTs-COOH-CHIT, the peak current of obtained electrode (Fig. 3c) was apparently increased. The current response of the electrode which was modified with $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ had an obvious increase (Fig. 3d) compared to the monolayer MWCNTs-COOH-CHIT-modified electrode (Fig. 3c), demonstrating the second layer of MWCNTs-COOH-CHIT was immobilized stably and favorably on the electrode interface through the electrostatic force with PDDA. However, after antibody (Fig. 3e) and BSA (Fig. 3f) were absorbed on the electrode surface, the current response was reduced obviously, which attributed to antibody and BSA partially blocking the electron transfer between $[Fe(CN)_6]^{3-/4-}$ solution and the electrode.

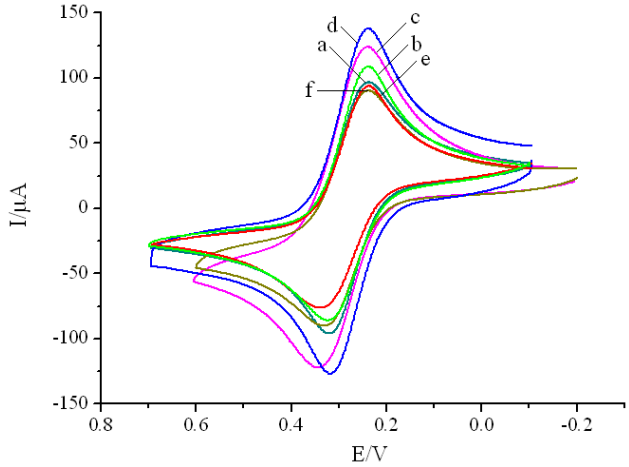


Fig. 3. CVs of modified GCE recorded in 0.1 M PBS (pH 7.0) containing 5.0mM $[Fe(CN)_6]^{3-/4-}$ and 0.1M KCl: (a) bare GCE; (b) GNPs/GCE; (c) MWCNTs-COOH-CHIT/GNPs/GCE; (d) $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE; (e) anti-chlorpyrifos/$\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE; (f) BSA/anti-chlorpyrifos/$\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE.

3.3. Electrochemical Impedance Spectroscopy

Fig. 4 illustrated the EIS obtained from different modified electrodes using $[Fe\ (CN)_6]^{3-/4-}$ as the redox probe, and the results were in agreement with CV results. Significant differences in the impedance spectra were observed during stepwise modification of the electrodes. It can be seen that a well defined semicircle at high frequencies and a linear part at low frequencies were obtained at the bare GCE (Fig. 4a), indicated small interface impedance. Compared to the bare GCE, when GNPs (Fig. 4b), MWCNTs-COOH-CHIT (Fig. 4c) and $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$ (Fig. 4d) were assembled onto the electrode surface, Rct gradually decreased, attributed to the GNPs and MWCNTs-COOH-CHIT layers for serving as conductive layers and increasing the interfacial electron transfer between the electrode and the detection solution. When the electrode was modified with antibody, the EIS presented an apparent increase in resistance (Fig. 4e). A further increase was noticed (Fig. 4f) when the anti-chlorpyrifos/$\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE electrode was blocked with BSA, which corresponded to the characteristic of CV. On the basis of above results, it could be clearly confirmed that anti-chlorpyrifos and BSA were successfully immobilized on the electrode.

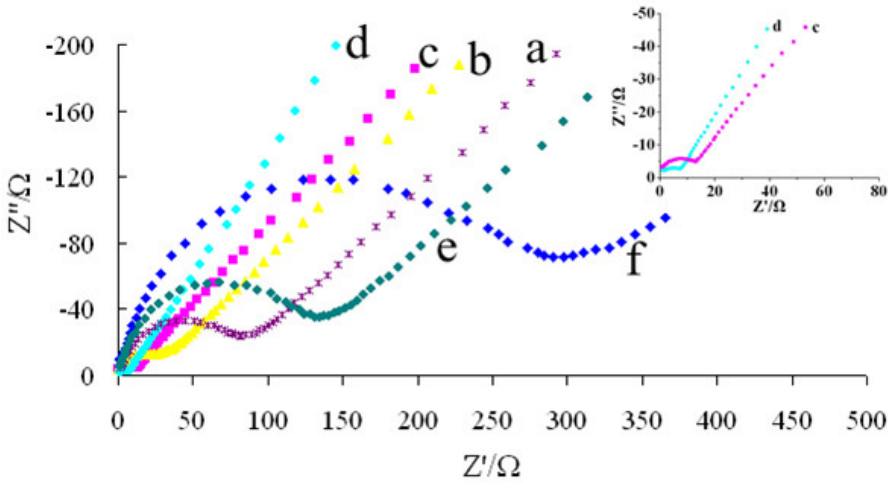


Fig. 4. EIS of modified GCE recorded in 0.1 M PBS (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl: (a) bare GCE; (b) GNPs/GCE; (c) MWCNTs-COOH-CHIT/GNPs/GCE; (d) $\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE; (e) anti-chlorpyrifos/$\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE; (f) BSA/anti-chlorpyrifos/$\{MWCNTs\text{-}COOH\text{-}CHIT\}_2$/GNPs/GCE.

3.4. Optimization Parameters of the Immunosensor Performance

3.4.1. Influence of Detection Solution pH

The effect of pH of the detection solution on response signals of the proposed immunosensor was studied in a series of 0.1 M PBS containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl with the pH from 5.0 to 8.5 by DPVs. As shown in Fig. 5A, the inhibition ratio, that is the relative change in current response, increased when pH value increased from 5.0 to 7.0. However, the inhibition ratio decreased continuously when the pH value was more than 7.0. The reason may be that antibody was not very stable and the biological activity of the antibody as protein declined in acid and alkaline solutions. The experimental results showed that the maximum inhibition ratio appears at pH 7.0. So the detection solution of pH 7.0 was chosen for further experiments.

3.4.2. Influence of Incubation Time on Inhibition

The amount of chlorpyrifos captured from the sample solution was depended on the incubation time before it reached the saturated equilibrium. In the incubation solution, when the pesticides reach the antibodies on the electrode surface of the immunosensor, it takes some time for the contacting species to form immunocomplexes. As displayed in Fig. 5B, by incubating with 200 ng/mL chlorpyrifos at 25°C, the inhibition ratio was rapidly increased with the duration of the incubation time up to 30 min, and then the curve trended to maintain a stable value. Therefore, the incubation time of 30 min was adopted in the subsequent work.

3.4.3. Influence of Incubation Temperature on Inhibition

The effect of temperature on the immunoreaction, which has been reported to be vital to the activity of the antibody and pesticides, was investigated at different temperatures ranging from 4 to 50 °C under the same experimental conditions using 200 ng/mL chlorpyrifos. As shown in Fig. 5C, it was found that the inhibition ratio gradually increased as the temperature increased from 4 to 25 °C and then began to decrease as the temperature increased above 25 °C. The reason may be attributed to the fact that during

the temperature increasing from 4 to 25 °C, more and more immunocomplex formed and inhibited the current response. It was well known that 37 °C could be an optimal temperature of immunoreaction [17]; nevertheless, long-time use in high temperature may damage the modified materials and affect the lifetime of the immunosensor. Thus, synthesis consideration of the lifetime, activity, and response characteristics of biomolecules, room temperature (about 25 °C) was recommended as the optimal incubation temperature for practical application.

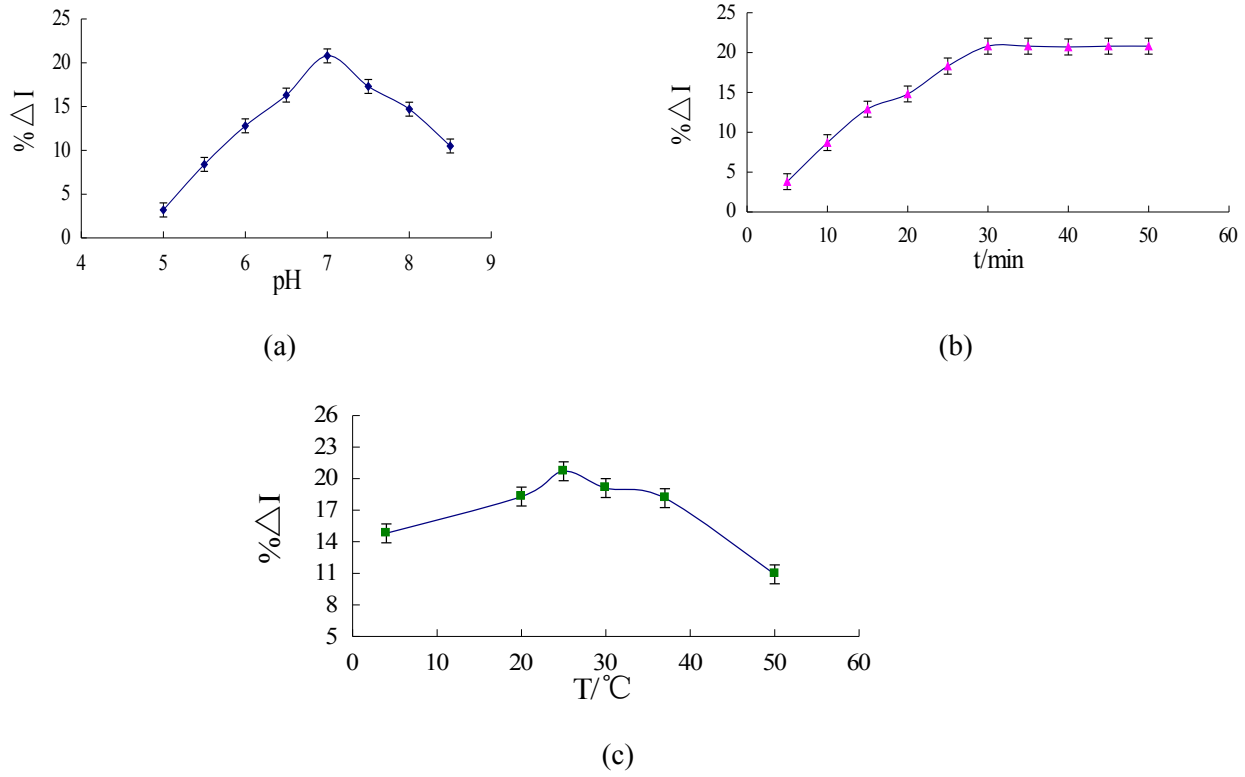
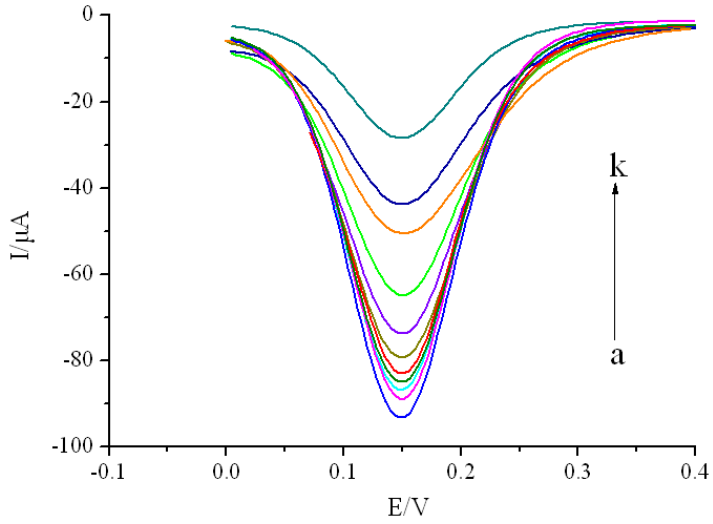


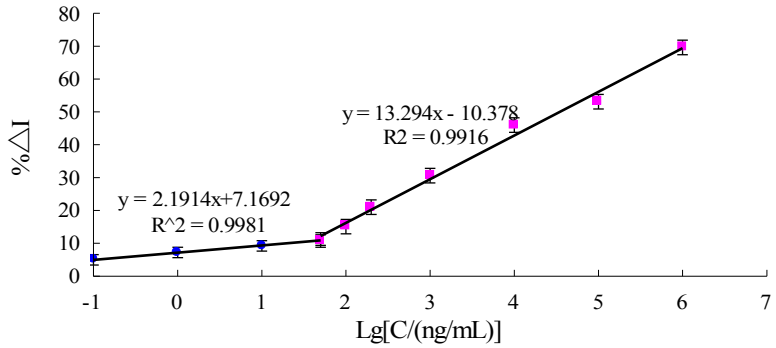
Fig. 5. Optimization of experimental parameters: (a) Influence of detection solution pH;
(b) Influence of incubation time on inhibition;
(c) Influence of incubation temperature on inhibition.

3.5. Detection of Chlorpyrifos

Under optimal experimental conditions, the immunosensors were carried out for the immunoreactions with the chlorpyrifos standard solution and the DPVs in 0.1 M PBS (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl and the calibration plot for chlorpyrifos detection with the proposed immunosensor was illustrated in Fig. 6. It was found that the current response decreased with increasing chlorpyrifos concentrations in Fig. 6a. It may be due to more chlorpyrifos binding to the immobilized antibodies in higher chlorpyrifos concentrations, which serves as a barrier for the electron transfer. As displayed in Fig. 6b, the inhibition of chlorpyrifos was proportional to its concentration in two ranges, from 0.1 ng/mL to 50 ng/mL and another linear relationship from 50 ng/mL to 1 mg/mL (Fig. 6b); the linear regression equation is %ΔI=7.1692+2.1914l gC (ng/mL) and %ΔI= -10.378+ +13.294l gC (ng/mL), with the correlation coefficients of 0.9981 and 0.9916, respectively. And the detection limit was estimated to be 0.069 ng/mL at a signal/noise of 3 (S/N=3) between the detection signal of low concentration samples and the noise of blank samples. The results indicated the feasibility and the superiority of the proposed immunosensor.



(a)



(b)

Fig. 6. (a) The DPVs of the immunosensor after incubation in different concentrations of chlorpyrifos standard solution (from a to k): 0, 0.1, 1.0, 10.0, 50.0, 1×10^2, 200, 1×10^3, 1×10^4, 1×10^5, 1×10^6 ng/mL under the optimal conditions; (b) The calibration curve of the relative changes in peak current of DPV (%ΔI) of the proposed immunosensor versus the logarithm of chlorpyrifos concentration.

As shown in Table 1, compared the performance of the immunosensor with other reported analytical methods for the detection of chlorpyrifos, the proposed immunosensor had a relative larger linear range and lower detection limit, indicating that the immunosensor was reliable for the determination of pesticides.

3.6. Performance of the Immunosensor

3.6.1. Reproducibility and Stability of the Immunosensor

The reproducibility of the immunosensor was investigated by evaluating the intra-assay and inter-assay precision. The intra-assay precision of the immunosensor was estimated by analyzing three concentration levels for five times under the same conditions, independently. The relative standard deviations (RSDs) were 5.1 %, 4.2 % and 4.3 % for 50 ng/mL, 200 ng/mL and 100 μg/mL chlorpyrifos, respectively. while the inter-assay precision was estimated by assaying five parallel measurements of three chlorpyrifos levels for different electrodes. The RSDs were 4.7 %, 4.5 % and 4.2 % for 50 ng/mL, 200 ng/mL and 100 μg/mL chlorpyrifos solutions, respectively. The results confirmed the well reproducibility and precision of the proposed immunosensor.

Table 1. Comparison of the analytical methods for the detection of chlorpyrifos.

Analytical methods	Linear range	Detection limit	References
immunochromatography	50-12150 ng/mL	132.91 ng/mL	[34]
A bi-enzymatic sensor(immobilizing both B394 and PTE)	11.22 ng/mL-1.437 μg/mL	-	[35]
[BMIM][BF_4]-MWCNT gel-modified CP electrode	3.505-350.5 ng/mL	1.402 ng/mL	[36]
PVA-SbQ polymer(Photo-co-polymerization)	0.0240–0.24 μg/mL	24 ng/mL	[37]
PPy-PVS/ITO(Electrochemical entrapment)	0.0016–0.02 μg/mL	0.0016 μg/mL	[37]
β-SPR biosensor system	-	45-64 ng/L	[38]
AChE-Fe_3O_4NPs/c-MWCNTs/ITO electrode	35.05 ng/mL-17.525 μg/mL	35.05 ng/mL	[39]
{MWCNTs-COOH-CHIT}$_2$/GNPs/GCE	0.1-50 ng/mL and 50 ng/mL-1 mg/mL	0.069 ng/mL	This work

Furthermore, the stability of the immunosensor was also examined. The prepared immunosensors were suspended over the PBS (pH 7.4) at 4 °C for 30 days, and measured the current response every day. As we all known, it is unavoidable that the steady state of the modified electrode will gradually decrease after storage and usage for some time due to the slow deactivation of protein and leakage of modified materials on the electrode. The result indicated that there were no obvious changes during the first 15 days and the initial current response changed less than 4.1 %, and after a 30 days storage period, the immunosensor retained over 83 % of its initial current response, demonstrating acceptable stability. The good stabilities could be attributed to the fact: firstly, MWCNTs-COOH-CHIT could be attached tightly on the electrode because of the strong chemical adsorption and electrostatic force; secondly, MWCNTs-COOH-CHIT nanocomposite possessed good stability and biocompatibility and the antibodies were attached firmly on the surface of composite.

3.6.2. Selectivity of the Immunosensor

Selectivity of the biological molecule for its analyte is one of the potential advantages of using biological molecules as recognition elements in biosensor. In order to investigate the selectivity of the fabricated immunosensor against the interferences arising from the other non-target molecules, the immunosensors were respectively incubated with 200 ng/mL chlorpyrifos coexisting with 200 ng/mL four other interfering substances which present widely in real samples including monocrotophos, carbaryl, carbofuran, carbofuran-3-hydroxy. As shown in Fig. 7, no conspicuous changes in peak current (%ΔI) were obtained for the interferences, indicating an acceptable selectivity of the proposed immunosensor based on the high specific antigen-antibody reaction.

3.6.3 Regeneration of the Immunosensor

Regeneration is a significant factor in the practical application of immunosensors. The regeneration of the prepared immunosensor was treated with a glycine-HCl buffer (pH 2.8) for about 5 min to dissociate the antibody-antigen complex and washed with PBS solution. Fig. 8 showed the relative change in peak current of DPV (%ΔI) of the immunosensor in 200 ng/mL chlorpyrifos solutions. It was found that the %ΔI gradually decreased with the increase of regeneration times and decreased sharply after regenerating 6 times. The result attributed to the fact that anti-chlorpyrifos antibody could gradually

shell off or denature during the regeneration and the binding activities between antibody and antigen was affected. The experimental results indicated the immunosensor had a good regeneration performance and could regenerate 6 times.

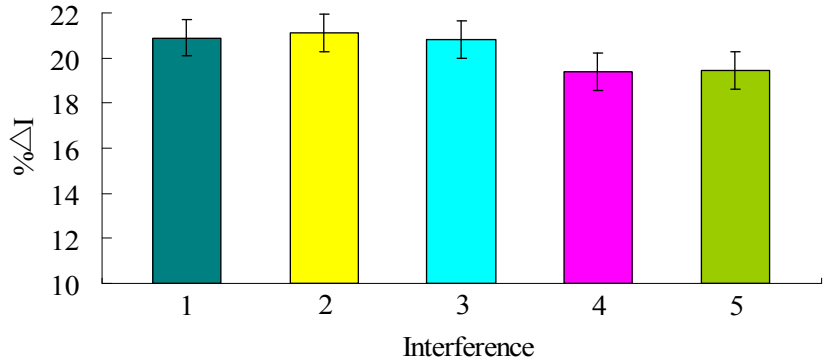


Fig. 7. The relative change in peak current (%ΔI) of the proposed immunosensor to: (1) 200 ng/mL chlorpyrifos; (2) 200 ng/mL chlorpyrifos + 200 ng/mL monocrotophos; (3) 200 ng/mL chlorpyrifos + 200 ng/mL carbaryl; (4) 200 ng/mL chlorpyrifos + 200 ng/mL carbofuran; (5) 200 ng/mL chlorpyrifos + 200 ng/mL carbofuran-3-hydroxy.

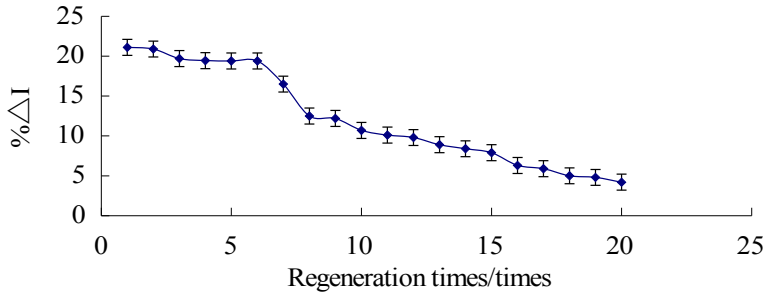


Fig. 8. Regeneration performance of the immunosensor.

3.7. Analysis of Real Samples

The usefulness and feasibility of the immunosensor for the analysis of real samples were examined under optimum experimental conditions by analysis of cabbage, carrot, green peppers and lettuce which were prepared by adding chlorpyrifos of different concentrations to these samples. The results in Table 2 showed that the recovery in the range 84.0 % - 108.6 % was in acceptable values, and the RSD between 3.54 % and 5.57 % were obtained, indicating that the proposed immunosensor can be used for direct analysis of practical samples.

4. Conclusions

In this paper, a simple and sensitive label-free immunosensor has been developed for the rapid and effective determination of chlorpyrifos residues. The proposed immunosensor was modified by {MWCNTs-COOH-CHIT}$_2$/GNPs nanocomposite films. Compared with a monolayer of MWCNTs-COOH-CHIT, the presence of {MWCNTs-COOH-CHIT}$_2$ films with good film-forming ability, conductive ability and excellent biocompatibility not only promoted electron transfer which could amplify the response of antigen-antibody interaction, but also increased the surface area to capture a large amount of antibodies, thus increased detection sensitivity. The fabricated immunosensor

possessed good reproducibility, long-term stability, acceptable selectivity and satisfactory regeneration. Furthermore, the immunosensor was well studied for the fast and direct detection of chlorpyrifos in real samples. Therefore, the proposed immunosensor has a promising application prospects for the trace detection of chlorpyrifos pesticides residues.

Table 2. The recovery of the proposed immunosensor in real samples.

Sample	Added (ng/mL)	Found (ng/mL)	RSD (%) (n=3)	Recovery (%)
Cabbage	10	10.50	4.36	105.0
	1×10^2	0.95×10^2	3.61	95.0
	1×10^3	1.06×10^3	4.18	106.0
Carrot	10	10.57	4.53	105.7
	1×10^2	0.90×10^2	3.54	90.0
	1×10^3	0.86×10^3	4.58	86.0
Green peppers	10	10.86	5.57	108.6
	1×10^2	0.93×10^2	5.05	93.0
	1×10^3	0.88×10^3	4.53	88.0
Lettuce	10	10.57	3.87	105.7
	1×10^2	0.84×10^2	4.18	84.0
	1×10^3	0.96×10^3	5.05	96.0

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.30972055, 31101286), Agricultural Science and Technology Achievements Transformation Fund Projects of the Ministry of Science and Technology of China (No.2011GB2C60020) and Shandong Provincial Natural Science Foundation, China (No.Q2008D03).

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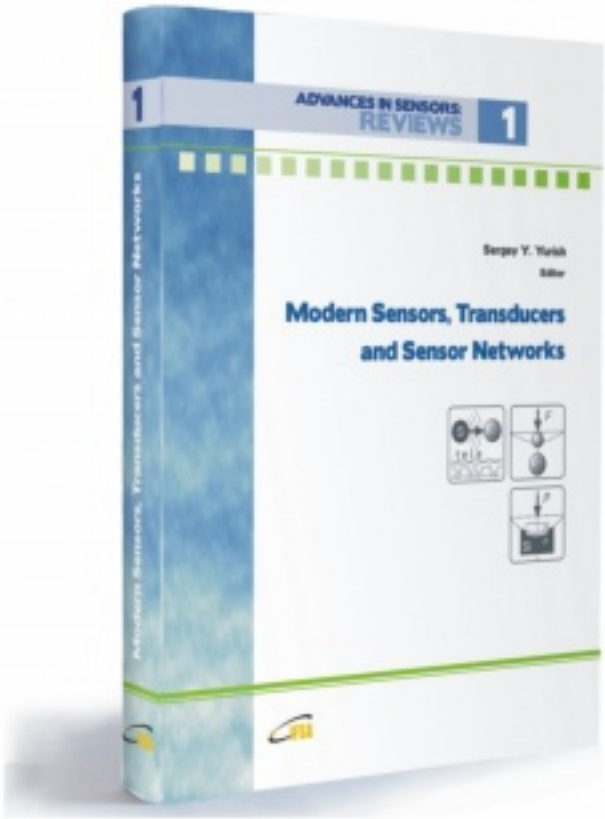

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