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Label-free impedimetric immunosensor for sensitive detection of 2,4-dichlorophenoxybutyric acid (2,4-DB) in soybean

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ABSTRACT

Electrochemical impedance immunosensor, with its high sensitivity from electrochemical impedance analysis and ideal specificity from the immunoassay, is increasingly used in the detection of a kind of phenoxy acid herbicides which is 2,4-Dichlorophenoxybutyric acid (2,4-DB). In this experiment, synthetic 2,4-DB antibodies were immobilized on the electrode by the crosslinking of L-Cysteine/glutaraldehyde, and 2,4-DB were measured by the increase of electron-transfer resistance when the immune reaction occurred, with $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ as the probe. Under optimal conditions, the change of resistance is in a linear relationship with the logarithm of the concentration in the range of 1.0×10^{-7}–1.0×10^{-3} g/L (R=0.994) with the detection limit of 1.0×10^{-7} g/L (0.1 ppb). This method bears such merits as simplicity in operation, high sensitivity, wide linear range, specificity, reproducibility and good stability. The actual soybean samples were analyzed with the recovery of 82.8%–102.3%.

1. Introduction

The chemical name of 2,4-DB is 2,4-dichlorophenoxybutyric acid which is an important species of the phenoxy acid herbicides [1] with good suction and ability to spread out. The structure is shown in Fig. 1. As for their control of broad leaf weeds in soybean fields, 2,4-DB and other phenoxy acid herbicides has been used for more than 60 years, and they still hold a large share in the market, which is the second largest category of selective herbicidal agent and the largest category of broad-leaved weed herbicide in the world [2]. This kind of herbicide can be widely used in rice, corn, soybeans, wheat and other crops [3]. Extensive use of 2,4-DB not only poses harm human bodies, but also causes serious environmental pollution [4]. 2,4-DB would cause long-term toxicity when in the water and soil [5], tissue damage for human when inhaled even a small amount [6] and harm to placenta for animals [7].

Different countries have different MRL which of 2,4-DB in Australia and the United States are respectively 0.02 and 0.2 mg/kg [8]. Organizations in many countries and world authority promulgated laws and technical standards to control the phenoxy acid pesticides residues. According to EU Directive 86/362/EEC (cereals), the MRL of phenoxy acid pesticides in rice for human consumption is 0.050 mg/kg [9]. The latest version of Oeko-Tex Standard 100 [10] set the total amount of pesticide residues and the products for babies to be less than 1.0 and 0.5 mg/kg respectively. And such provisions are also applied in China [11]. Internationally, the test for 2,4-DB focuses mostly on its concentration in the soil and water and other environmental samples [12–15] while there are few reports on the test of soybean and other cereals [16].

Most of the reported analysis techniques of 2,4-DB are conducted on chromatography [17–23], immunoassay [24–26] and so on. Generally, before tested by GC, 2,4-DB should be derived [27,28], and more reported HPLC [29,30], frequently takes liquid–liquid extraction [31], and requires a lot of organic solvents [32,33]. GC–MS has the merits of lower detection limits, improved sensitivity and suitable for quantitative analysis. However, 2,4-DB derivation must be carried out under dry conditions since 2,4-DB is not volatile, which easily leads to the increase in baseline noise and the detection limit. Since LC–MS is easy to produce matrix effects, the accuracy and precision of the method are not high [34]. Additionally, the above chromatography methods are of time-consuming, high cost, complex operation and cannot meet the requirements of on-site rapid test.

Electrochemical impedance spectroscopy (EIS) with immunosensors has been increasingly applied to the detection of viruses, bacteria and pesticide residues of small molecules in recent years [35]. Comparing with enzyme-linked immunosorbent assay

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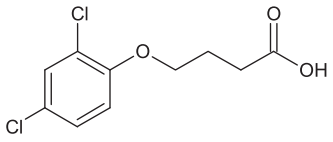

Fig. 1. The structural formula of 2,4-DB.

(ELISA), free-label impedance immunosensors have attracted the attention of many researchers. Khan and Dhayal [35] reported a detection of Ochratoxin-A with impedance immunosensor. Navrátilová and Skladal [36] immobilized antibody on gold electrode and detected 2,4-Dichlorophenoxyacetic acid by EIS. These tests indicated that electrochemical impedance spectroscopy has such advantages as high accuracy, good reproducibility, high sensitivity and selectivity, rapidity and convenience [37–40]. But the determination of 2,4-DB in soybean with this method has not been reported.

In this paper, synthetic 2,4-DB specific antibody was immobilized by the crosslinking of L-Cysteine/glutaraldehyde and determined the 2,4-DB residue with electrochemical impedance spectroscopy measurement with linear range of 1.0×10^{-7}–1.0×10^{-3} g/L and the detection limit of 1.0×10^{-7} g/L (0.1 ppb). A sensitive, accurate and simple electrochemical method was established to detect 2,4-DB residues. The method has the merits of simplicity, convenience, high sensitivity, wide linear range, specificity, reproducibility, stability and comparatively cheap equipments, which is less demanding for the expertise and greatly simplifies the pretreatment of samples or even eliminates it.

2. Experimental

2.1. Chemicals

2,4-DB antibodies (Ab) and 2,4-DB antigens(Ag) were provided by the panel of Prof. Shuzhao Liu from College of Environmental Science and Engineering in Yangzhou University. Gelatin (Gel) was from Sino-American Biotechnology Company (Shanghai, PR China). L-Cysteine (L-Cys) and glutaraldehyde (GA) were purchased from Shanghai Chemical Reagent Company. PBS is 0.02 M phosphate buffer solution and unless otherwise indicated the pH is 7.0. All chemicals were used as received without further purification. The water used throughout the experiment was purified through a Millipore system.

2.2. Apparatus

An Autolab Electrochemical Analyzer (Ecochemie, Netherlands) equipped with a one-compartment three-electrode cell was employed to conduct the electrochemical impedance spectroscopic experiments with a chemically modified Au electrode as working electrode, a saturated calomel reference electrode (SCE) as reference electrode and a platinum wire electrode as counter electrode. The frequency range of 10^{-1}–10^{6} Hz was applied to electrochemical impedance spectra. An open circuit potential and a single modulated AC potential of 10 mV were adopted for impedance measurement with 5.0 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl solution as the test solution. The surface morphology and roughness was examined by atomic force microscopy (AFM), using a Nasnoscope III device (Veeco, USA).

2.3. Fabrication of immunosensor

2.3.1. Pretreatment of the gold electrode

Polish the gold electrode (Au, $\Phi=3$ mm) with a finer piece of sandpaper and subsequently with Al_2O_3 powder with the diameter of 0.3 and 0.5 mm on the silk and rinse it with redistilled water. And ultrasonicate it with doubly-distilled water, ethanol (1:1, V/V), and HNO_3 (1:1, V/V), followed by the cyclic voltammetry experiment in 0.5 M H_2SO_4 over the potential range from -0.5 to $+1.4$ V at the scan rate of 100 mV/s until the reproducible background was yielded. Then the electrode was dried with a nitrogen flow.

2.3.2. Self-assembly of L-cysteine onto gold electrode (denoted as L-Cys/Au)

After pretreatment, the clean gold electrode was subsequently soaked in 2.0 mM L-Cys solution (containing 0.08 M hydrochloric acid) at 4 °C for 24 h, followed by rinsing with ethanol and doubly-distilled water to remove the physically absorbed L-Cys. There is a mercapto group in the L-cysteine molecule which can combine with the gold through strong Au–S covalent bond. Finally the electrode was dried in nitrogen atmosphere.

2.3.3. Self-assembly of glutaraldehyde onto L-Cys/Au

The L-Cys modified electrode was immersed into 5% glutaraldehyde solution at 4 °C for 4 h (stored in a shady place). Afterwards the remaining liquid was washed out with doubly-distilled water and ethyl alcohol.

2.3.4. Immobilization of antibody protein on GA/L-Cys/Au

The modified electrode was incubated in 0.2 mL PBS (pH 7.0) with 20 μg/mL monoclonal antibody at 4 °C for 12 h and then rinsed with PBS to remove unbound antibody. Then the as-prepared electrode (denoted as Ab/GA/L-Cys/Au) was immersed in 5 mg/mL BSA solution at 37.0 °C for 1 h to block the non-specific sites (denoted as BSA/Ab/GA/L-Cys/Au). To remove the redundant amount of BSA on the surface of BSA/Ab/GA/L-Cys/Au electrode, redistilled water was used to rinse the electrode surface. The electrode obtained was stored in PBS at 4 °C.

2.4. Measurement procedure

The antibody-coupled electrode was immersed in PBS solution with different concentration of 2,4-DB antigen for 180 min at 37.0 °C before each measurement. The electrode obtained was rinsed with redistilled water and PBS solution respectively. The relative change in impedance of the immunosensor was measured in 0.1 M KCl in 5.0 mM $K_3[Fe(CN)]_6/K_4[Fe(CN)_6]$ with the AC impedance method.

2.5. Preparation for real samples

Soybeans were ground into powder and separated by means of quartering with the each amount as 10 g. Put them respectively into 50 mL tubes with a plug and added 2,4-DB standard sample at three levels with 0.001, 0.1 and 10 μg (2,4-DB standard samples taken were 10 μg/mL 1 mL, 1 μg/mL 0.1 mL and 0.1 μg/mL 0.01 mL) and made a blank solution (without 2,4-DB standard samples). Then 30 mL petroleum ether was added and oscillated for 20 min and centrifuged at 6000 rpm for 8 min. The petroleum ether layer was discarded and this operation was repeated once. 30 mL acetonitrile and 13 mL 50 mM HCl were added, and then oscillated it for 20 min, centrifuged, and the upper layer of the solution was collected. These steps were repeated again. The solutions were merged together and transferred to a flask to remove acetonitrile by vacuum rotary evaporator to nearly dry. The sample obtained was dissolved with 1 mL methanol and diluted with PBS to make the concentration 1/10 of the original value.

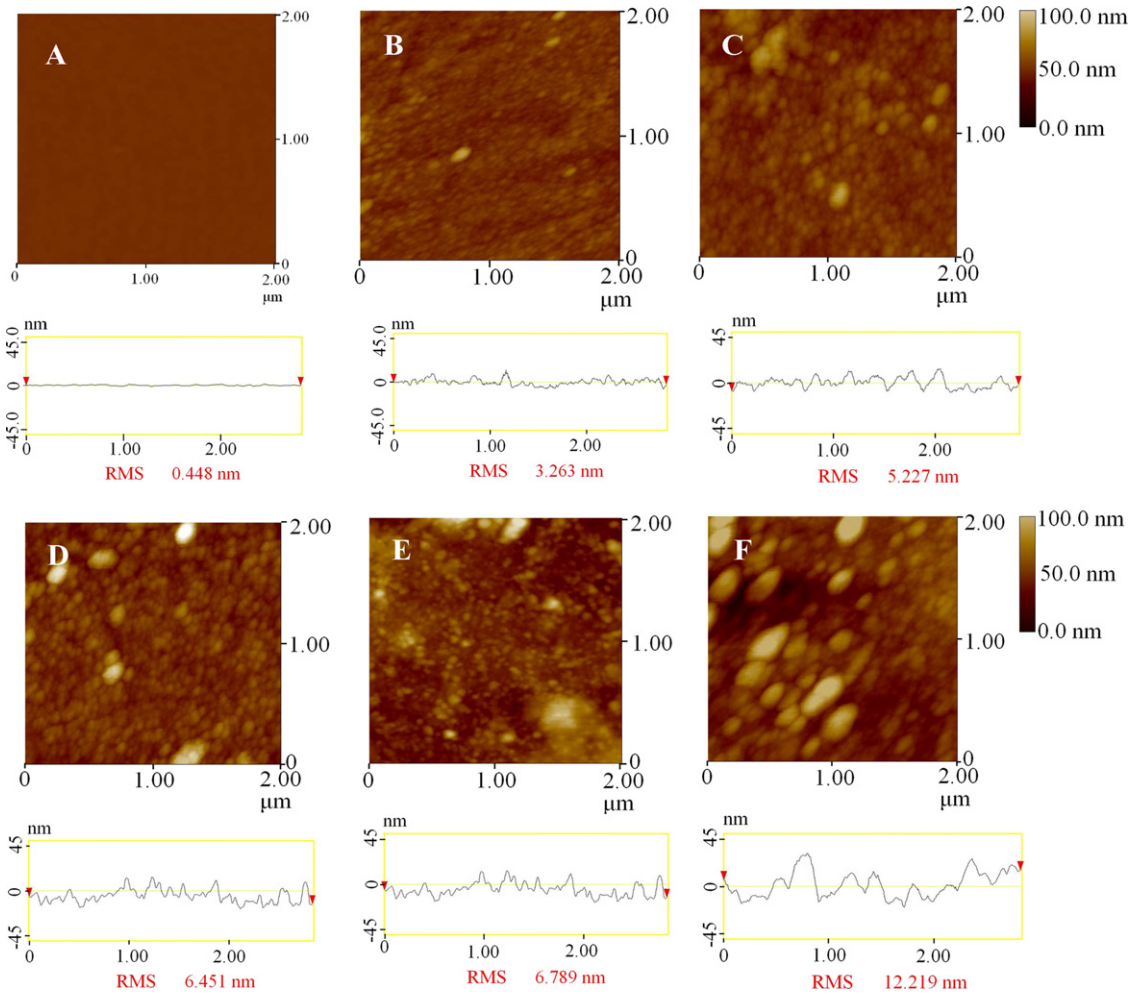

Fig. 2. AFM images (A) bare Au electrode, (B) L-Cys/Au, (C) GA/L-Cys/Au, (D) Ab/GA/L-Cys/Au, (E) BSA/Ab/GA/L-Cys/Au, and (F) 2,4-DB/BSA/Ab/GA/L-Cys/Au. The concentration of 2,4-DB was 1.0×10^{-5} g/L.

3. Results and discussion

3.1. AFM and EIS characterization of the immunosensor fabrication

Soak the conductive glass sprayed with gold, instead of gold electrode, in the L-Cysteine hydrochloride solution (containing 0.08 M hydrochloric acid), 5% glutaraldehyde solution and 2,4-DB antibody in succession for 24, 4 and 12 h at 4 °C, respectively. Fig. 2(A–F) show the AFM images observed in the modification process. Fig. 2(A) indicates that the bare electrode is smooth and without obvious protrusions. Fig. 2(B–D) are the images of the electrode modified with L-Cysteine, glutaraldehyde and 2,4-DB antibody in sequence, which show that the amount of the substance on the surface raised with the roughness increased from 0.448 to 6.451 nm. The above results prove that 2,4-DB antibody can be immobilized on the gold electrode by the cross-linking of L-Cysteine and glutaraldehyde. Fig. 2(E–F) are the images of the obtained electrode blocked by BSA and sequentially immunoreacted at 37 °C, which show more undulation of Fig. 2(F) than that in Fig. 2(E) with the roughness raised from 6.789 to 12.219 nm, indicating that 2,4-DB antigens were successfully combined on the surface of BSA/Ab/GA/L-Cys/Au.

Electrochemical impedance spectra (EIS), as is well known, could be applied to the characterization of the assembly of the immunosensor by investigating the interface properties of chemically modified electrode surface. The typical EIS is presented in the form of Nyquist plot which consists of a semicircle structure and a linear part. The semicircle structure at higher frequencies is in accordance with the electron-transfer limited procedure, while its diameter matches the electron-transfer resistance (R_{ct}) which dominates the electron-transfer kinetics of the redox sensor on the electrode interface. Fig. 3(A) shows the EIS observed upon the modification process and the reaction process between 2,4-DB antigen–antibody. On the bare gold electrode (curve *a*), a straight line could be observed, indicating a low transfer resistance and the reaction mainly controlled by the diffusion. After L-Cys self-assembly, the diameter of semicircle was increased (curve *b*). This can be accounted for by the formation of L-Cys monolayer on the gold electrode, implying that the film of L-Cys obstructed the electron-transfer in the electrochemical reaction. Similarly, the self-assembled glutaraldehyde monolayer on the surface of L-Cys/Au revealed that comparing with L-Cys modified gold electrode R_{ct} (curve *b*), R_{ct} (curve *c*) has slightly increased.

Subsequently, the adsorption of 2,4-DB antibodies via self-assembly of the L-Cysteine and GA made the diameter increase (curve *d*), demonstrating that 2,4-DB antibodies had been successfully immobilized on the surface of modified electrode (denoted as Ab/GA/L-Cys/Au), which is due to the molecular structure of antibodies hindered the electron-transfer, it also

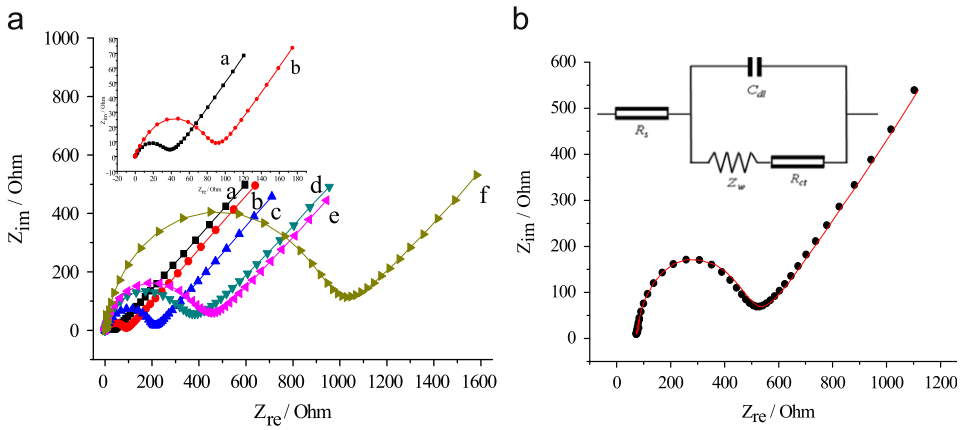

Fig. 3. (A) Nyquist plot (Z_{im} vs. Z_{re}) for Faradaic impedance measurements in the presence of 0.02 M phosphate buffer solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KNO_3 at: (a) bare Au electrode, (b) L-Cys/Au, (c) GA/L-Cys/Au, (d) Ab/GA/L-Cys/Au, (e) BSA/Ab/GA/L-Cys/Au, and (f) 2,4-DB/BSA/Ab/GA/L-Cys/Au. The frequency range is from 10^{-1} to 10^6 Hz with a signal amplitude of 10 mV. (B) Fitted (solid line) and experimental (scattered line) Nyquist plots of impedance spectra. The inset is the equivalent circuit applied to fitted the impedance spectra in the presence of the redox probe of $Fe(CN)_6^{3-/4-}$.

Table 1
Electrochemical impedance results for stepwise assembled electrodes obtained from Fig. 3A.

Electrode	R_{ct} (Ω) (SD)	R_s (Ω)	n	Q (F)	Z_w
Bare Au electrode	37.9(0.11)	139.2	0.8701	2.572×10^{-7}	2.656×10^{-4}
L-Cys/Au	93.6(2.98)	144.1	0.8932	2.389×10^{-7}	2.249×10^{-4}
GA/L-Cys/Au	214.2(3.54)	140.2	0.8973	2.739×10^{-7}	2.341×10^{-4}
Ab/GA/L-Cys/Au	383.4(4.07)	139.5	0.8971	2.295×10^{-7}	2.385×10^{-4}
BSA/Ab/GA/L-Cys/Au	444.8(4.51)	141.1	0.9017	2.637×10^{-7}	2.636×10^{-4}
2,4-DB/BSA/Ab/GA/L-Cys/Au	1028.2(5.94)	142.7	0.9012	2.902×10^{-7}	2.789×10^{-4}

proved that antibodies were successfully immobilized on the gold electrode. After Ab/GA/L-Cys/Au was blocked by BSA, R_{ct} increased slightly (denoted BSA/Ab/GA/L-Cys/Au, curve *e*), and R_{ct} also dramatically increased when the immunosensor was used to detect 2,4-DB (denoted as 2,4-DB/BSA/Ab/GA/L-Cys/Au, in curve *f*). The phenomenon was possibly caused by the impeded diffusion of ferricyanide towards the electrode surface brought by the protein layer of the electrode, which acted as the inert electron and mass-transfer blocking layer. The results demonstrated that 2,4-DB antibodies not only can be modified on gold electrode surface through crosslinking of L-Cysteine and glutaraldehyde, but also can produce a characteristic immune response.

A Randles equivalent circuit was obtained according to the impedance data based on FRA software of Autolab (Fig. 3B), which includes the electron-transfer resistance (R_{ct}), the Warburg impedance (Z_w), constant phase element (Q) and the solution resistance (R_s).

Randles parameters of the fitting value of a variety of procedures of the impedimetric immunosensor modification of the electrode were presented in Table 1, which demonstrates that R_{ct} was a suitable signal for sensing the interfacial properties of prepared immunosensor.

The relative change in R_{ct} ($\%\Delta R_{ct}$) is calculated by the following equation:

$$\%\Delta R_{ct} = \frac{R_{ct(Ag-Ab)} - R_{ct(BSA)}}{R_{ct(BSA)}} \times 100 \quad (1)$$

where $R_{ct(Ag-Ab)}$ is the value of the electron-transfer resistance after 2,4-DB antigens coupling with the immobilized antibodies on the prepared electrode, and $R_{ct(BSA)}$ denotes the impedance value after blocking the remaining adsorption-reactive sites with BSA.

The detailed process of preparation of novel impedimetric immunosensor and the interaction between antibody and antigen was shown in Fig. 4. Initially, L-cysteine molecules with a mercapto groups combined with the gold substrate through strong Au–S covalent bond. And then, the glutaraldehyde with one of aldehyde groups, as a coupling agent, combined with above mentioned L-cysteine, and the another aldehyde group could connect with the free amino of 2,4-DB antibody, subsequently. After that, non-specific adsorption sites on the surface of gold were blocked by the bovine serum albumin. The 2,4-DB acts as an antigen role react with the antibody, and the 2,4-DB consequentially was detected according to the electrochemical impedance responses.

3.2. Optimization of the experimental conditions

The interface properties of electrodes could be controlled by the immobilization of proteins on the GA/L-Cys/Au electrode, resulting in a change of R_{ct}. Generally, the more the antigen was absorbed, the more change of R_{ct} would be obtained. The antigen adsorption could be influence by various factors, such as immobilization conditions for antibody protein amount, and the time, temperature and pH of the incubation.

3.2.1. Effect of antibody concentration

The response of the antigen to the 2,4-DB antibody is related to the amount covered. With more antibodies in a given area, the electrode bears more sites which can be combined with antigen. In the preparation of the sensor, great difference in its property shows when the amount of the antibody varies. As can be seen from Fig. 5, the signal increases with the amount of antibody and the maximal signal is obtained when the concentration is

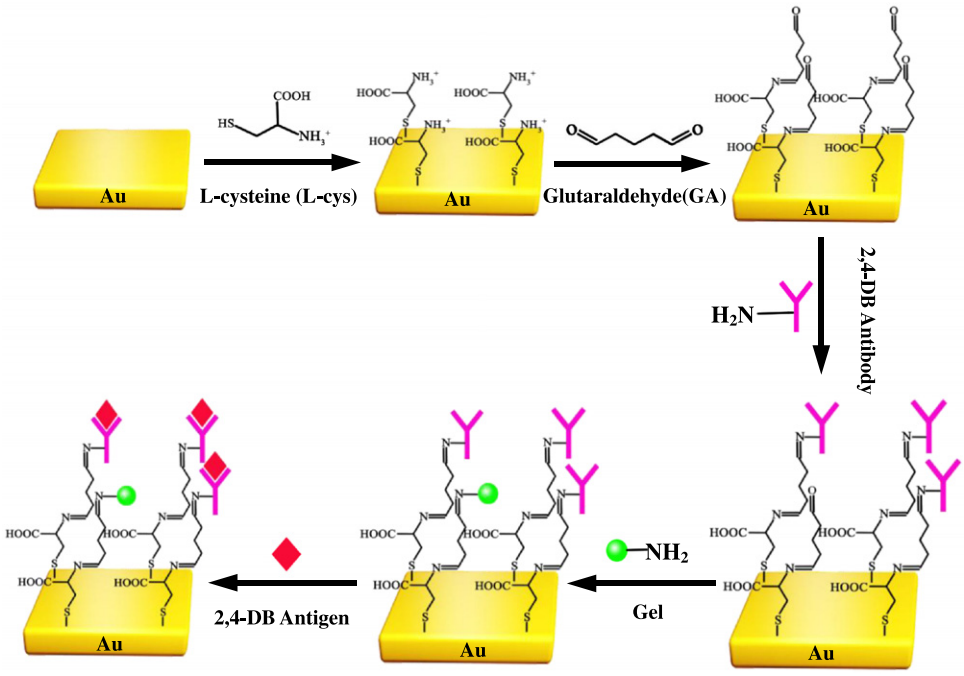

Fig. 4. The schematic illustration of preparation of novel immunosensor and the interaction between antibody and antigen.

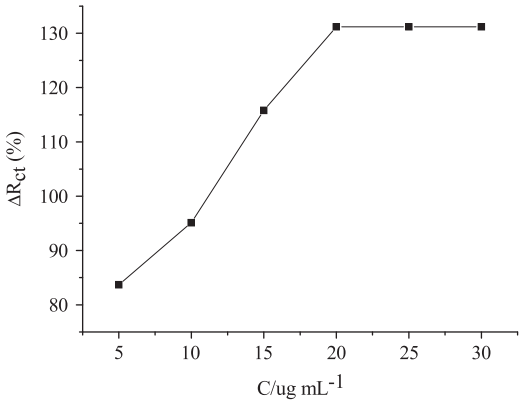

Fig. 5. Effect of the amount of immobilized antibody.

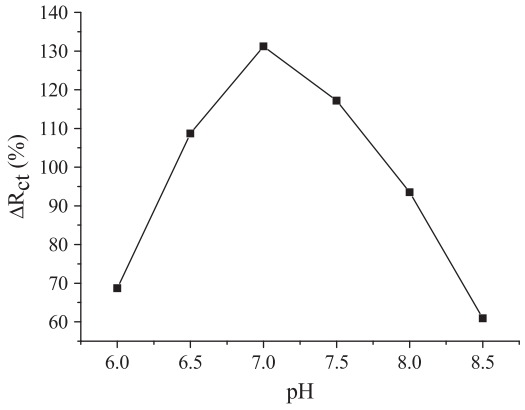

Fig. 6. The influence of incubation pH between antibody and antigen interaction.

20 μg/mL. There is little change even when the concentration continues to rise since the amount of the active sites for immunoreaction is limited. Thus the proper concentration of the antibody is 20 μg/mL.

3.2.2. *Effect of pH of the antibody–antigen reaction*

The influence of pH during the binding reaction was investigated between pH 6.0 and 8.5 with the same concentration of 2,4-DB antibody solution (1.0×10^{-5} g/L) containing 0.02 M PBS for 180 min. The experimental results showed that the relative change in impedance ($\%\Delta R_{ct}$) increased with pH value from 6.0 to 7.0, and then decreased when pH value continue to increase from 7.0 to 8.5 (shown in Fig. 6). Thus, phosphate buffer solution of pH 7.0 was selected as the buffer solution in the binding reaction.

3.2.3. *Influence of incubation time*

The incubation time for immunoassay has influence on the binding reaction. In order to optimize the incubation time, the Ab-immobilized electrode was incubated in the 1.0×10^{-5} g/L 2,4-DB in 0.02 M PBS for varied time, and the relative change in impedance ($\%\Delta R_{ct}$) was tested (shown in Fig. 7). When the incubation time was about 180 min, the relative change in impedance was observed. After incubation time for 180 min, the adsorptive process of antibody proteins would reach the equilibrium. Thus, the incubation time of 180 min was selected for the whole immunoassay.

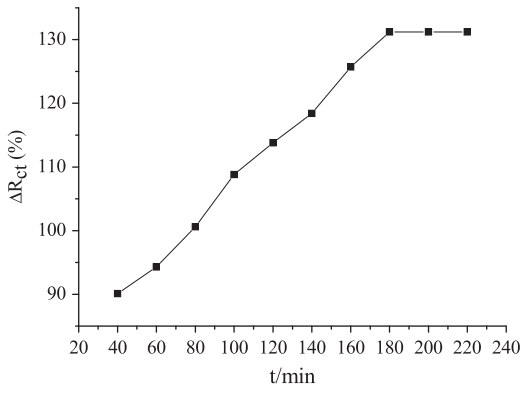

Fig. 7. The influence of incubation time between antibody and antigen interaction. The concentration of 2,4-DB was 1.0×10^{-5} g/L.

3.2.4. The influence of incubated temperature

The formation of immuno-complex on electrode surface depends on the incubation temperature. When the incubation temperature increased from 20 to 45 °C (shown in Fig. 8), the maximal relative change in impedance (%ΔR_{ct}) was observed at the temperature of 37.0 °C, which was used as the optimum incubation temperature.

3.2.5. Effect of PBS (base liquid) on the reaction of antigen–antibody

The electrode modified with antibody was blocked and immersed in 0.02 M PBS (pH 7.0) buffer for 180 min. Repeat above process for seven times with the same electrode and the result showed the RSD as 0.78%, indicating the PBS has no effect on the immunoreaction.

3.3. Performance of the immunosensor

3.3.1. Calibration curve

Faradaic impedance spectra of the immunosensor incubated with various concentrations of 2,4-DB in 0.02 M PBS (pH 7.0) and 0.1 M KCl containing 5.0 mM $Fe(CN)_6^{3-/4-}$. Experimental results indicated that the diameter of the Nyquist circle increased following the adding of the 2,4-DB as antigen (Ag). This may be caused by more 2,4-DB binding to the immobilized antibodies in higher concentration of 2,4-DB, which performs as a kinetic electron-transfer barrier. In the range from 1.0×10^{-7} to 1.0×10^{-3} g/L (shown in Fig. 9B), a linear relationship between the change in R_{ct} and logarithm of 2,4-DB concentration was obtained, with the linear regression equation of $\Delta R_{ct}(\%)=391.504+51.474\log[2,4\text{-DB}]$ (g/L) (S/N=3), in which case, a correlation coefficient of 0.994 and a detection limit of 1.0×10^{-7} g/L (0.1 ppb) were obtained. Therefore, it is clear that within the detection range the relative change in impedance (%ΔR_{ct}) grew with the increase of the antigen concentration. However, the increases of %ΔR_{ct} were not apparent at high antigen concentration due to steric hindrance or the saturation of coupled antigen molecules. The 2,4-DB concentration could be detected quantitatively according to the linear equation. Table 2 shows electrochemical impedance results for antigen–antibody interaction from Fig. 9A).

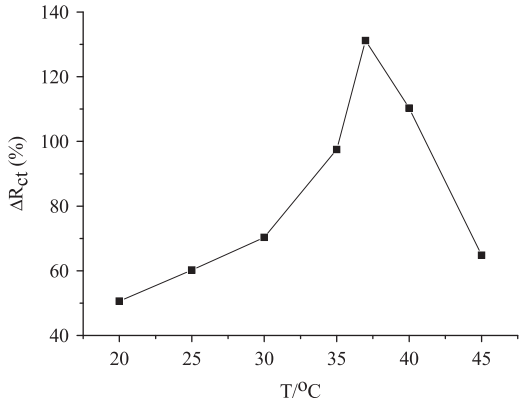

Fig. 8. Effect of the incubation temperature on relative change in impedance of the immunosensor.

3.3.2. Reproducibility and stability of immunosensor

The reproducibility of the proposed immunosensor was evaluated by analyzing 2,4-DB antigen for five replicate measurements. The intra-assay coefficients of variation with the above method were 1.8% and 2.7% at 2,4-DB concentration of 1.0×10^{-6} and 1.0×10^{-5} g/L, respectively. The fabrication reproducibility was evaluated by determining 2,4-DB using five immunosensors made independently at the same electrode. The fabrication reproducibility was 3.2% and 5.2% at 2,4-DB concentration of 1.0×10^{-6} and 1.0×10^{-5} g/L, respectively. The above experimental results indicated that the proposed immunosensor beared good reproducibility.

The proposed immunosensor was stored in the refrigerator at 4 °C. The relative change in impendence was tested at the same 2,4-DB concentration of 1.0×10^{-5} g/L every 4 days. The experimental results showed that the impendence remained the same during the first two months, and decreased gradually and retained 92.7% of initial value after 4 months' storage. The good stability of proposed immunosensor is supposed to be caused by two factors. On one hand, L-Cysteine and glutaraldehyde has provided a biocompatible microenvironment around the

Table 2
Electrochemical impedance results for antigen–antibody interaction from Fig. 3.

Log(2,4-DB)/g/L	R_{ct} (Ω)	ΔR_{ct} (Ω)	ΔR_{ct} (%)
−3	1529.8	1085	243.9
−4	1239.2	794.4	178.6
−5	1028.2	583.4	131.2
−6	810.8	366.0	82.28
−7	599.1	154.3	34.69

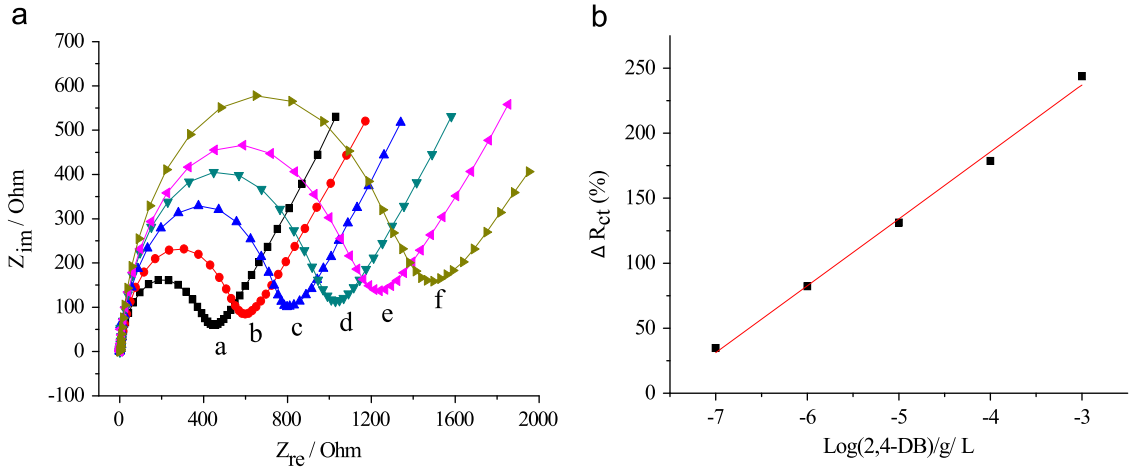

Fig. 9. (A) Faradaic impendence spectra that the immunosensor incubated with different concentration of 2,4-DB in 5.0 mM $Fe(CN)_6^{3-/4-}$ solution containing 0.1 M KCl, curves a–h represent 2,4-DB/BSA/Ab/GA/L-Cys/Au and 1.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4} and 1.0×10^{-3} g/L 2,4-DB, respectively. (B) Calibration curve for the immunosensor.

Table 3
Impedance change of the proposed immunosensor to 1.0×10^{-7} g/L 2,4-DB containing different concentration of interfering antigen.

Interfering antigens	C[interfering antigens] (g/L)				Mean (Ω)	RSD(%)
	0	1.0×10^{-7}	1.0×10^{-6}	1.0×10^{-5}		
2,4-D	595	603	609	612	605	1.2
2,4-DP	599	610	625	630	616	2.3
2,4,5-TP	597	607	617	625	612	2.0

Table 4
The recovery of three samples by the proposed method obtained in the PBS (pH 7.0).

Samples	Spiked amount (g/L)	Determined (g/L)	Average recovery (%)	RSD (%)
Soybean	0	ND*	–	–
	1.0×10^{-7}	0.878×10^{-7}	82.8	6.4
	1.0×10^{-5}	0.955×10^{-5}	95.5	3.7
	1.0×10^{-3}	1.023×10^{-3}	102.3	9.2

* ND: Not Detected.

biological molecules. On the other hand, the protein molecules were attached firmly to the surface of composite matrix.

3.3.3. Nonspecific interactions

In order to confirm the specificity of this immunosensor, the most common used phenoxy acid herbicides in the other three antigens were used as interfering substances and the results were shown in Table 3. Table 3 showed the impendence change responses of the proposed immunosensor in 1.0×10^{-7} g/L 2,4-DB solutions containing different concentration of interfering antigens. It can be seen that there is only a slight variation on the impendence change with the increase of 2,4-D, 2,4-DP, 2,4,5-TP concentration, and the RSD value is less than 3.0%. Such small changes of impendence change of the nonspecific adsorption are acceptable. These results clearly confirmed that the observed change in impedance all originated from specific antibody-2,4-DB interactions.

3.4. Preliminary analysis of real samples

The real soybean samples, which were treated according to Section 2.5, were diluted to appropriate concentration (1.0×10^{-3}, 1.0×10^{-5} and 1.0×10^{-7} g/L) with PBS (pH 7.0) and then examined with the proposed electrochemical immunosensor. The samples were determined by standard addition method and tested average recovery. All the measurements were carried out five times (shown in Table 4). It could be seen that the relative deviations between the two methods were in the range of 3.7% and 9.2%. Therefore, the proposed method could be applied to the determination and detection of 2,4-DB in real samples.

4. Conclusion

In this experiment, self-assembled L-Cysteine monolayer was used to crosslink with glutaraldehyde on the gold electrode which was subsequently immobilized with 2,4-DB monoclonal antibodies to prepare the labeling-free electrochemical impedance immunosensor. 2,4-DB antigens were measured qualitatively and quantitatively by detecting the change of impedance value. The experimental results show that the immunosensor prepared, with its high sensitivity, good reproducibility, ideal specificity and low costs, provides a reliable basis for the application of impedance immunosensor. It is believed that impedance immunosensor will be a kind of convenient and specific immunosensor in the near future.

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