

## A NEW IMMUNOSENSOR FOR RAPID DETECTION OF GIBBERELLIN ACID

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### INTRODUCTION

Monitoring and controlling GA in crops can ensure efficient development of the crops, which is very significant to agriculture and horticulture. Since the content of a phytohormone in a plant is very low, and easily decomposed by heat, light, and oxygen, the traditional methods such as GC, HPLC, ELISA, and radioimmunoassay are usable for the phytohormone assay, but some of them require sophisticated instrumentation or radioactive chemicals or are time consuming. So, exploring simple and convenient methods for phytohormone assay is of considerable interest to the phytohormonal research.

This paper reports the use of immunological technology, in conjunction with the square wave anodic stripping voltammetry (SWASV) detection, to design an immunosensor for the assay of GA. The principle of this immunosensor as shown in Figure 1 is based on the competitive immunoreaction. This reaction takes place between the  $\text{Cu}^{2+}$  labeled antigen ( $\text{GA-BSA-Cu}^{2+}$ ) as the signal producer and GA as the analyte on the glassy carbon electrode (GC) surface assembled with the anti-GA IgG by the Staphylococcal protein A (SPA) that was absorbed on the nano-Au particles loaded by the amine groups on the surface of the silanized GC (20, 21). This immunosensor can carry out a rapid, selective, sensitive, accurate, and inexpensive assay for GA detection. GA was detected in the range of 1-150  $\mu\text{g/mL}$ .

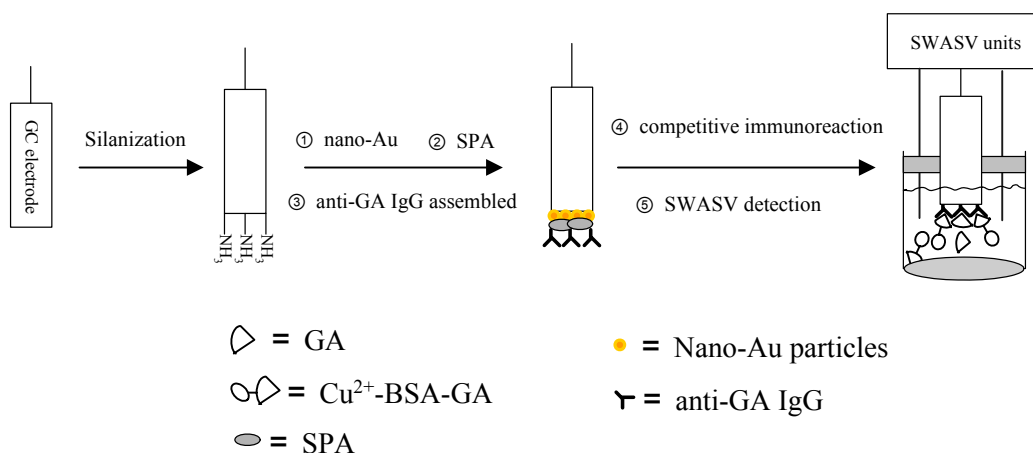


Figure 1. Schematic diagram of the working principle of the immunosensor

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## MATERIALS AND METHODS

**Reagents and Apparatus.** (Aminopropyl)triethoxysilane (APTES) was purchased from Wuda Chemicals Co. Dimethylformamide (DMF), hydrofluoric acid (HF), sodium-potassium tartrate, and cupric sulfate were purchased from Beijing Chemicals Co. (Beijing). *Staphylococcal* protein A (SPA) was obtained from the Shanghai Institute for Bioreagents (Shanghai). Bovine serum albumin (BSA) was from Lizhu Biotechnology Company (Shanghai). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and polyvinyl-pyrrolidone polymer (PVPP) were obtained from Sigma. Gibberellin acid was the product of ICN Biomedical Inc. All other reagents were of analytical grade, and doubly distilled water was used throughout.

Quantitative determination of copper was carried out by square wave anodic stripping voltammetry (SWASV) in a three-electrode system consisting of an immunosensor. All potentials are referenced to Ag/AgCl. The assay cell with a stir bar was placed in a CJJ78-1 magnetic stirrer of Jintan Instruments (Jiangsu, China). A model CSS501 thermostat (Chongqing) was used to control the temperature.

**Preparation of Coating Antigen** GA (100 mmol) and EDC (140 mmol) were dissolved in 200  $\mu\text{L}$  of 50% DMF (pH 5.3 borate buffer) and stirred for 15 min at room temperature. Then, the pH was adjusted to 6.5 with 0.1 M NaOH, and the mixture was added to 500  $\mu\text{L}$  of 0.5% BSA (w/v, in 50% DMF solution). The reaction was carried on for 4 h under nitrogen atmosphere at 4  $^{\circ}\text{C}$ . The coating antigen of GA-BSA conjugate inside the dialyzer was obtained from the mixture solution purified by dialysis against 10% DMF at 4  $^{\circ}\text{C}$  overnight followed by lyophilization.

**Antigen Labeled by  $\text{Cu}^{2+}$**  Sodium hydroxide (4.0 g) and sodium potassium tartrate (4.5 g) were dissolved in 400 mL of water, with 3.0 g of  $\text{CuSO}_4$  added and diluted to 500 mL. 1 mL of the mixture solution was mixed with 1 mL of antigen (BSA-GA) with the concentration of 40 mg/mL (w/v, in 0.9% NaCl solution). The solution was kept at 4  $^{\circ}\text{C}$  for 2 h and dialyzed in 50 mM phosphate buffered saline (PBS, pH 7.0) for 24 h. Finally, the labeled antigen complex solution inside the dialysis tube was stored at 4  $^{\circ}\text{C}$ .

**Preparation of the Immunosensor** The glassy carbon electrode was polished in a water slurry of 0.3  $\mu\text{mol}$  of  $\text{Al}_2\text{O}_3$  and cleaned in 70% nitric acid, followed by being washed in water and acetone.

**Immunosensor Assay Procedure.** The assembled GC was immersed into 1 mL of 0.9 % NaCl (w/v), in which 100  $\mu\text{L}$  of the 50  $\mu\text{g}/\text{mL}$   $\text{Cu}^{2+}$ -BSA-GA solution and 50  $\mu\text{L}$  of sample solution were added. After being incubated for 30 min at 35  $^{\circ}\text{C}$ , the quantitative determination of copper ions was carried out by square wave anodic stripping voltammetry (SWASV) with the peak current recorded as  $I_x$ . When no GA existed in the competitive reaction, the peak current was recorded as  $I_0$ . The percent current reduction (CR %) of the competitive immunoreaction is given by

$$\text{CR}\% = \frac{I_0 - I_x}{I_0} 100$$

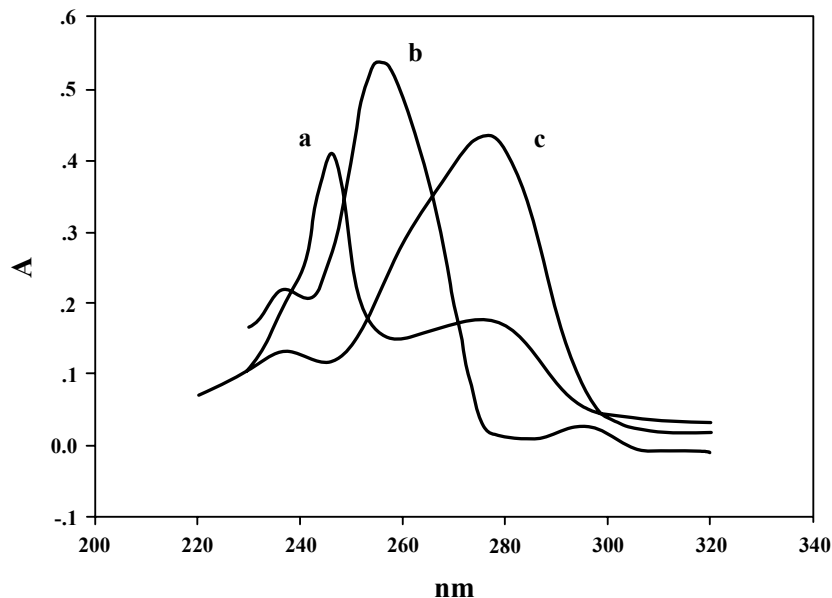
A series of standards of GA ranging from 1 to 150  $\mu\text{g}/\text{mL}$  was used to construct the calibration curve, which could be used for the determination of GA in unknown samples.

## RESULTS

**Conjugated Ratio in Antigen Preparation.** Having a strong absorbance at 280 nm, UV spectrophotometric determination of the conjugated compound indicates that the optimal ratio of GA conjugated to BSA in immunization is 12:1. The conjugated ratio ( $R$ ) was given by

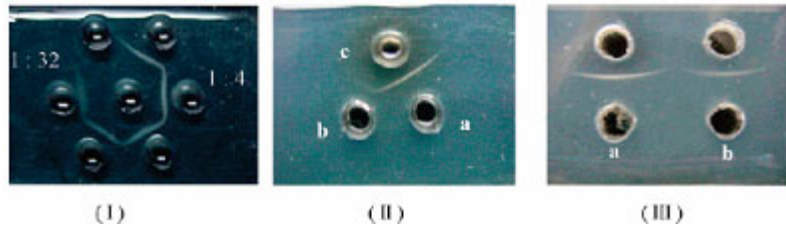
$$R = \frac{A_{(\text{BSA-GA})} - A_{\text{BSA}}}{A_{\text{GA}}}$$

Figure 2 shows the change of the UV spectrum when the GA is conjugated to the BSA.



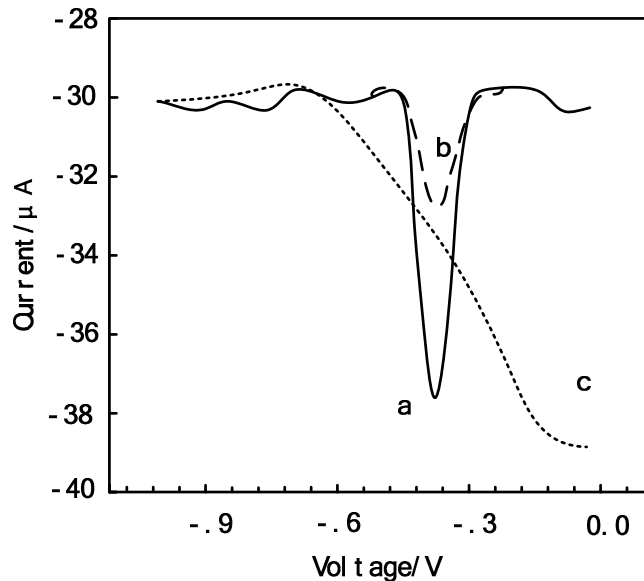
**Figure 2.** UV spectrum of a: GA; b: GA-BSA; and c: BSA.

**Characterization of Antiserum.** The titer of the antiserum increases with the immunization schedule and reached 1:32 after 8 weeks from the first injection as Figure 3(I) shows. When the antibody is allowed to diffuse toward its antigen in a gel matrix, they will form a visible precipitin band at their optimal concentration, but there is no precipitin band when the antibody meets with the antigen without the affinity. In Figure 3 (II), the antiserum from well c with a dilution of 1:100 (v/v, in 0.9% NaCl solution) formed a precipitin band with 20  $\mu\text{g}/\text{mL}$  BSA-GA from well b, while no precipitin band appeared between well c and well a of 20  $\mu\text{g}/\text{mL}$  BSA-IAA. In Figure 3 (III), when 500  $\mu\text{g}/\text{mL}$  GA blended in 20  $\mu\text{g}/\text{mL}$  BSA-IAA in well b, the precipitin band became weak due to GA inhibiting BSA-GA combining to the antibody in the antiserum, indicating that the antibody in the antiserum has a very good specificity character.



**Figure 3.** Specific characteristic of the antibody (I). 8 weeks after the first injection. (II) a: 100  $\mu\text{g/mL}$  BSA-GA; b: 100 $\mu\text{g/mL}$  BSA-IAA; and c: 1:100. antiserum (v/v). (III) a: the deposition line of 100  $\mu\text{g/mL}$  BSA-GA combining with 1:100 antiserum (v/v); b: the same with 500  $\mu\text{g/mL}$  GA in 100 $\mu\text{g/mL}$  BSA-GA solution.

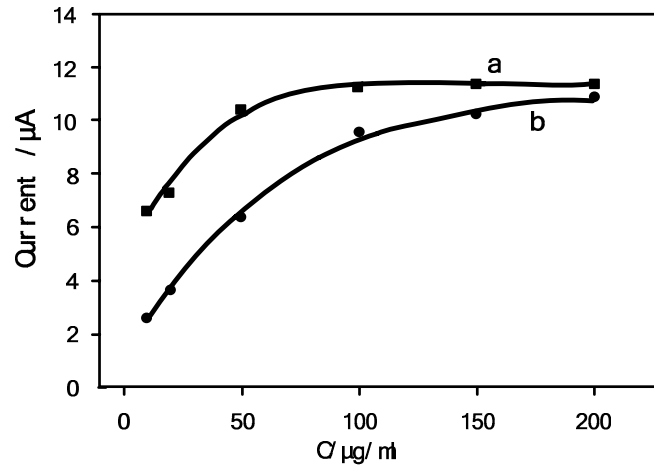
**Characteristic Performance of the Immunosensor.** SWASV is one of the sensitive electroanalytical techniques that is employed to detect reversible redox systems. The copper ion, which was labeled on the antigen, was adsorbed onto the electrode surface by the immunoreactions and electrodeposited as copper. The electrodeposited copper was stripped from the electrode by anodic oxidation bringing a peak current that depended on various parameters such as the potential step increment, square wave amplitude, and frequency. Figure 4 shows the square wave anodic stripping voltammograms obtained with the biosensor immersed in 0.9% NaCl (w/v) solution.



**Figure 4.** Square wave stripping voltammetry of the immunosensor in 0.9% NaCl solution after the competitive immunoreaction between 100 $\mu\text{L}$  of 50 $\mu\text{g/mL}$   $\text{Cu}^{2+}$ -BSA-GA and 50 $\mu\text{L}$  of a: 20 $\mu\text{g/mL}$  GA; b: 100  $\mu\text{g/mL}$  GA; and c: washed by a glycine-hydrochloric buffer of pH 2.4 after immunoassay.

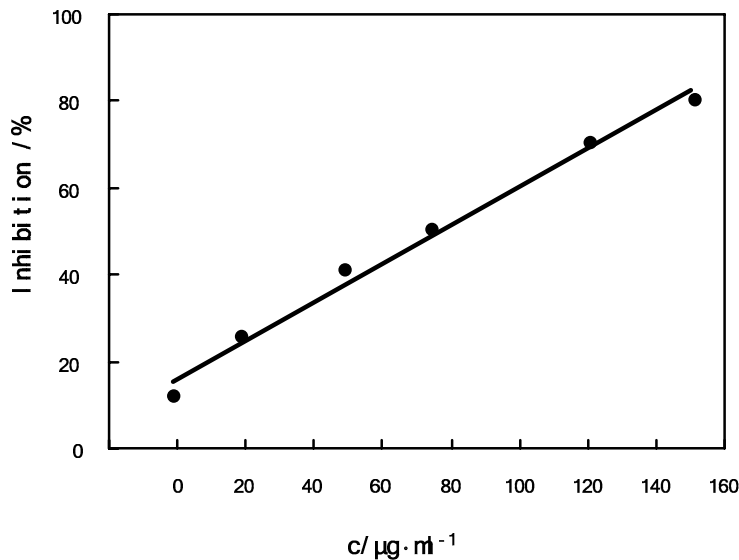
**Optimum Experimental Conditions for Immunosensor Assay.** The current response of the immunosensor increased with the concentration of  $\text{Cu}^{2+}$ -BSA-GA in the assay solution as shown in Figure 5. Since the amount of GA in the sample is very small, to provide a nice competition environment with  $\text{Cu}^{2+}$ -BSA-GA, the concentration of the latter should not be too high in the reaction medium. We employed 50  $\mu\text{g/mL}$   $\text{Cu}^{2+}$ -BSA-GA in the experiment to guarantee both a

sufficiently large current signal and a significant current change due to the competitive immunoreaction.



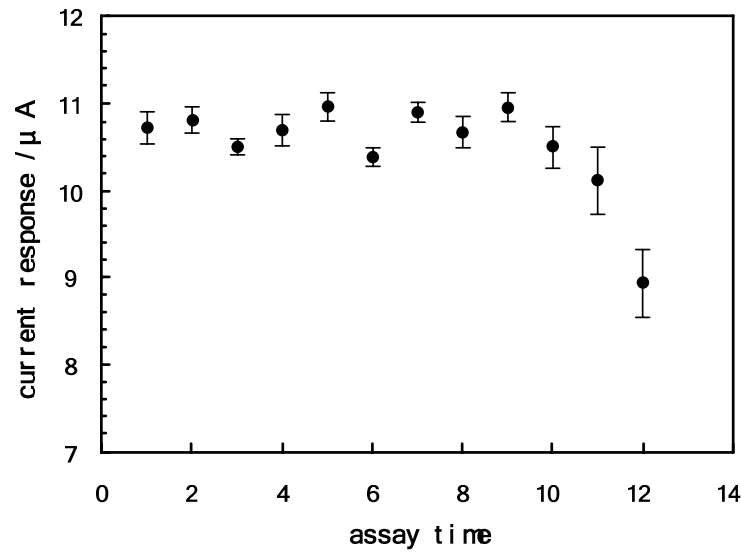
**Figure 5.** Peak current responses of the immunosensor incubating with different concentrations of  $\text{Cu}^{2+}$ -BSA-GA: a, without GA and b, with 50 $\mu\text{g/ml}$  GA competing.

**Calibration and Reproducibility of the Immunosensor.** The calibration curve for GA was based on CR % versus [GA] as shown in Figure 6. The CR % (y) is linearly related to the concentration of GA (x) in the 1  $\mu\text{g/ml}$  to 150  $\mu\text{g/ml}$  range with a regression equation of the form  $y = 0.44x + 15.59$  and correlation coefficient of 0.99.



**Figure 7.** Calibration curve under the optimal experimental conditions

The immunosensor can be repeatedly used for 10 times with a RSD of 1.8% before the assembled layer becomes loose as shown in Figure 7. Five immunosensors made independently yielded a mean current change of  $10.6 \pm 1.2 \mu\text{A}$ .



**Figure 8.** Repeated use of the immunosensor after regeneration. The immunosensor was incubated with  $50\mu\text{g/mL}$   $\text{Cu}^{2+}$ -BSA-GA under the optimal assay condition for each time.

## CONCLUSION

The immunosensor described in this paper provides an alternative tool for determining GA in the plant samples. Its assay speed and efficiency allowing a rapid throughout of the extracted sample are particularly useful for the simultaneous analysis of large numbers of samples. The reliability of the method is shown by the satisfactory agreement of the analytical results with those obtained by HPLC and ELISA.

## Acknowledgements

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