Co_3O_4/PAn MAGNETIC NANOPARTICLE-MODIFIED ELECTROCHEMICAL IMMUNOSENSOR FOR CHLORPYRIFOS

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In this study, Co_3O_4/PAn nanoparticles modified electrochemical immunosensor was produced, and applied to rapidly detect chlorpyrifos residues in agricultural products. Chlorpyrifos(CPF)-BSA artificial antigen was coupled to the surface of working electrodes coated with the Co_3O_4/PAn nanoparticles thin layer. The competitive reaction is launched between the CPF in the samples and the CPF coupled to the electrodes with CPF monoclonal antibodies in the test system. After the antigen-antibody signal is amplified by silver nanoparticles, the electrolytic current under a certern voltage is detected, which was called as voltammetry. After establishing basic detection system, a series of indices including the concentration of immobilized membrane, materials coated on the electrode surface, buffer, the concentration of coupled antigen and secondary antibodies were optimized. Then the concentration of CPF in standands, green vegetables and apples was detected. The results show that the immune sensor has good sensitivity and high accuracy; Lower Limit of Detection (LLD) is 0.01μg/mL for CPF, recovery rate is more than 82%, test time is less than 2 h, and variable coefficient is less than 5%. The sensor is economical because it can be reused after reusing treatment. The study contributes to realize rapid detection of pesticide residues in agricultural products.

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1. Introduction

Chlorphrifos (CPF) is a kind of organophosphorus pesticide with moderate toxicity, and is widely applied in modern life and agriculture. Currently, the production and usage of virulent organophosphorus pesticides was strictly controlled in the world, leading to excessive use of substitutes such as CPF, which severely affect human health and environment. Therefore, the sensitive and efficient screening and detecting methods for CPF residues should be developed urgently. Conventional methods for detecting residual CPF are usually gas chromatography and high pressure liquid chromatography over the world. However, these methods are not only time-consuming and costly, but also require specific instruments, complex operations and specialized training, which are difficult to satisfy the rapid detection on the spot [1, 2].

In recent years some immunoassay based on antigen-antibody reaction have been applied widely in the detection for pesticide residue in food and environment, and these methods are high

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sensitive, specific, economical and gradually replaced chromatography [3]. In immunoassay of pesticide residues, labeled active enzymes are used to amplify the signal of antigen-antibody reaction, and the signal is measured by enzyme-labeled instrument, whose inconveniences restrict the application in rapid detection of pesticide residue. Most of molecular structures of organophosphorus pesticides contain the functional group with electrochemical activity, which are very suitable for electrochemistry experiment. The methods would be largely developed in rapid detection of organophosphorus pesticides in future due to their rapidness, high sensitivity, conveniency and miniaturization [4, 5]. Lin et al prepared high sensitive ZrO_2/Au nano film electrode to detect the residual sulfur and phosphorus of organophosphorus pesticides, and the results showed that this method was simple and sensitive [6]. Nano-materials are generally considered as the most promising materials in the 21st century for the granule and surface effect, which present distinct physical and chemical characteristics. It has been reported that nano technology plays a pivotal role in antibacterial material, drug carrier, biosensor and environment monitoring [7, 8]. Electrochemical sensors using metal/metal oxide detect the concentration of samples by measuring the changes of current, and conductance based on the electrochemical reaction between reference and working electrode. As an important metal oxide ceramic material, Co_3O_4 is widely used in electrochemistry, magnetics and catalysis field, and its application in the super capacitor has become a hotspot research [9, 10]. The micro structures of Co_3O_4 such as grain size and crystal morphology are the key parameters to determine its performance.

Based on the strong selective adsorption of Co_3O_4 nanocomposites to phosphate group, our study established a sensitive, rapid, cheap and simple detecting method for organophosphorus pesticides residue by a self-made working electrode with Co_3O_4/PAn magnetic nanoparticles, which measured CPF concentration by voltammetry. The optimization of the preparation process and detecting methods for CPF were also discussed.

2. Experimental

2.1 Preparation of Co_3O_4 nanoparticale

The chemical reagents used in this study were analytical pure (purchased from Tianjin Chemical Reagent No.6 Plant). Co_3O_4 nanoparticale was prepared by chemical coprecipitation. The specific steps were as follows: 0.2 g $Co(NO_3)_2$ was dissolved in 100 mL of distilled water and stirring for 30 min by agitating magnetically. Afterwards, ammonium hydroxide was added dropwise to adjust pH to 9.0. After aging for 12 h, the solution was filtered, and washed thrice with distilled water and ethyl alcohol alternately. Following that, Co_3O_4 precursor was obtained by drying for 4h at 60℃ in a vacuum oven. Finally, the precursor was calcined at 200℃ for 4 h in a muffle furnace.

2.2 Preparation of Co_3O_4 composite (Co_3O_4/PAn) coated with polyaniline

The specific steps were as follows: 0.36 g CTAB was dissolved in 50 mL of distilled water, and a certain amount of Co_3O_4 nanoparticale was added. After ultrasonic dispersion and agitation for 5 h, 180 μL of aniline was added and stirred for 1 h, and then 0.6g citric acid was added. After stierring for 10 h, 0.7g ammonium persulfate was added and stirring for 12h. The products were filtered by a vacuum pump and the filter cake was washed several times with deionized water and absolute ethyl alcohol until the filtrate was colorless. At last, filter cake was dried at 60℃ for 4 h in the vacuum.

2.3 Preparation of silver nanoparticles

The specific steps were as follows: 0.1 mL of $AgNO_3$ (1 mM) was diluted to 1 mL of solution, and 5 mL of CTAB (0.2 M) was added. Then, 0.1 g $NaBH_4$ was added and stirred magnetically at 30℃ for 2 min. After aging for 5 h at 28 ℃, the seed solution was prepared. 5 mL of CTAB (0.2 M) was added in a beaker, and 5 mL of $AgNO_3$ (1 mM) was respectively added in three small beakers. After shaking for 1 min, 100 μL of ascorbic acid (0.1 M) was added. After stirring magnetically for 2h, 60 μL of $AgNO_3$ (0.01 M) was added and mixed well in 28 ℃

thermostatic water bath, which was the growth solution. After 10 min of thermostatic water bath, 5 mL of the seed solution was added and shaked for 5 min, and was then standing at 28 ℃ in a water bath for 5h to obtain silver nanoparticle solution.

2.4 Enzyme-labeled second antibody labeled by silver nanoparticles

The PH of 5 mL of silver nanoparticle solution (0.05 g/L) was adjusted to 8 with NaOH solution (0.1 M). 20 μL of the mixture of IgG-HRP and HRP (1 mg/mL) was added in the silver nanoparticle solution, and stirring for 30 min. Until now, the majority of IgG-HRP antibodies have been absorbed on the surface of silver nanoparticles. The solution was centrifuged at 10000 rpm for 20 min, so as to remove the uncombined enzyme-labeled second antibodies. After washing several times with PBS (0.01M, pH=7.4), AgNRs-IgG-HRP was dispersed into 1 mL PBS buffer, and stored at 4℃ for future use.

2.5 Preparation of immunoelectrode

ITO conductive glasses were cut into 24 cm slices, sonicated in acetone and ethanol and deionized water, and was drying. After the area of probe was sealed to 1 cm^2, ITO was sonicated respectively in methylbenzene, acetone, ethyl alcohol and deionized water for several minutes. After drying in nitrogen, 100 μL of Nafion solution was added on the working area, and dried in a drying oven. 100 μL of dilute Co_3O_4/Pan nanocomposites solution was added to the surface of ITO electrode and dried in a drying oven. And then ITO electrode was activated by 100 μL of 0.25% glutaraldehyde solution for 2h at room temperature. Activated ITO electrode was washed with double-distilled water and dried in nitrogen. 100 μL of diluted chlorpyrifos artificial antigen was added and incubated at 37℃ for 3 h. The electrode was washed with PBS for several times after overnight. After drying, 100 μL of BSA (5 mg/mL) was added to eliminate nonspecific adsorption. The electrode was blocked at 37℃ for 5 h, and then washed with PBS for several times. Finally, the prepared sensor probe was preserved at 4℃ for future use.

2.6 Electrochemical behavior test in the electrode-assembling process

Nafion solution was dripped onto pre-treated ITO electrode to form a layer of immobilized membrane, which was used to adsorb Co_3O_4/PAn nanoparticles. After Co_3O_4/PAn nanocomposites were assembled to the electrode, glutaraldehyde as the cross-linking agent covalently linked chlorpyrifos-BSA to the electrode. The carboxyl of chlorpyrifos-BSA artificial antigen was able to have an amidation with the amino group of activated Co_3O_4/PAn nanocomposites, so that chlorpyrifos monoclonal antibodies could specifically bind to chlorpyrifos-BSA.

2.7 Electrochemical detection

In the experiment, an indirect competitive immunoassay was used to determine chlorpyrifos. 100 μL of the mixed solution of chlorpyrifos monoclonal antibodies and diluted chlorpyrifos standards was added to the blocked electrode, and incubated at 37℃ for 1 h. After washing with PBS buffer, 100 μL of silver nanoparticles-labeled IgG-HRP antibodies was added to it and incubate at 37℃ for 45 min. After washing with PBS, pre-prepared working electrode, Ag/AgCl electrode and platinum electrode were placed in 4 mL of potassium ferricyanide mixture (1 mM) in a 10 mL beaker. Cyclic voltammetry scan was conducted at the rate of 50 mV/s in a range of -0.8 to 0.8 V. 10 μL of H_2O_2 (0.2 M) was added while stirring, and 10μL for each time in a total of 40 μL was added. With the increase of H_2O_2, the cathodic current declined constantly. Preparation and measuring principle of immunoelectrode have been shown in Fig.1.

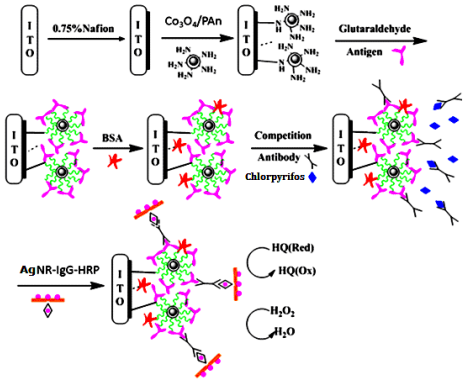

Fig.1. Preparation and measuring principle of chlorpyrifos immunosensor

2.8 Sample characterization

Japan JSM-5600LV Low Vacuum Scanning Electron Microscope (SEM) and Japan JEOL JEM-2010 Transmission Electron Microscope (TEM) were used to observe the morphology and size of the samples. CHI660D electrochemical workstation (Shanghai Chen Hua Instruments Inc.) was adopted for electrochemical test.

2.9 Pre-treatment of the tested sample

5 g green vegetables and apples were ground into the syrup, and 0 to 10 μg/mL standard solution of chlorpyrifos was added to the sample. Each sample was extracted with methanol for three times, and then was merged and concentrated and was diluted to 10 mL with PBS buffer.

3. Results

3.1 SEM characterization of Co_3O_4/PAn nanocomposites

Fig.2 showed the scanning electron microscope images (SEM) of prepared Co_3O_4/PAn nanocomposites. As shown in the figure, Co_3O_4/PAn nanocomposites were present mainly in the form of nanoparticles, and some particles connected with each other; meanwhile, local agglomeration was observed, and the size of the particles was about 80 nm, accumulated fluffily. The illustration indicated that prepared Co_3O_4 was granular, about 40 nm, and accumulated fluffily. SEM test showed that Co_3O_4 and polyaniline formed constitutionally stable composite. Co_3O_4/PAn nanocomposites with such structure and morphology helped to improve the conductivity and the dynamic performance of the electrode process.

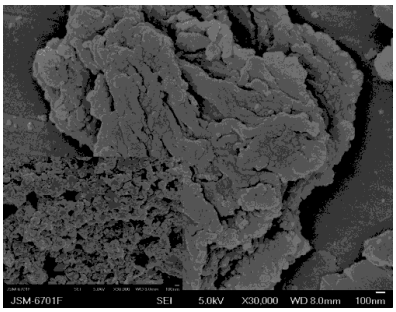

Fig. 2. Scanning Electron Microscope Image of Co_3O_4/PAn nanocomposites

3.2TEM characterization of silver nanoparticles

Fig. 3 showed TEM characterization of silver nanoparticles. From this figure, ascorbic acid-reduced Ag was present in the form of nanoparticles, with the diameter of about 350 nm and a

good dispersion. The selected-area diffraction indicated that prepared silver nanoparticles had a good crystallinity. The structure of such nanoparticles was very beneficial to contacts and tags of the antibody.

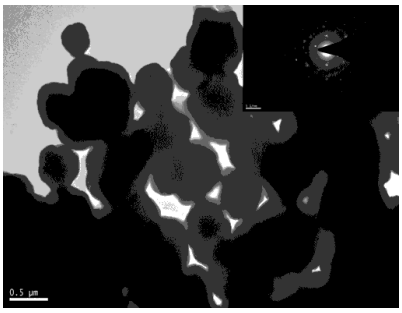

Fig.3. TEM characterization of silver nanoparticles

3.3 Cyclic voltammetry behaviour

The electrochemical property of the electrode in self-assembly processes was investigated by using the cyclic voltammetry. The results showed the cyclic voltammetry behaviours of the immunosensor in 4 mL of potassium ferricyanide at different assembly phases. As shown in Fig.4, curve a showed the cyclic voltammogram of redox current in the bare ITO electrode. When the electrode was modified with Nafion membrane, the current of the cyclic voltammetry curve further declined, which suggested that Nafion membrane hindered the electron transfer (curve b). According to curve c, after ion exchange occurred between Co_3O_4/PAn and Nafion membrane, the redox peak current of the modified electrode in the potassium ferricyanide solution was further increased, indicating that Co_3O_4/PAn in Nafion membrane can effectively transfer the electrons. After the re-modified electrode was coupled with chlorpyrifos BSA, the redox peak (curve d) declined compared with curve c, which was caused by adsorption effect of the protein molecules to impede electron transport. In particular, after the chlorpyrifos artificial antigen and chlorpyrifos monoclonal antibody were specifically combined, antigen-antibody complex blocked more channels on the surface of modified electrode and increased the resistance across the membrane, thus the response current decreased (curve e).

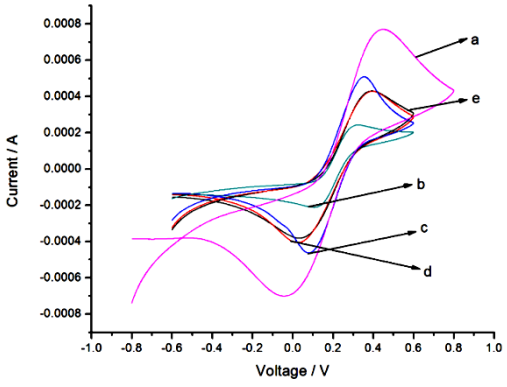

Fig. 4. Cyclic voltammograms in electrodes. (a) Bare electrode. (b) Nafion-modified electrode. (c) Co_3O_4/PAn-modified electrode. (d) chlopyrifos artificial antigen-modified electrode. (e) chlorpyrifos monoclonal antibody-modified electrode.

3.4 Optimization of reaction conditions

It is very important to optimize conditions in the process of immunization for the property of immunosensors. For example, dilution ratio of the antigen buffer for immobilization, dilution ratio of the antibody buffer binding to antigen, the amount of antibody binding to hapten, and the

duration and temperature of immunoreaction all have a great impact on the accuracy and sensitivity of the sensor. In this study, we focused on optimization of concentration of Nafion solution, dilution ratio of Co_3O_4/Pan solution, dilution ratio of antigen and antibody and pH value of diluted potassium ferricyanide buffer.

3.5 Optimization of immobilized membrane concentration

As shown in Fig. 5 (a), with the increasing of Nafion concentration, the corresponding change of electric current increased and then decreased. It is indicated that the increase of Nafion helped coupling more Co_3O_4/Pan nanoparticles, but when the concentration reached 1.25%, the Nafion membrane on the ITO surface was too thick for electronic transmission. Therefore, 1.25% was chosen as the optimum concentration for immunosensors in this experiment.

3.6 Selection of Co_3O_4/PAn nanocomposite concentration

The concentration of nanoparticle determined immobilization of antigen in the late experiment. Influences of different concentration ratios of Co_3O_4/Pan nanoparticles on the immunosensors have been shown as in the Fig. 5 (b). The electric current had no significant change in dilution ratio between 1:100 and 1:400 of Nifion, but suddenly enhanced in dilution ratio between 1:400 and 1:800, and reached the peak in the dilution ratio of 1:800. However, the current decreased when dilution ratio continued to increase. This indicated that when dilution ratio of Nifion is 1:800, the binding force between Nifion and Co_3O_4/Pan nanoparticles was the strongest, showing the maximum binding number. Besides, either too high or too low dilution ratio could not be good for electric currency change. Therefore 1:800 was chosen as the optimum dilution rate.

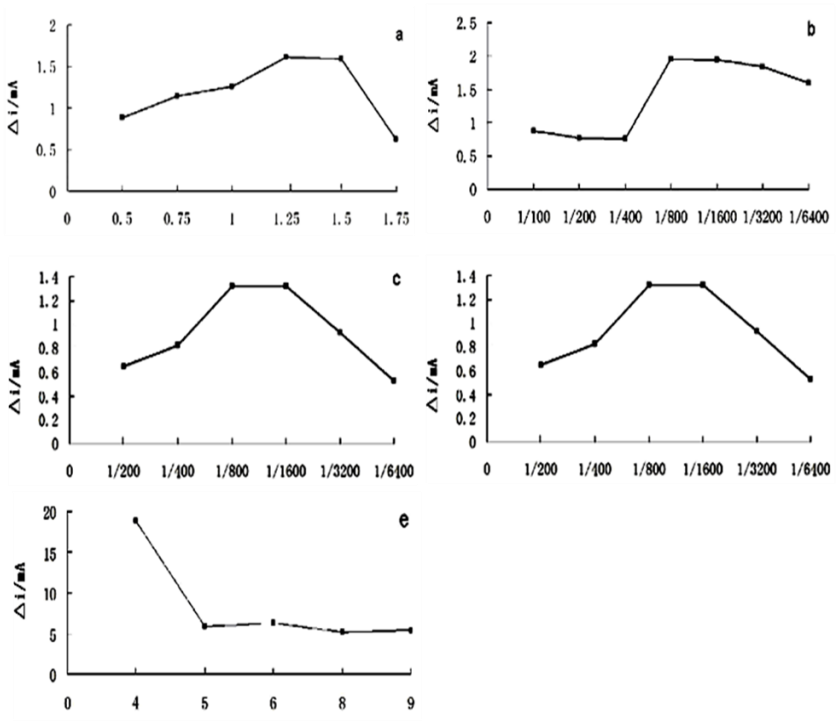

Fig. 5. Optimization and selection of base buffer. (a) Optimization curve of Nafion concentration. (b) Optimization curve of nano-Co_3O_4/PAn solution dilution ratio. (c) Optimization curve of antigen dilution ratio. (d) Optimization curve of antibody dilution ratio. (e) Optimization curve of pH of the test system.

3.7 Optimization of antigen concentration

The number of antigen immobilized on the electrode surface could directly affect sensitivity of the sensor. As shown in Fig. 5(c), with the increase of artificial antigen concentration,

the electric currency also increased. When the dilution ratio ranged from 1:800 to 1:1600, the amount of immobilized antigen became too large and a larger steric hindrance was produced to decrease antigen-antibody binding ratio. Thereby, the electronic signal change of the sensor was reduced. Therefore, 1:1600 was chosen as the fixed dilution ration for artificial coupling antigen.

3.8 Optimization of antibody concentration

Before indirect competitive immunoassay was performed, the response of chlorpyrifos antibodies at different titers was investigated for the tested sample. As shown in Fig. 5 (d), the electric current decreased with the increasing dilution ratio of chlorpyrifos antibody. A linear relationship has been shown then the dilution ratio ranging from 1:100 to 1:200 and from 1:4000 to 1:8000. This indicated that in the two concentration range, the increase of antibody concentration was proportional to binding rate. However, the increase of antibody concentration between 1:200 and 1:1000 had little effect on binding ratio, suggesting antigen supersaturation. Therefore, 1:1000 was selected as the optimum dilution ratio of chlorpyrifos antibody.

3.9 Optimization of pH value

Antigen and antibody are usually amphoteric matters possessing a certain isoelectric point. Specially, the spatial structures and the ionic station of their groups are different under different pH values. Therefore, pH values have a significant influence on the charging and affinity. The study investigated the current responses of the immunosensor in the test system at pH 4-9. According to Fig. 5 (e), the electrode current of the sensor reached the highest and the enzyme activity was also in the best condition at pH 4.0. Therefore, PBS (1/15 M) at pH 4 was selected as the base solution.

3.10 Application of multi-labeled secondary antibody

Transforming immune signals into electrochemical signals through the catalysis of labeled HRP on the secondary antibody is a common method of electrochemical immunosensor. However, the signal achieved by traditional single-labeled method is low and not good to improve sensitivity of the sensor. The multi-labeled antibody is prepared with silver nanoparticles mainly because silver nanoparticles have good biocompatibility and conductivity, and the surface effect could increase the amount of absorbed enzyme. When using nano-materials, a secondary antibody molecule could be linked to multiple enzyme molecules. When the concentrations of samples are the same, the system built by multi-labeled secondary antibody could obtain much larger signals than single-labeled system. Thus, amplification of the effective signal is achieved to greatly improve the sensitivity of the sensor. Fig. 6 showed the system diagram built with single-labeled secondary antibody and multi-labeled secondary antibody. As shown in the cyclic voltammogram, after adding 25 μL 0.48 M H_2O_2, redox peak current was respectively 0.09 mA and 0.31 mA built by single-labeled secondary antibody and multi-labled secondary antibody. This proved multi-labeled secondary antibody amplified the signal of the enzyme and further improved the sensitivity of the sensors.

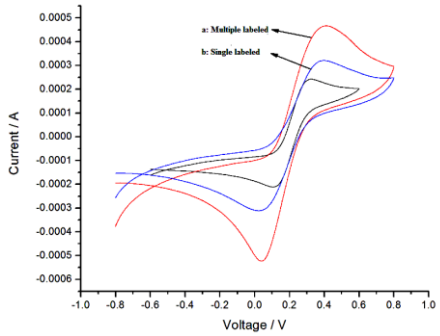

Fig. 6. Optimization and selection of secondary antibody labeling. a: Multi-labeled secondary antibody sensor, b: single-labeled secondary antibody sensor.

3. 11 Sensitivity and standard curve of electrochemical immunosensor

CPF standard solution was diluted to a series of solutions at different concentrations, and the standard curve was obtained by indirect competitive assay. PBS buffer was used as the control. As showed in Fig. 7, a significant liner relationship has been shown between frequency response and log of CPF concentration in the range from 0 to 10 μg mL^{-1}, i.e., I=58.6 X +32.4 (R^2=0.9812), and the limit of detection was 0.01 μg/mL.

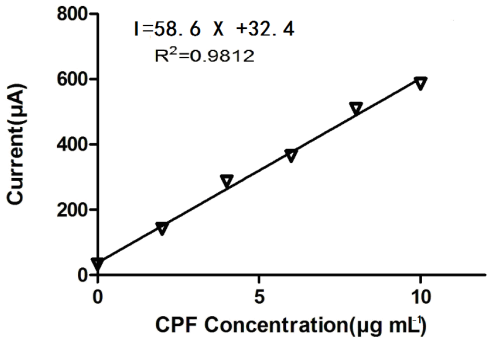

Fig.7. Standard curve of CPF standards measured by Co_3O_4/PAn sensors

3.12 Accuracy and regeneration of electrochemical immunosensor

The formula, recovery rate= (measured value of spiked samples - measured value of the sample)/ spiked samples × 100%, is used to evaluate the coincidence between the measured value and real value. The added recovery rates measured in apple and green vegetables samples have been shown in Table 1. According to Table 1, as for the two agricultural products, the recovery rate was over 85%, and the variable coefficient was less than 5%. The recovery rate over 100% might be caused by the experimental error or base affection. The recovery rate of the standards at the low concentration region might be affected by the base value of the samples. In brief, the lower base value of the sample was, the higher the recovery error of the standards may be. After the prepared immunoelectrode was used to determine the samples of the standards, green vegetables and apple, the immunosensor electrode was washed for 3 times by glycine-ydrochloric acid buffer solution. Subsequently, it was used again to determine the standards, and the result indicated the sensor could measure CPF concentration of the samples.

Table.1 Added recovery rate of chlorpyrifos in green vegetables and apples by Co_3O_4/PAn sensor

Sample	Added standards (μg mL^{-1})	Measured value (μg mL^{-1})	Recovery rate (%)	Variable coefficient (%)
Green vegetables	0	0.008	-	-
	0.01	0.012	106.79	4.9
	0.1	0.091	89.21	3.9
	1	0.972	90.84	2.8
Apples	0	0.006	-	-
	0.01	0.008	82.84	4.6
	0.1	0.089	84.92	3.9
	1	0.923	89.19	3.8

4. Conclusions

This paper has built a kind of immune-electrochemistry sensor system based on the indirect competition method which can be used in determining CPF residue amount of agricultural products, water and soil. The basic principle is that CPF artificial antigen is coupled on the surface of the working electrode coated with Co_3O_4/PAn magnetic nanoparticle thin layer, and coupling antigens of free CPF and CPF on the working electrode are bound competitively to CPF monoclonal antibodies in the solution. The immune reaction between antibody and coupling antigen are measured by voltammetry. The immunosensor is used to detect the pesticide residues of agricultural products like green vegetables and apples. Moreover, the sensitivity, accuracy and reproducibility have been evaluated. The standard curve in the range of 0 to 10 μg/mL: I(μA)=58.6 x (μg/mL) +32.4, with the correlation coefficient of 0.9812, limit of detection of 0.01 μg mL^{-1}, the recovery rate of > 82% and variable coefficient of < 5%. In addition, the prepared sensor still possesses a reliable sensitivity after regeneration through glycine-hydrochloric acid buffer solution.

The electrode developed in this study based on Co_3O_4/PAn magnetic nanoparticle thin layer and present reliable performance. It is promising in the application that markedly increased the detecting sensitivity to organophosphorus pesticides such as CPF. Our objective is to prepare thin layer electrode to improve the detecting sensitivity and limit of the analyte, and realize the integration and miniaturization of electrochemistry detection system [11, 12]. Therefore, on the basis of this research, micro-electrical mechanical system and microfluidic system are adopted to integrate the prepared Co_3O_4/PAn thin layer electrode. Meanwhile, because of favorable physical and chemical performance, various special catalytic and modified sensor functions, Co_3O_4/PAn electrode possesses excessively high effective specific surface area. Hence, further expansion of the Co_3O_4/PAn electrode application is one of the subsequent studies of this subject [13, 14].

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