Electrochemical immunosensors for 2,4-dichlorophenoxyacetic acid based on 3-aminopropyltrimethoxysilane modified electrodes

Rui Xue[1], Tianfang Kang[2, b, *], Xin Rui[1, c]

1. China Electronics Engineering Design Institute, Beijing 100142, P. R. China.
2. Key Laboratory of Beijing on Regional Air Pollution Control, College of Environmental and Energy Engineering, Beijing University of Technology, Beijing 100124, P. R. China
a. xuerui@sdic.com.cn, b. kangtf@bjut.edu.cn, c. ruixin@sdic.com.cn
***corresponding author**

Abstract: A novel electrochemical immunosensor prepared by direct coating of small molecular hapten on glassy carbon electrode (GCE) is reported for the detection of 2,4-dichlorophenoxyacetic acid (2,4-D). The glassy carbon electrodes were activated by electrochemical method and then were further treated with 3-aminoprpyltriethoxysilane (APS) to functionalize the GCE surface with amino groups for covalent linkage to small molecular hapten of 2,4-D with carboxyl groups. The electrodes were characterized by X-ray photoelectron spectroscopy (XPS). The principle of this immunoassay is based on an indirect competitive immunoassay. Analytes of 2,4-D and hapten of 2,4-D immobilized on glassy carbon electrode (GCE) compete for 2,4-D antibodies labeled by horseradish peroxidase (HRP-anti-2,4-D). After complete immunoreaction, the GCE attached HRP-anti-2,4-D tracers was transferred to a substrate solution containing hydroquinone (QH2) and hydrogen peroxide for square wave voltammetry (SWV) detection. Determination conditions were optimized. The SWV anodic peak current decreased linearly with the increase of 2,4-D concentration over the range from 0.10 to 15.0 mg/L 2,4-D with a detection limit of 0.01mg/L. The performance of this electrochemical immunoassay was successfully evaluated with river water spiked with 2,4-D, indicating that this convenient, rapid, and sensitive technique offers great prospect for the monitoring of trace 2,4-D in environment.

Keywords: Electrochemical Immunosensor; 3-aminopropyltrimethoxysilane; 2,4-Dichlorophenoxyacetic acid

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is a common environmental hormone that is widely used in agriculture as herbicide and plant growth regulator. Its residues in agricultural products, water and soil could cause various abnormal phenomena in endocrine, immune and nervous systems, even produce sexual dysfunction [1]. The effects of environmental hormone pollutants on environment and disturbance of endocrine have attracted considerable attention in recent years.

The World Health Organization (WHO) has proposed a provisional guideline value of 0.03mg/L 2,4-D in drinking water [2]. 2,4-D is commonly detected by gas chromatography (GC) [3], high performance liquid chromatography (HPLC) [4,5] or high performance liquid chromatography-mass spectrometry technology (HPLC-MS) [6]. These techniques are very sensitive and reliable, but have disadvantages, such as consumed long analysis time and trivial sample handling. Compounds containing a carboxylic group such as 2,4-D must usually be derivatized for gas chromatography because they are thermally unstable and lack volatility. In addition, these methods need expensive instrument, have to be performed by a highly trained technician, and are not suitable for rapid analyses in site. Therefore, to develop sensitive, rapid, simple and cheap detection method for 2,4-D is of considerable interest. Electrochemical immunosensor is an important technique for the detection of toxic pollutants in the environment due to its specific immunity, high sensitivity, short analysis time and minimal sample pretreatment when compared with classical instrumental methods.

There are some reports about immunoassay for 2,4-D, such as electrochemical impedance [7], surface plasmon resonance [8,9], and quartz crystal microbalance [10,11], enzyme-linked immunosorbent assay

(ELISA) [12] etc. A few labeled immunoassays are usually performed by indirect methods to detect the signal of tracer connected covalently to antigen [13-15]. In recent year, some determination methods of 2,4-D based on molecular imprinted polymers for 2,4-D are also reported [16, 17].

In this research, we describe a method for generating amino groups on the surface of a GCE using simple one-step aqueous silanization method. An immunosensor for detecting 2,4-D in water was fabricated, which was based on the covalently immobilizing 2,4-D on the surface of a 3-aminopropyltriethoxysilane modified glassy carbon electrode. The indirect competitive immunoassay format was adopted. The analyte of 2,4-D and the hapten of 2,4-D immobilized on glassy carbon electrode (GCE) compete for 2,4-D antibodies labeled by horseradish peroxidase (HRP-anti-2,4-D). After a complete immunoadsorption, the GCE attached HRP-anti-2,4-D tracers was transferred to a substrate solution containing hydroquinone (QH_2) and hydrogen peroxide for electrochemical detection. Square wave voltammetry (SWV) was adopted to determine 2,4-D. The proposed immunosensor possesses the properties of fast and sensitive, etc.

2. Experimental

2.1. Reagents

3-Aminopropyltriethoxysilane (APS, 98%), 2,4-dichlorophenoxyacetic acid, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) were purchased from J&K Scientific Ltd., China. Stock solutions of 5.00g/L 2,4-D were prepared in ethanol and kept at ~ 4 ℃ for use. 2,4-D antibodies labelled by horseradish peroxidase was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. Stock solutions of 0.10g/L HRP-anti-2,4-D were prepared by dissolving a certain amount of HRP-anti-2,4-D in 0.01mol/L pH7.0 phosphate buffer solution (PBS) and kept at -20 ℃ for use. N-hydroxysuccinimide (NHS) was obtained from Sinopharm Chemical Reagent Beijing Corporation. All other reagents were analytical regent grade and all solutions were prepared with double distilled water.

2.2. Apparatus

Electrochemical experiments were performed on Model CHI 842b Electrochemical Analyzer (Chen Hua Instrumental Corporation, Shanghai, China) with a conventional three-electrode system. Glassy carbon electrodes modified by different methods were used as working electrodes, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode. X-ray photoelectron spectroscopy (XPS) analysis was carried out on Quantera SXM Scanning X-ray Microprobe (ULVAC-PHI INC).

2.3. Preparation of immunosensors

The preparation process of the immunosensor was shown in Fig.1. The silanization of GCE was carried out according to the method reported in the literature [18] with a slight modification. A GCE was polished to a mirror with 0.5 μm alumina slurries. After successive sonication in ethanol and double distilled water, the electrode was activated by repeatedly linear potential scan from 0.0 to +2.0 V (vs. SCE) in 0.2 M H_2SO_4 for 7 times in order to obtain hydroxyl group on the electrode surface, rinsing with distilled water and drying by nitrogen gas fluid. The activated electrode was put in 5% APS solution, kept in 37℃ to incubate for 15 min. A polymer film with $-NH_2$ was modified on the activated glassy carbon electrode by C-O-Si bonds, rinsing with distilled water and drying by nitrogen gas fluid. The obtained electrode was denoted as APS/GCE.

EDC (2.0mg) and NHS (3.0mg) were added in 5.00mL 0.5000g/L 2,4-D solution and the mixture was stirred for 15min at room temperature in order to activate 2,4-D. APS/GCE was immersed in the activated 2,4-D solution in 37℃ to incubate for 10 min. Hapten of 2,4-D was formed on the surface of the electrode by the reaction between the amino-group of APS and the carboxyl group of 2,4-D. The electrode was denoted as 2,4-D/APS/GCE. After 2,4-D/APS/GCE was rinsed with distilled water and was dried with nitrogen gas fluid, the electrode was immersed in the HRP-anti-2,4-D solution or the mixture solution of analyte 2,4-D and HRP-anti-2,4-D, kept in 37℃ to incubate for 30 min, the resulted electrode was denoted as HRP-anti-2,4-D/2,4-D/APS/GCE (Fig. 1).

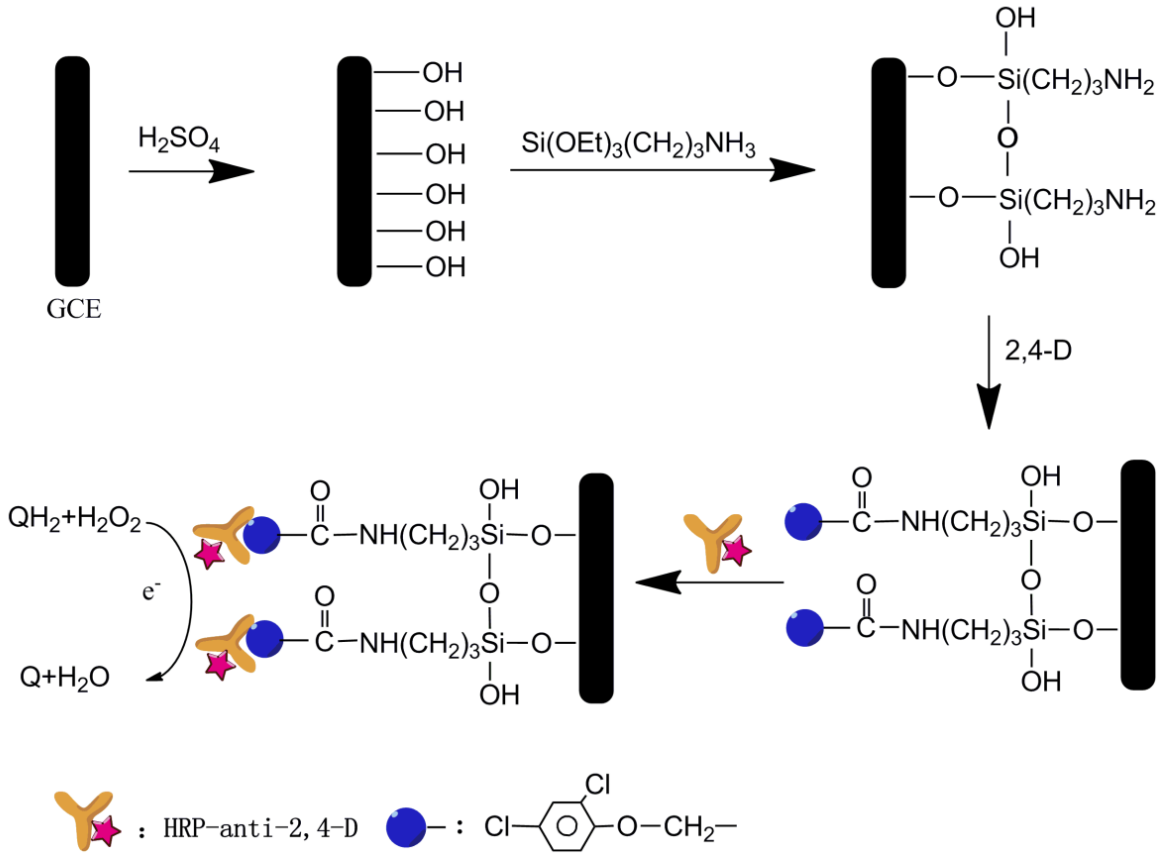

Fig. 1 Illustration of the modification process

2.4. Analytical procedures

A 5.0 mL aliquot of 0.01 M pH 7.0 phosphate buffer solution was placed in a voltammetric cell, and the required volumes of QH_2 and H_2O_2 solutions were added. A potential scan of cyclic voltammetry was performed from -1.0V to 1.0V (vs.SCE) at scan rate of 100mV/s, or a potential scan of square wave voltammetry from -0.4V to 0.6V (vs.SCE) with a frequency of 25Hz and an amplitude of 0.02V.

Quantitative detection of 2,4-D was carried out by competitive immunoassay. The hapten modified on the electrode competes with analyte to combine HRP-anti-2,4-D due to the special recognition between antigen and antibody. The response of anodic peak current of QH_2 at the HRP-anti-2,4-D modified electrode increased with the decrease of 2,4-D concentration at a certain concentration range. The concentration of 2,4-D was determined through the electrooxidation of QH_2 catalyzed by HRP.

3. Results and discussion

3.1. XPS characterization of 2,4-D/APS/GCE

The differences in the elemental composition of electrodes surface were characterized by XPS. Compared with the situation on bare GCE (Fig. 2A), the peak intensity of oxygen element on the surface of activated GCE increased obviously, i.e. atomic mole percentages of O/C on the electrode surface increased from 13.31% to 24.78% (Fig. 2B). It demonstrated that hydroxyl was produced on the surface of the electrode successfully by electrochemical activation. After the electrode was incubated in APS solution for 15 min, N element and Si element appeared in the image of XPS (Fig. 2C). After the APS/GCE was immersed in activated 2,4-D solution in 37°C to incubate for 10 min, the chlorine element was detected on the 2,4-D/APS/GCE (Fig. 2D). The experimental results confirmed that 2,4-D was linked on the electrode surface by covalent bond to form the hapten of 2,4-D.

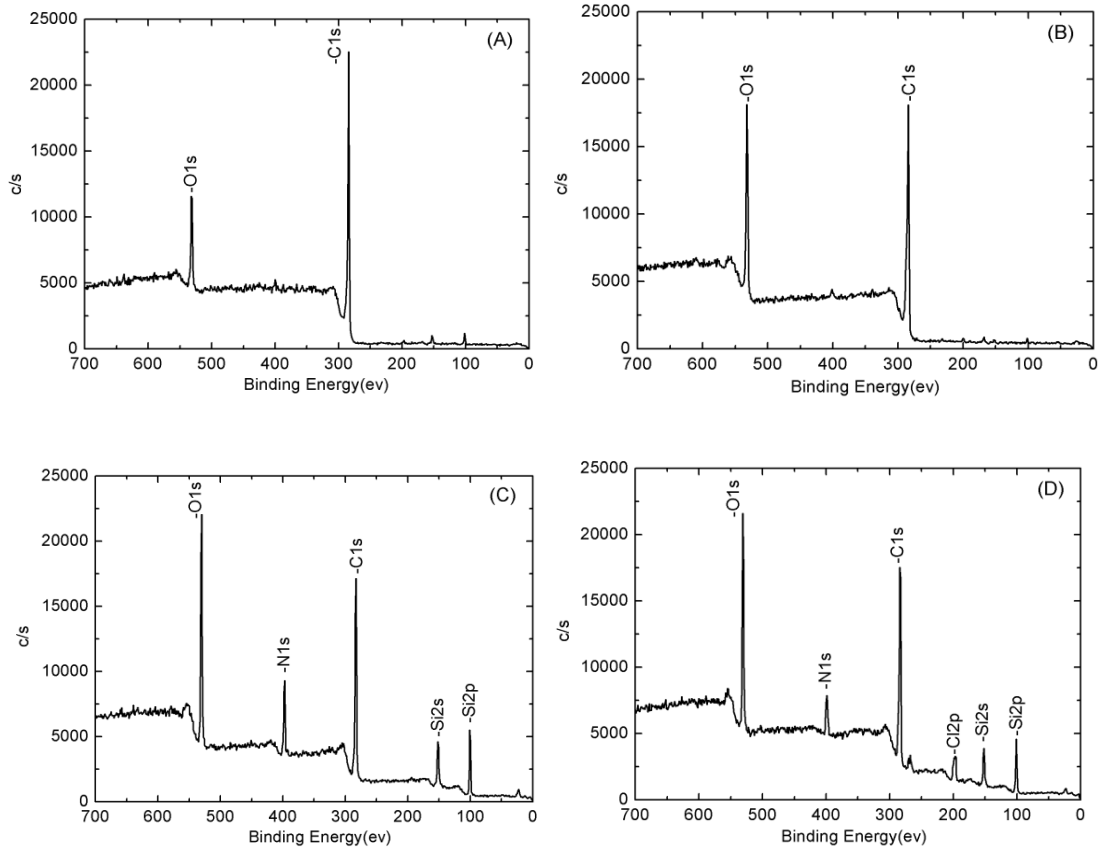

Fig. 2 XPS spectra of bare GCE(A), activated GCE(B), APS/GCE(C) and 2,4-D/APS/GCE(D)

3.2. Electrochemical behavior of HRP-anti-2,4-D/2,4-D/APS/GCE

The electrode of HRP-anti-2,4-D/2,4-D/APS/GCE was fabricated by immersing 2,4-D/APS/GCE in 1.00mg/L HRP-anti-2,4-D solution and incubating for 30 min in a thermostat container at 37℃. As shown in Fig. 3, the cyclic voltammogram of HRP-anti-2,4-D/2,4-D/APS/GCE in 1.6 mM H_2O_2 - 1.0 mM QH_2 - 0.01M PBS (pH 7.0) solution displayed a pair of well-defined peaks at 0.249V and 0.308V, which were caused by the redox reaction of HRP and QH_2. The reactions that occurred on the surface of the immunosensor were as follows [19]:

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow HRP(I) + H_2O \quad (1)$$

$$HRP(I) + QH_2 \rightarrow HRP(II) + Q \quad (2)$$

$$HRP(II) + QH_2 \rightarrow HRP(Fe^{3+}) + Q + H_2O \quad (3)$$

$$Q + 2e \rightarrow QH_2 \quad (4)$$

HRP(I) and HRP(II) represent +5 valence and + 4 valence oxidation state of HRP, respectively, which are intermediate products during the enzymatic catalytic reaction and can oxidize QH_2 into its oxidation state benzoquinone (Q).

In this paper, the anodic peak current of hydroquinone was recorded by square wave voltammetry in order to investigate the quantity of HRP-anti-2,4-D combined on the electrode. In a certain concentration range, the more the analyte concentration is, the more the HRP-anti-2,4-D combined with analyte, and then the less the HRP-anti-2,4-D combined with hapten modified on the electrode. The anodic peak current of hydroquinone will therefore decrease with the increase of 2,4-D concentration in a certain 2,4-D concentration range and 2,4-D in solution thereof can be determined. Fig. 4 showed square wave

voltammograms of HRP-anti-2,4-D/2,4-D/APS/GCE in the presence (curve a) and absence (curve b) of H_2O_2 and QH_2.

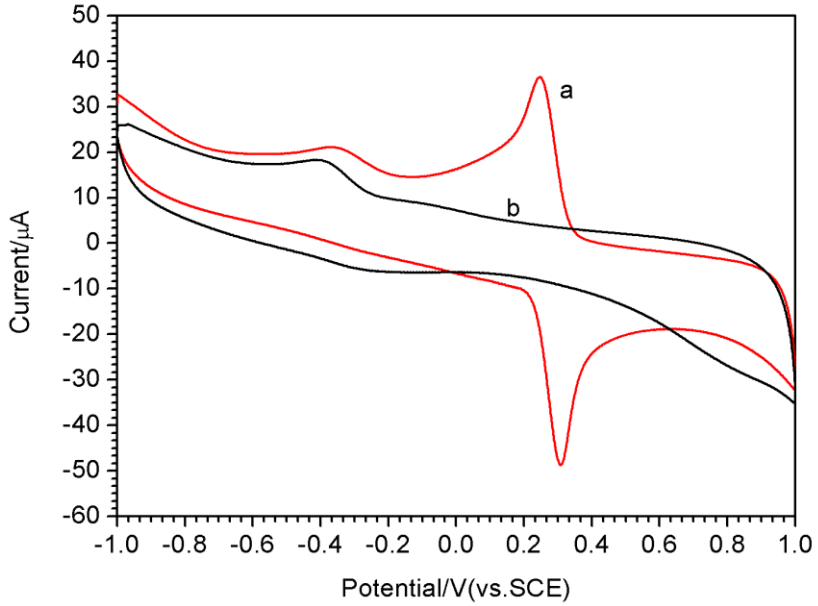

Fig.3 Cyclic voltammograms of HRP-anti-2,4-D/2,4-D/APS/GCE in (a) 0.01M PBS(pH 7.0) containing 1.0mM QH_2 and 1.6mM H_2O_2, (b) 0.01M PBS(pH 7.0)
scan rates: 100 mV/s

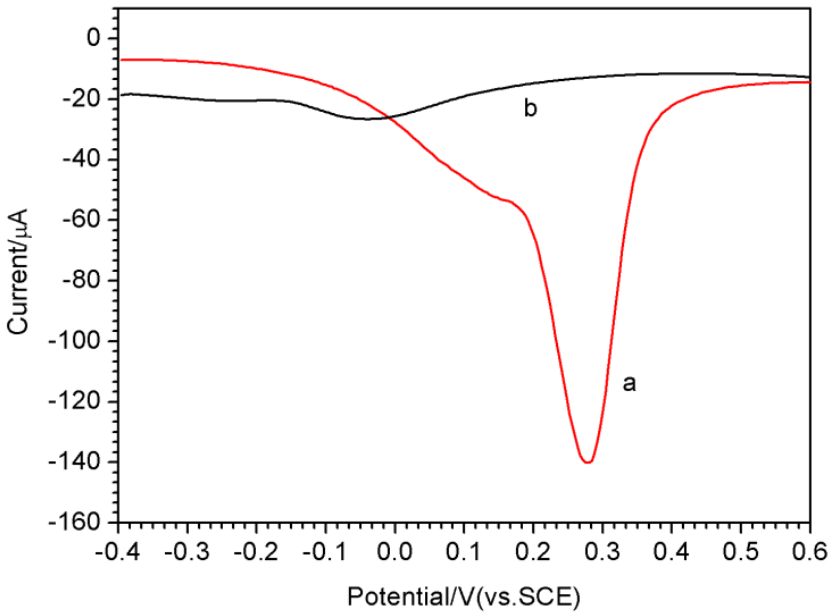

Fig.4 Square-wave anodic voltammograms of HRP-anti-2,4-D/2,4-D/APS/GCE in (a) 0.01M PBS(pH 7.0) containing 1.0mM QH_2 and 1.6mM H_2O_2, (b) 0.01M PBS(pH 7.0)

3.3. Optimization of analytical conditions

The effect of H_2O_2 concentration on the response of HRP-anti-2,4-D/2,4-D/APS/GCE to 1.0mM QH_2 was examined. It can be seen from Fig.5(A) that the anodic peak current value increased with the increase of H_2O_2 concentration up to 1.6mM H_2O_2 in the absence of analyte. After then the peak current value decreased with the increase of H_2O_2 concentration. As shown in Fig. 5(B), the anodic peak current attained to a

maximum value at 1.0mM QH_2. The supporting electrolyte solution of 0.1 M PBS (pH 7.0) containing 1.6 mM H_2O_2 and 1.0 mM QH_2 was therefore used as in the subsequent experiments.

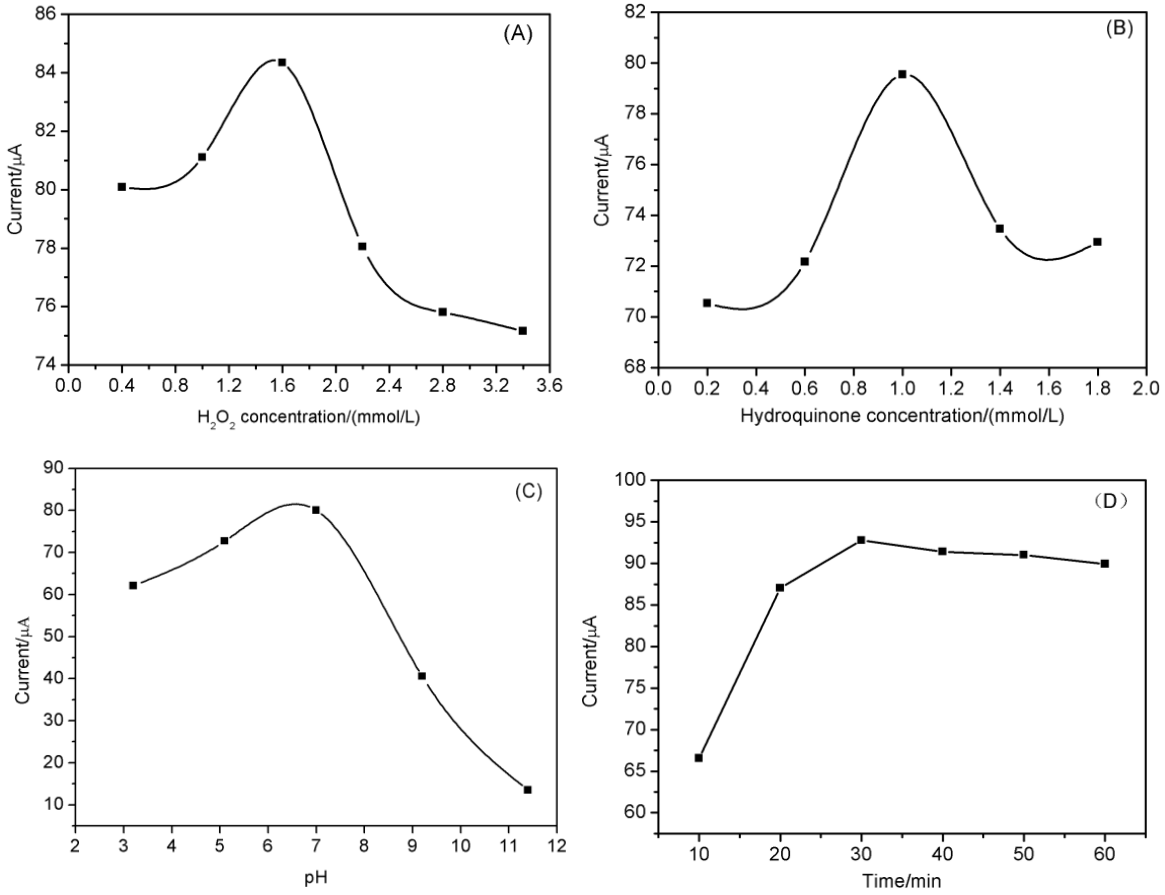

Fig. 5 Effects of H_2O_2 concentration (A), hydroquinone concentration (B), pH (C), and incubation time (D) on the anodic peak current

The effect of pH on the oxidation peak current was investigated using a HRP-anti-2,4-D/2,4-D/APS/GCE electrode in 0.1 M pH 7.0 PBS containing 1.6 mM H_2O_2 and 1.0 mM QH_2 over the pH range from 3.2 to 11.4. As shown in Fig. 5(C), the anodic peak current attained to a maximum value at approximate pH 7.0. In the range of pH 4.5 to 7.2, antigen can combine with antibody steadily. It is more beneficial to conserve the activity of HRP-anti-2,4-D and in neutral solution. Therefore, 0.1 M PBS (pH 7.0) was selected as the supporting electrolyte solution in the subsequent experiments.

The effect of incubation time on response current was examined in this paper. The 2,4-D/APS/GCE electrode was immersed in 1.0 mg/L HRP-anti-2,4-D solution and was incubated in a thermostat container at 37 ℃ for a certain time. Then SWV measurement in 0.1 M PBS (pH 7.0) containing 1.6 mM H_2O_2 and 1.0 mM QH_2 was performed. The anodic peak current versus incubating time is shown in Fig. 5(D). The peak current value increased with incubation time up to 30 min and afterwards the peak current values decreased slightly with incubation time. Thus, the incubation time of 30 min was chosen for 2,4-D determination.

3.4. Determination of 2,4-D

Under these optimized conditions, an inmunosensor for 2,4-D detection was fabricated. The electrode of 2,4-D/APS/GCE was immersed 1.00mg/L HRP-anti-2,4-D solution containing different concentration analyte and was incubated for 30 min in a thermostat container at 37℃. Peak current of anodic square wave voltammetry was measured using the immunosensors. As shown in Fig. 6, in the range of 0.10~15.0 mg/L 2,4-D, the peak current decreased linearly with the increase of 2,4-D concentration and the linear regression equation was i_p (μA) = -1.267c (μg/mL) + 75.19 with a correlation coefficient of r = 0.9973. The detection

limit was 0.01mg/L (S/N=3) and the detection limit is lower than 0.03mg/L which is the provisional guideline value of 2,4-D in drinking proposed by the World Health Organization (WHO) [2].

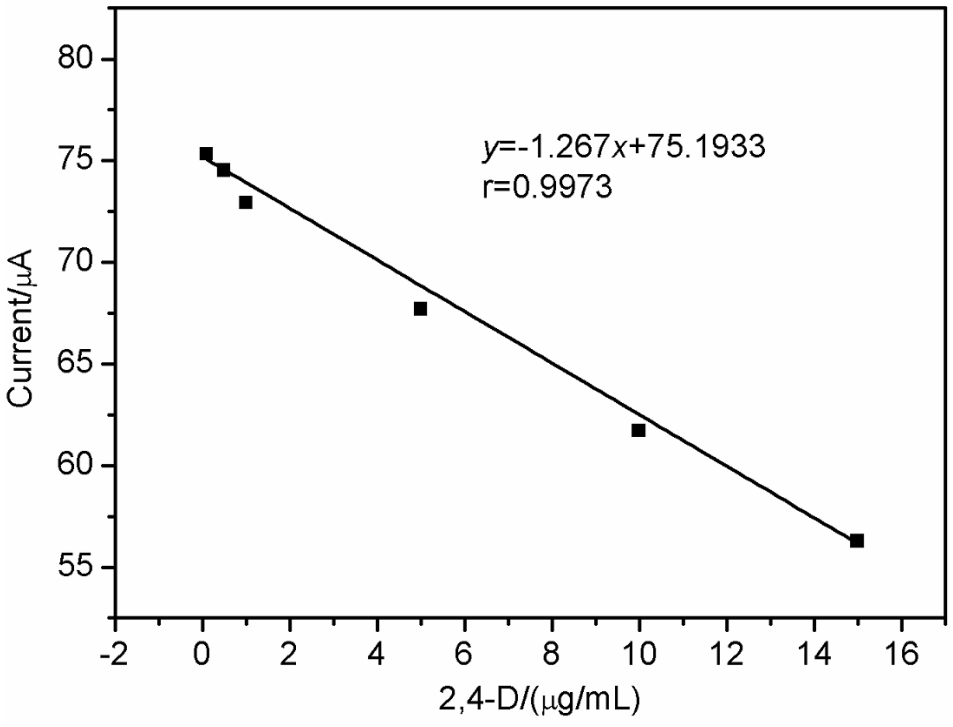

Fig.6 The calibration curve of 2,4-D determination by SWV

3.5. Comparison of the biosensor for determination of 2,4-D with others

The analytical range and detection limit values of the biosensors fabricated by us are better than many methods reported in recent publications. The immunosensors is easy to prepare than other some sensors fabricated with the materials such as quartz crystal microbalance[11] and TiO_2 nanotube [20]. The immunosensor also exhibited lower detection limit than that reported in references [11, 21, 22]. Moreover, the method can be applied to the determination of 2,4-D in real water samples.

3.6. Specificity of immunosensor and applications

Interference studies were carried out with organic species. No interference could be observed for the following materials: benzoic acid (190), 4-chlorophenoxyacetic acid (12), phthalic acid (14), nitrobenzene (9), where the data in brackets denote the molar ratio of the interfering compound to 3.5×10^{-7} M 2, 4-D. Interferents such as 2,4-D structural analogues had no remarkable effect on 2,4-D determination, illustrating that immunosensor has high specificity to 2,4-D.

To evaluate the feasibility of the prepared immunosensor for analytical application, the immunosensor was used to determine 2,4-D in the real samples which were obtained from Beijing Tonghui river. After the water samples were filtered with a 0.45-μm filter membrane to eliminate particulate matters, the determination was proceeded. No SWV response corresponding to 2,4-D was observed when the real water samples were determined. Thus different quality of 2,4-D was added to the samples, respectively. Standard-addition methods was adopted to estimate the accuracy of the method, and the results were presented in Table 1. It can be seen that the results were satisfactory.

Table 1 Determination of 2,4-D in real samples(n=5)

Sample	Added (mg/L)	Found (mg/L)	Mean recovery (%) ± S.D. (n=5)

1	1.000	1.040	104.0±4.5
2	5.000	4.820	96.4±5.7
3	10.00	9.870	98.7±3.2

4. Conclusions

In this paper, indirect competitive immunoassay format was adopted for 2,4-D determination. The immunosensor appears good biocompatibility, selectivity and stability. The proposed method possesses the properties of fast, and sensitive, simple, etc. In addition, the standard addition recovery of real water samples also indicated that the immunosensor possesses good application prospects in environmental monitoring.

5. Acknowledgments

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