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An Amperometric Immunosensor Based on Graphene Composite Film and Protein a for Chlorpyrifos Detection

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Abstract: In this paper, an immunosensor was designed for chlorpyrifos detection, which was based on graphene-multi-walled carbon nanotubes-gold nanoparticle-chitosan (GR-MWCNTs-AuNPs-CHIT) nanocomposite film. Protein A (SPA) can combine with gold nanoparticles, which made anti-chlorpyrifos antibody immobilized orientedly, eventually the modified immunosensor was developed for the detection of chlorpyrifos residues. Under the optimized conditions, a regression equation: y=9.5676 lgC (ng/mL) +18.164 (R^2=0.9976) was obtained with a detection limit as low as 0.037 ng/mL. The proposed chlorpyrifos immunosensor exhibited high reproducibility, stability, and good selectivity and regeneration, it has the potential of real sample detection. *Copyright © 2014 IFSA Publishing, S. L.*

Keywords: Graphene, Protein A, Chlorpyrifos, Immunosensor.

1. Introduction

Pesticides have made major contributions to agriculture and disease control, but widespread use has created serious concerns regarding their effects on the environment and on human health [1]. Organophosphorus (OP) insecticides constitute a large family of structurally related pesticides that share a common mode of toxico-logical action, high acute toxicity and a wide range of biological activities [2-3]. In high doses, OP pesticides can cause respiratory, myocardial, and neuromuscular impairments [4-5]. Chlorpyrifos, as a broad-spectrum insecticide and one of the most frequently used organophosphate pesticides, is widely used to control insect and arthropod pests on agricultural and vegetable crops such as grains, cotton, nuts and fruits [6].

Immunoassays have been developed for analysis of the parent compound or metabolite residues in food [7-8], agrochemicals [9-11] and environmental samples [12-13] Many kinds of immunosensors such as electrochemistry [14-16], chemiluminescence [17-18], piezoelectricity [19-20], and impedance biosensing chips [21-24] have been developed. Therein, electrochemical immunosensors are widely used because of their simple preparation, fast detection, high sensitivity, and low cost.

Graphene (Gr)-based nanocomposites have attracted considerable attention due to their electrical conductivity, high surface area, good mechanical strength, high thermal conductivity and high mobility of charge carriers [25-26]. These properties make Gr an ideal two- dimensional catalyst support to semiconductor catalyst nanoparticles, offering versatile selective catalytic or sensing performances

[27-28]. Based on these merits, graphene-based nanomaterials also attract considerable attention for developing electrochemical biosensors in recent years [29-30]. Multi-walled carbon nanotubes (MWCNTs) have attracted an explosion of interest due to their fascinating physical and chemical properties such as their great chemical stability, large aspect ratio, electrical conductance, and extremely high mechanical strength and the possibilities of functional large surface area [31-34]. In addition, to improve the response sensitivity, protein A is commonly used as a binding material by binding with the Fc part of the antibody for the construction of a well-defined antibody surface [35-36]. Therefore, protein A has been used in the biosensors [37-38].

As described above, Graphene is considered as an excellent support material due to their high surface area, remarkable mechanical stiffness. Multi-walled carbon nanotube determines the unique structure of its excellent mechanical, electrical, adsorption, thermodynamic properties, etc., and carbon nanotubes and other material made of composite material of the above properties will be more prominent. However, untreated Gr and MWCNTs are extremely hydrophobic and tend to assemble into bundles, which make them tricky to process. Therefore, it is necessary to find effectual dispersants for MWCNTs. CHIT can dissolve GR and MWCNTs composite adequately because of its excellent film forming and adhesion ability. AuNPs has the ability of providing the suitable site for the protein A through covalent forces. Moreover, there are studies that nanohybrid often can combine the merits of each component and exhibit enhanced properties, such as GR-MWCNT [39], GR-AuNPs [40], MWCNTs-AuNPs [41]. Besides, protein A mediated antibody immobilization leads to highly efficient immunoreactions and enhances detection system performance. In the paper we explore a novel immunosensor with the ingenious combination of GR-MWCNTs-AuNPs-CHIT composite and facilitating orientation-controlled immobilisation of antibody on the electrochemical immunosensor by using protein A. The immunosensor provided a simple, economic, sensitive and specific method for chlorpyrifos detection. Moreover, real sample analysis was performed to evaluate the proposed immunosensors.

2. Experimental

2.1. Materials

The cyclic voltammetry (CV) and different pulse voltammetry (DPV) measurements were performed with a CHI 660D electrochemical workstation (Shanghai Chenhua Co., Shanghai, China). The working electrode was the bare golden electrode (d=1 mm), a saturated calomel electrode (SCE) and a platinum electrode were used as reference and auxiliary electrodes, respectively.

2.2. Apparatus

Anti-chlorpyrifos monoclonal antibody and chlorpyrifos were purchased from Lifeholder. Bovine serum albumin (BSA, 96-99 %) and protein A were purchased from sigma. Graphene was from Nanoon (Hebei, China). MWCNTs were obtained from Nanotech Port Co. (Shenzhen, China). $HAuCl_4$ was purchased from Shanghai Sinopharm Chemical Reagent Co. Ltd., China. CHIT (95 % deacetylation), ethanol and other chemicals were used of analytical grade. 0.01 M phosphate buffer solution (PBS, pH 7.5, high-pressure sterilization) was used for dissolving anti-chlorpyrifos monoclonal antibody; 0.1 M PBS with various pH values was prepared with stock standard solution of Na_2HPO_4/NaH_2PO_4. All chemicals and solvent used were of analytical grade and were used as received without further purification.

2.3. Preparation of the AuNPs

Firstly, 2.5 mL of 1 % sodium citrate solution was quickly added to 100 mL of 0.01 % $HAuCl_4$ while the solution was brought to boiling with vigorous stirring. Then the color changed from pale yellow to claret-red. The solution was boiled for 10 min, followed by continual stirring until the solution reached room temperature. The prepared colloidal gold nanoparticles were stored in a dark bottle at 4 °C before use.

2.4. Fabrication of the GR-MWCNTs-AuNPs-CHIT Composite

Then, the mixture CHIT solution (0.5 wt %) that contained 0.4 mg/mL GR and 0.4 mg/mL MWCNTs was prepared and mixed to obtain a suspension solution, the 1 mL abstained GR-MWCNTs-CHIT were added dropwise into 1 mL of the as-prepared AuNPs. The mixture was allowed to react at room temperature under stirring for 24 h, followed by centrifugation. The resulting GR-MWCNTs-AuNPs-CHIT nanocomposites were washed with water and then re-dispersed in 1 mL of PBS (pH 7.5) buffer and stored at 4 °C before use.

2.5. Stepwise of the Immunosensor

The gold electrode (Au) (d=1 mm) was first polished sequentially with Al_2O_3 powder of 0.3 and 0.05 μm, then, it was cleaned ultra-sonically with distilled water, 6 M nitric acid, absolute ethanol and

distilled water 5 min and dried with nitrogen for further use.

2.5 μL of the prepared GR-MWCNTs-AuNPs-CHIT composite was firstly dropped onto the pretreated gold electrode and air-dried to prepare the modified electrode GR-MWCNTs-AuNPs-CHIT/Au. Next, 2 μL 100 μg/mL SPA solution was added onto the surface GR-MWCNTs-AuNPs-CHIT/Au to immobilize the 2.5 μL 10 μg/mL anti-chlorpyrifos antibody onto the electrode surface orientedly. Subsequently, the resulting immunosensor was incubated with 2.5 % BSA solution for 1 h at room temperature to block possible remaining active sites against nonspecific adsorption. The modified BSA/anti- chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au immunosensor was thus obtained and stored at 4°C when not in use. A schematic illustration of the stepwise procedures for the fabrication of the immunosensor is shown in Fig. 1.

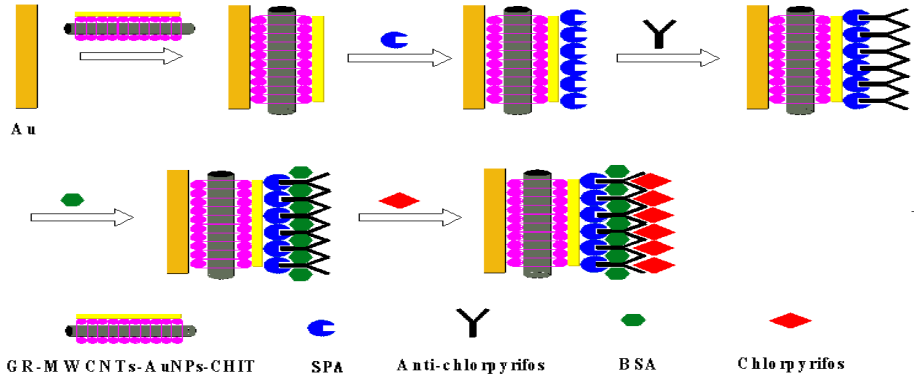

Fig. 1. Schematic illustration of the immunosensor fabrication process.

2.6. Electrochemical Measurements

The modified electrode was first incubated with different dilutions chlorpyrifos for 30 min at room temperature. Then, the electrode was rinsed with ultrapure water to remove the unbound chlorpyrifos. Finally, the steps of the immunosensor fabrication procedures were investigated by CV with a CHI 660D electrochemical system in 0.1 M pH 7.5 PBS containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl.

The relative change in peak current (%ΔI) of the immunosensor before and after interaction between anti-chlorpyrifos antibody and chlorpyrifos was measured by DPV. %ΔI was calculated as follows:

$$\% \Delta I = \frac{I_{p,\,BSA} - I_{p,\,chlorpyrifos}}{I_{p,\,BSA}} \times 100\% ,$$

where $I_{p,BSA}$ and $I_{p,chlorpyrifos}$ were the peak current of the DPV before and after reaction to the antigen, respectively.

3. Results and Discussion

3.1. Characteristics of GR, GR-MWCNTs-CHIT and GR-MWCNTs-AuNPs-CHIT Composite

Fig. 2A shows the typical flake-like wrinkled shapes illustrating the existence of graphene. In Fig. 2B, we can see image of the GR-MWCNTs-CHIT, with a tubular structure indicating the presence of multi-walled carbon nanotubes and GR, which displayed a loose and homogeneous appearance suggestting that CHIT successfully prevented the aggregation of GR. There is a more pultaceous morphology observed with many globular features in Fig. 2C, implying the successful adsorbing of the AuNPs has conjugated with the GR-MWCNTs-CHIT through amino-Au affinity, which was favorable for the immobilization of protein.

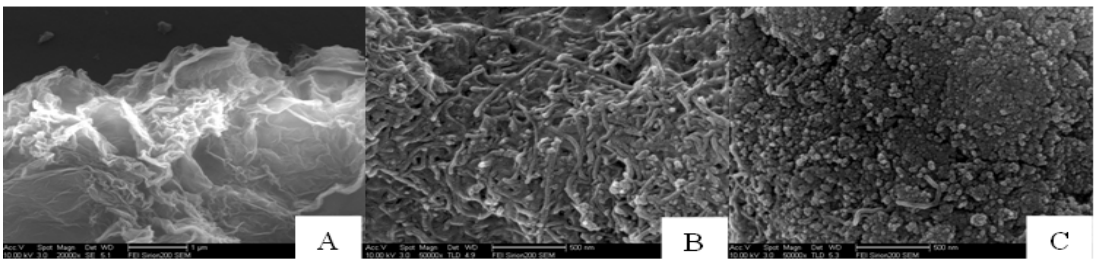

Fig. 2. SEM images of (A) GR, (B) GR-MWCNTs-CHIT and (C) GR-MWCNTs-AuNPs-CHIT.

3.2. Electrochemical Impedance Analysis

The electron-transfer behaviors of the prepared immunosensor in preparation processes were performed by cyclic voltammetry (CV). Fig. 3 displays the CV of the immunoassay in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl at 50 $mV \cdot s^{-1}$. The bare Au electrode showed a pair of redox peaks (Fig. 3a). And the increase of redox peaks (Fig. 3b) reflected the attachment of GR-MWCNTs-AuNPs-CHIT composite on the Au electrode. A decrease in the CV value (Fig. 3c) was observed after the modification of protein A. Similarly, when the antibody, BSA and antigen were subsequently fixed on the electrode, a sharp decline in peak current occurred (Fig. 3d-f). The assembly of GR-MWCNTs-AuNPs-CHIT composite on the Au electrode could provide a large surface coverage for the immobilization of biomolecules, and AuNPs, acting as a conducting wire or a supporter, increased the anodic peak and cathodic peak currents, which increased the signal amplification [42].

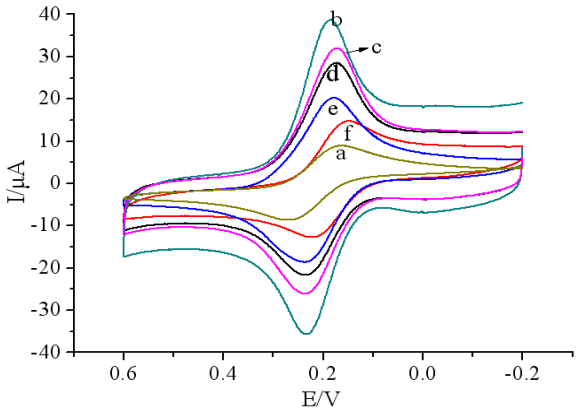

Fig. 3. Cyclic voltammograms: (a) bare Au; (b) GR-MWCNTs-AuNPs-CHIT/Au; (c) SPA/GR-MWCNTs-AuNPs-CHIT/Au; (d) anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au; (e) BSA/anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au; (f) chlorpyrifos/BSA/anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au recorded in pH 7.5 PBS containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl.

3.3. Effect of Concentrations of Concentration of GR-CHIT and MWCNTs-CHIT Composite

For preparing GR-MWCNTs-CHIT conjugates, the volume ration of GR-CHIT and MWCNTs-CHIT was an important parameter because it could influence the immobilization amount of SPA on the electrode surface, and further influence the detection sensitivity. As shown in Fig. 4, the value of peak current of $[Fe\ (CN)\ _6]^{3-/4-}$ increased quickly with decreasing the volume ration from 1:3 to 3:1. The current response of the electrode was first increased to the maximum at 1:1. With the increase of GR concentration improved, the conductivity of the modified immunosensor strengthened. While, the conductivity could be influenced by the solubility of compounds due to a mount of GR was dissolved differently. Then, it trended to level off. Therefore, the volume ration of 1:1 was selected.

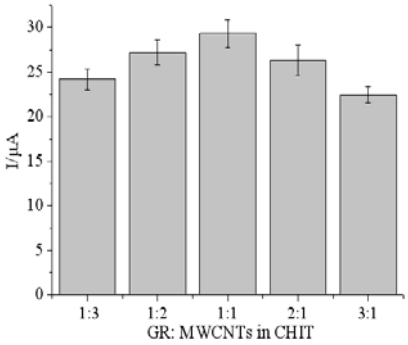

Fig. 4. Effect of the concentrations of GR-CHIT and MWCNTs-CHIT on the immunosensor.

3.4. Optimization of Experimental Conditions

When antigens bound with the antibodies, it would take some time to make sure that the formation of immunocomplexes reached a saturated state, which meant that the incubation time of chlorpyrifos was very significant. As can be seen in Fig. 5(a), the signal increased with the increasing incubation time and reached a maximum value at 30 min. The effect of pH on the electrochemical response of antigen-antibody reaction over the pH range 5.5 to 8.5 in 0.1 M phosphate buffer solution was illustrated in Fig. 5(b). We can see the optimal signal was achieved at pH 7.5.

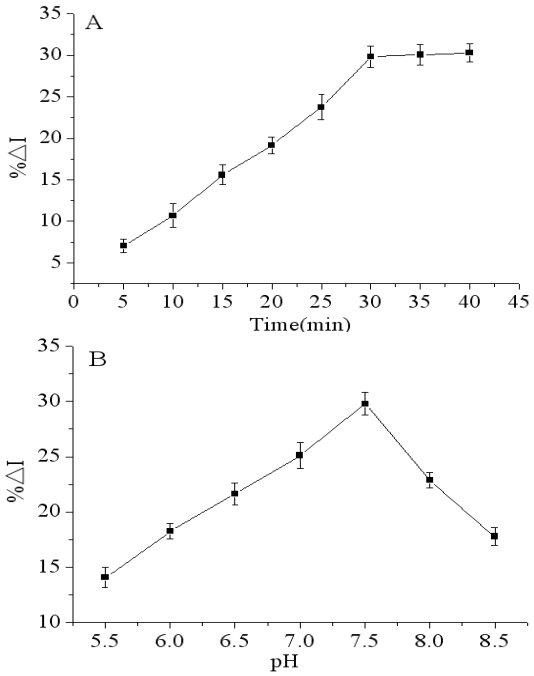

Fig. 5. Effect of incubation time (a) and pH of the detection solution (b) on the immunosensor.

3.5. Performance of the Proposed Immunosensor for Chlorpyrifos Detection

Fig. 6 (a) presented the peak of the current of immunosensors incubated to different concentrations of chlorpyrifos solutions, it also can be seen that the electron-transfer resistance increased with a rise of chlorpyrifos concentration as shown in curves (a–h), the reason was that the immunocomplex would inhibit the electron transfer, which obstructed the transfer of the electron. The calibration plots between %ΔI and different concentrations of chlorpyrifos were shown in Fig. 6 (b), curve a shows the linear curve ranging from 0.1 ng/mL to 1.0×10^5 ng/mL with a regression equation: y=9.5676 lgC (ng/mL) + 18.164 (R^2=0.9976).

The detection limit was 0.037 ng/mL (S/N =3). As comparison, the proposed BSA/anti- chlorpyrifos/ AuNPs/GR-MWCNTs-CHIT/GCE sensor exhibited the detection limit of 0.057 ng/mL (curve b), which showed that SPA-modified electrode leads a higher sensitivity. The immunosensor had a good detection limit compared with other reported methods in Table 1. The results can be attributed to the SPA can effectively and firmly combine the Fc part of the antibody onto solid surfaces, and GR and MWCNT composite used with large surface area and good conductivity, and their interaction to from a 3D hybrid.

So a better electrochemical response could be found on the proposed BSA/anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au sensor.

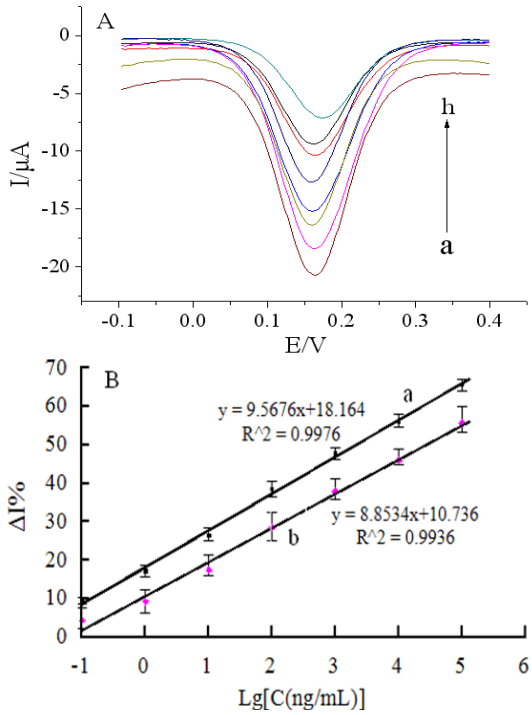

Fig. 6. (a) The DPVs after the immunosensors incubated in different concentrations of chlorpyrifos standard sample solution (from a to h):0, 0.1, 1.0, 10.0, 1.0×10^2, 1.0×10^3, 1.0×10^4 and 1.0×10^5 ng/mL under the optimal conditions; (b) The calibration plots of the relative change in peak current of DPV (%ΔI) of the proposed immunosensor versus the logarithm of chlorpyrifos concentration under optimal conditions: **a** BSA/anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/Au, **b** BSA/anti-chlorpyrifos/AuNPs/GR-MWCNTs-CHIT/GCE.

Table 1. Comparison with other immunosensors for the detection of chlorpyrifos.

Electrode	Liner range, (ng/mL)	Detection limit, (ng/mL)	References
PPy-PVS/ITO(Electrochemical entrapment)	1.6-20	1.6	[43]
AChE/[BMIM][BF_4]/MWCNT/CP	3.5-350	1.4	[2]
dsCT-DNA/PANI-PVS/ITO	0.5-200	0.5	[44]
SPR	0.02-200	0.05	[45]
BSA/anti-chlorpyrifos/SPA/GR-MWCNTs-AuNPs-CHIT/GCE	0.1-1.0×10^5	0.037	This work

3.6. Stability, Reproducibility, Selectivity and Regeneration of the Immunosensor

The stability of the newly developed immunosensor was studied under continuous cyclic potential scans for 10 cycles. From Fig. 7 (a), the results indicated that the proposed immunosensor had excellent stability. The good stability was attributed to the reasons: Chitosan showed a high chemical stability and excellent film-forming property. In addition, GR-MWCNTs-AuNPs-CHIT film provided a good electron transfer tunnel and a nice matrix for loading proteins.

The decay of current response of the immunosensor was also investigated by comparing with the stripping currents after an immunoreaction complex (Fig. 7 (b)). About 86 % of the original reaction activity retained after 21 days storage of the BSA/anti-chlorpyrifos/SPA/ GR-MWCNTs-AuNPs-CHIT/Au sensor in PBS at 4 °C indicating a good stability for the immunosensor.

The reproducibility of the immunosensor was investigated by determining 100 ng/mL chlorpyrifos levels for five replicate measurements. The intra-assay and inter-assay coefficients of variation with the above method were 3.7 % and 4.5 %, indicating satisfactory reproducibility of the proposed immunosensor.

The selectivity of the immunosensor was investigated using carbofuran, phoxim, carbaryl, 3-hydroxycarbofuran as interference pesticides. The specificity was studied by using an incubation

solution containing the known chlorpyrifos standards (100 ng/mL) and interfering agents. As seen from Fig. 7 (c), no obvious difference of stripping peak current was observed toward various concentrations of interfering agents in comparison with the results obtained in the presence of only chlorpyrifos. Moreover, the increase of the concentration of interfering agents did not lead to a significant current shift to some extent. So the selectivity of the as-prepared immunosensor was acceptable.

The sandwich immunocomplexes could be dissociated with little reactivity loss of immobilized antibody and be reused after a simple regeneration step [18, 46].

$$RE = [1-(RT-B)/T]\times 100\%,$$

where RT represents the electrochemical signal obtained after the elution cycle, T is the signal before applying the regeneration step and B is the signal for blank. The antibody-antigen complex can be dissociated by lowering the pH value of reaction solution. Generally, we choose 0.1 M glycine-HCl solution (pH 2.8) as the elution reagents to reactivate the proposed immunosensor, which allowed a fast and complete dissociation of the immunocomplexes with REs of 97.14 %. This supports the conclusion that the immunosensor developed in this study has high regeneration towards the chlorpyrifos.

3.7. Analysis of Real Samples

To evaluate the application potential of the proposed immunosensor for real sample analysis, cabbage, lettuce, Chinese chives and carrot samples were cleaned three times using double-distilled water and were spiked with chlorpyrifos solutions of different concentration. After 24 h, samples weighing 10 g were chopped and meshed. Then the samples were extracted with 10 mL mixed solution of acetone/0.1 M pH 7.5 PBS (1/9, v/v) by shaking for 45 min. Then the extract was separated from the insoluble materials by centrifugation for 10 min at 10,000 rpm and the supernatants were directly detected by DPV without extraction or preconcentration. The results were listed in Table 2, which showed relative errors less than 5.32 % for chlorpyrifos detection and recoveries between 87.4 % and 104.0 % indicated that the proposed method could be satisfactorily applied to detect the chlorpyrifos residues.

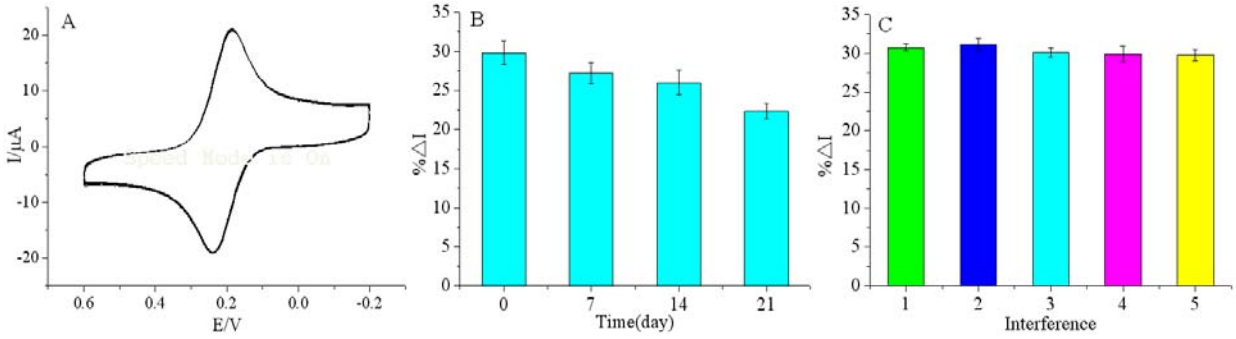

Fig. 7. (a) The stability analysis of immunosensor. (b) Decay of current response of the immunosensors with time. (c) The relative change in peak current (%ΔI) of proposed immunosensor to:
(1) 100 ng/mL chlorpyrifos+100 ng/mL phoxim, (2) 100 ng/mL chlorpyrifos+100 ng/mL carbofuran,
(3) 100 ng/mL chlorpyrifos+100 ng/mL carbaryl, (4) 100 ng/mL chlorpyrifos+100 ng/mL 3-hydroxycarbofuran,
(5) 100 ng/mL chlorpyrifos.

Table 2. The recovery of chlorpyrifos in real samples.

Sample	Taken, (ng/mL)	Found, (ng/mL)	Recovery, (%)	Relative error, (%) (n=3)
Lettuce	10	9.63	96.3	4.78
	100	104	104.0	4.63
	1,000	986	98.6	4.87
Cabbage	10	10.3	103	5.15
	100	89.7	89.7	4.68
	1,000	958	95.8	4.59
Chinese chives	10	10.2	102.0	5.12
	100	93.6	93.6	4.93
	1,000	894	89.4	4.79
Carrot	10	9.82	98.2	4.43
	100	94.1	94.1	5.32
	1,000	964	87.4	4.72

4. Conclusions

In this work, a label-free amperometric immunosensor based on BSA/anti-chlorpyrifos/ SPA/GR-MWCNTs-AuNPs-CHIT nano- composite film has been developed for chlorpyrifos detection. The synergy of graphene and MWCNTs can greatly enhance the electron transfer between the electrolyte and electrode. Furthermore, the GR-MWCNTs-AuNPs-CHIT nanocomposites film provides lots of carboxylic and amino groups to immobilize protein A, which would be very useful for immobilizing antibody more efficiently. More significantly, the ordered state of protein A in the self-assembled layer increases its binding capacity to antibody, which increases chlorpyrifos binding and make anti-chlorpyrifos antibody immobilized orientedly. The proposed biosensor possessed a wider linear range and a lower detection limit compared with other methods. So the biosensor offers the advantages such as good stability, high electrocatalytic ability and simply preparation procedure. It will be useful for the development of electrochemical immunosensors and could easily be adapted for the detection of other pesticide residues.

Acknowledgements

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