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Research Article

The Development of an Electrochemical Immunosensory for 1-naphthylacetic Acid

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Abstract: A new detection method for 1-naphthylacetic acid (NAA) based on the electrochemical immunosensor was introduced in the study, in which anti-NAA polyclonal antibody was used. An monolayer-molecular, self-reassemble membrane was made from 3-mercaptopropionic acid on the surface of gold electrodes, on the membrane the anti-NAA antibody was immoblize through ester-activated method with addition of 1-(3-Dimethylaminopropyl)-3- ethylcarbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS). Based on the three-electrode system, an NAA immunosenory detection method was established with using the signal from cyclic voltammetry. The standard curve could be represented as $\Delta E = 0.0407\lg c+0.0701(R^2 = 0.9984)$ with the range from 0.05 μg/mL to 2 μg/mL and with the detection limit was 0.035 μg/mL. The sensory method had very low cross-reactivity, lower than 3%, to NAA similarities such as phenylacetic acid and indolyl-3-acetic acid. In addition, the sensor could be used with high stability as long as 30 days (room temperature). It indicated that a specific, stable electrochemical immunosensory detection method for 1-Naphthaleneacetic acid was successfully developed.

Keywords: 1-naphthylacetic acid, electrochemical immunosensor, self-assembled monolayer membrance

INTRODUCTION

1-Naphthylacetic Acid (NAA) is a naphthalene derivative extensively used in the world. Although it was first successfully synthesized by Boessneck in 1883, it has been used in the United States since the 1960s (Xue *et al*., 2012). As a kind of synthetic commercial plant growth regulators, it can effect seed germination, root growth, preharvest fruit and quality of fruit (Robert, 1939; George, 1942; Liang *et al*., 2013). Regarding to toxicity, NAA is considered as unlikely hazardous by the World Health Organization (WHO) and as highly toxic by the U.S. Environmental Protection Agency (U.S. EPA) (Esparza *et al*., 2013). NAA is not believed to be mutagenic neither carcinogenic but shows low acute toxicity so its Maximum Residue Levels (MRLs) have been established. A MRL value of 50 μg/kg for NAA in fruits and vegetables was set up by the European Union (EU), while 100 μg/kg in Japan and U.S.A. As a kind of low toxicity, efficient plant growth regulator, it would be more and more widely used in China. Chinese government is paying attention to the NAA residue in the food, but has no limit standard with NAA. So the limitation standard of NAA was proposed to be established in China and the monitoring for NAA. The existing detection methods for NAA are fluorimetry (Alberto *et al*., 1997; Dong *et al*., 2002), phosphorimetry (Jose *et al*., 2012; Xu and Zhu, 2009), Capillary Electrophoresis (CE) (Chen *et al*., 2015), Chemilumine scence (Gao *et al*., 2013), HPLC (Liu *et al*., 2007; Kong *et al*., 2010), GC (Nagayama *et al*., 2003; Wang *et al*., 2003), GC-MS (Wu *et al*., 2014) and LC-MS (Chen *et al*., 2012; Guan *et al*., 2011). But these methods require expensive instrumentation, tedious sample pretreation and preparation, as well as highly trained personal.

In recent years, immunosensor has drawn the interest of researchers because of their advantages, such as low cost, easy operation, fast response and high sensitivity, stability and reproducible (Keay and McNeil, 1998; Halamek *et al*., 2001; Sun *et al*., 2014). It becomes a hot spot to research the sensor for residues' detection. The electrochemical immunosensor is one kind of immunosensors, which is first studied and many have been prepared. In this study, a NAA detection method was introduced with combination of electrochemical sensor and immunological technique. The sensor was based on anti-NAA polyclonal antibody that is prepared at our lab. A self-assembled monolayer film with 3-thiopropionic acid (MPA) was attached to the gold electrodes, then coated by an antibody against NAA, which could be used for detection of NAA.

MATERIALS AND METHODS

Chemicals and instruments: 1-naphthylacetic acid standard material (99.0% purity), phenylacetic acid standard material (99.0% purity), indole-3-acetic acid (IAA) standard material (99.0% purity), 3-

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mercaptopropionic acid (analytical reagent), 1-(3-Dimethyl aminopropyl) -3- ethylcarbodiimide hydrochloride (EDC, analytical reagent) and *N*-Hydroxysuccinimide (NHS, analytical reagent) were purchased from Aladdin, American. Other reagents were analytical reagent and provided by sinopharm chemical reagent Co. Ltd., (Shanghai, China). The anti-NAA polyclonal antibodies were prepared at our lab. The cherry tomatoes were purchased from local supermarket. The Chi400 Electrochemical Quartzcrystal Microbalance (EQCM) was used as detection instrument which was purchased from Shanghai Chenhua Instruments Co. Ltd., (Shanghai, China). Termovap Sample Concentrator was purchased from Hangzhou Aosheng Instruments Co. Ltd., (Hangzhou, China).

Buffers and solution: Phosphate buffered solution (PBS pH7.4) containing 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4. 0.001 mol/L $Fe(CN)_6^{-3/-4}$ solution with addition of 0.001 mol $K_3Fe(CN)_6$. All of the standard solutions were diluted by PBS.

The preparation of sensor: The preparation of the gold electrode was modified based the methods described by Mohamed *et al*. (2012) and Limbut *et al*. (2006): The gold electrodes were rinsed with 0.1 M NaOH and 0.1 M HCl three times, immersed in Piranha solution (a mixed solution of 30% H_20_2 and concentrated H_2S0_4, volume ratio l:3) for 30 min, rinsed ultrasonically with water and absolute ethanol for 3 min. After that, they were polished successively with 0.3 and 0.05 µm Al_2O_3 powder on micro cloth pads and thoroughly rinsed with ultrapure water 5 min, followed by absolute ethanol and then dried in a flow of nitrogen. Subsequently, the electrodes were incubated in a MPA (10 mM) solution made-up with ethanol for 12 h to form the SAMs and rinsed with ethanol and PBS. After the terminal carboxylic acid (-COOH) groups were activated in a solution of NHS/EDC (0.1 M/0.1 M) for 1 h at room temperature, the electrodes were incubated for 1 h in a 0.5 mg/mL solution of anti-NAA polyclonal antibodies. The terminal amine groups on the antibody enable covalent bonding to occur through the activated carboxylic functions from MPA functionalized NHS/EDC. Finally, after rinsed with PBS, the electrodes were reacted in a 10mM 1-dodecanethiol ethanolic solution for 20 min to block the bare spots on the electrode surface and dried in a flow of nitrogen. The experiments were done at room temperature unless otherwise stated.

Characterization of electrochemistry: Detected by Sensor Chi400, the electrochemical cell consisted of three electrodes where gold electrode as the working electrode, Pt as the counter electrode and an Ag/AgCl electrode as the reference electrode. Before and after the modification, the gold electrodes were tested by using cyclic voltammetry technique in a degassed 0.001 mol/L $Fe(CN)_6^{-3/-4}$ solution at scan rate 100 mV/s with the potential range from -0.3V to 0.5 V. The Cyclic V-A curves of three kinds of gold electrodes: the bare gold electrode, that modified with MPA and modified with antibody gold electrode were obtained.

Response of potential:

The detection method of immunosensor for NAA: The sensor was based on three-electrode system where modified with antibody gold electrode as the working electrode, Pt electrode as the counter electrode and an Ag/AgCl electrode as the reference electrode. The three electrodes were inserted in a degassed 0.01 mol/L PBS at scan rate 100 mV/s with the potential range from -0.3V to 0.5 V. When the potential remain stable, it was detected and set as blank potential, called E_0. Then the electrode was respectively inserted into the gradient NAA standard solutions that were diluted to the appropriate concentrations with PBS (pH 7.4). When the potential value was stable, the value was recorded, called E_n. The difference between E_n and E_0 was set as the response value, called $\triangle E(\triangle E = E_n-E_0)$. The $\triangle E$ respectively responded for its concentration of NAA standard solution. The data of condition, optimization and calibration curve were the average of three measurements. All measurements were performed at room temperature.

RRSULTS AND DISCUSSION

Characterization of electrochemistry: Cyclic voltammetry technique was used to test three kinds of electrode (bare gold electrode, modified with MPA gold electrode and modified with antibody gold electrode) and the result showed in Fig. 1. Curve a denoted the CV of bare gold electrode, where the current peak reached 6.683 µA. Curve b denoted the electrode modified with MPA, where the current peak dropped to 5.813 µA, indicated that the Fe(II)/Fe(III) redox peaks have decreased after the functionalization of the MPA monolayer. This can be attributed to the decrease of the electron transfer rate that was created by the compactness of the formulated SAMs. Curve c denoted the gold electrode modified with antibody. After antibody binding, the redox peaks decrease even more. This was due to an increase of the biolayer thickness that was developed on the gold surface. And it more seriously prevented the electrode to exchange electric charge with $Fe(CN)_6^{-3/-4}$ solution.

The establishment of standard curve: The three-electrode system was inserted into the NAA standard solution with the concentration range from 0.02 to 5.00 µg/mL. After stability, it was performed. Figure 2 showed that the Cyclic V-A curves was detected in the different concentration of NAA standard solutions.

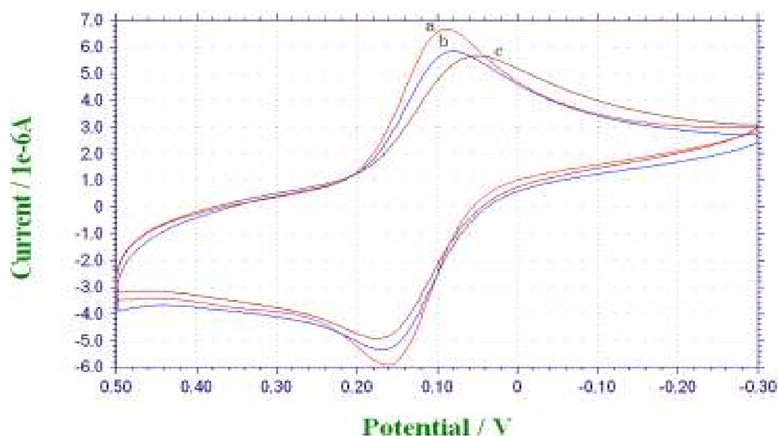

Fig. 1: Cyclic V-A curve of not modified gold electrode (curve a), modified with MPA gold electrode (curve b), modified with MPA and anti-NAA gold electrode (curve c)

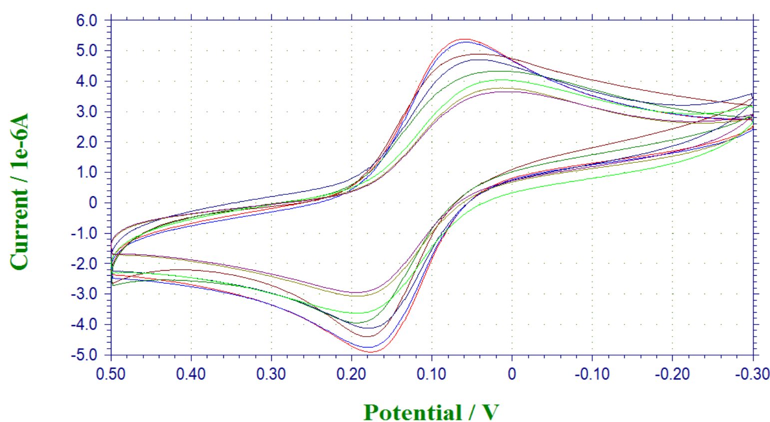

Fig. 2: Cyclic V-A curves of different NAA concentration (With the reduction of current peak, the Cyclic V-A curves respectively represented the result of NAA standard solution: 0.02, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00 µg/mL)

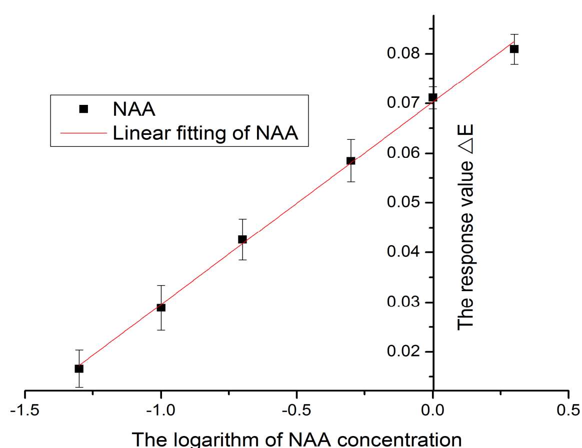

Fig. 3: Standard curve of NAA

Then all of ∆E were respectively calculated out. Aizawa thought membrance potential was linear with the logarithm of analyte's concentration (Tang *et al*., 2004). So according to the logarithm values of NAA standard solution's concentration as abscissa and response value ∆E as coordinate, the NAA standard curve was successfully established. From Fig. 3, the linear equation of NAA standard curve was: $\Delta E = 0.0407lgc+0.0701$(c means NAA standard solution's concentration, $R^2 = 0.9984$). The linear relation between the logarithm values of NAA standard solution's concentration and ∆E were good within the NAA concentration range from 0.05 to 2 µg/mL.

Detection limit of the immunosensor: The gold electrode modified with antibody was detected for PBS solution without NAA 20 times. Then the standard deviation of their ∆E was set as the background value, calculated as 0.0036 V. And the signal response was set as three times of the background value (Sun *et al*., 2011). So the value of lowest signal response is 0.0108 V. The detection limit of 0.035 µg/mL was worked out by using the NAA linear equation.

The specificity of the immunmosensor: The phenylacetic acid and IAA were respectively diluted to a series of gradient concentration by PBS. With three-

Table 1: The specificity of the NAA electrochemical immunosensory

Concentration (μg/mL)	Phenylacetic acid (ΔE)	IAA (ΔE)
0.05	0.0024	0.0036
0.2	0.0035	0.0048
0.8	0.0054	0.0062
2	0.0067	0.0070

Table 2: The reproducibility of the NAA electrochemical immunosensory

Concentration of NAA (μg/mL)	ΔE_1	ΔE_2	ΔE_3	ΔE_4	ΔE_5	ΔE_6	RSD (%)
0.05	0.016	0.0164	0.0165	0.0172	0.0163	0.0166	2.4
0.10	0.0281	0.0281	0.0302	0.0287	0.029	0.0284	1.7
0.50	0.0579	0.0599	0.0588	0.058	0.0581	0.0597	1.5
1.00	0.0718	0.0715	0.0721	0.0708	0.0701	0.0708	1.0

Table 3: The recovery rate of the NAA electrochemical immunosensory

Concentration of NAA (μg/mL)	0.05	0.1	0.5
Recovery rate	90.9±3.0%	95.8±2.3%	101.9±3.1%

electrode system, these solutions were tested by using cyclic voltammetry. The result showed in Table 1. From Table 1, all of the response values were lower than the value of lowest signal response. It showed that cross reaction rate was low and the specificity was strong.

The reproducibility and stability of the immunosensor: The Relative Standard Deviation (RSD) was used to determine whether the reproducibility was good or not. Different time points and different batches of NAA electrochemical immunosensor were prepared to detect the same concentration of NAA standard solution. The result showed in Table 2. From Table 2, it showed that all of response value was lower than 3%. It indicated that the reproducibility was good.

Immunosensor was a kind of fast detection method. Its stability was an important characteristic because it is a key factor to influence the application of method. In this method, the stability largely depended on the stability of unimolecular layer membrane on the surface of the gold electrode and the activity of antibody. The gold electrodes modified with antibody were sealed to store at 4°C. The electrode was used to detect 0.10 μg/mL NAA standard solution every three days. Before 30 days, the response value of the electrode kept mostly unchangeable. The thirty-third days, the response value reduced to only 87.3% of initial electrode. Thus, the gold electrode modified with antibody could keep good characteristic for 30 days.

The recovery of the immunosensor: Referring to sample pretreatment method of Wang *et al*. (2014), the cherry tomatoes were used as sample to detect the recovery of the sensor. The appropriate and known concentration of NAA was added into samples. The NAA in samples was extracted by PBS contain 5% methanol. The result of experiment showed in Table 3. The recovery was between 70 and 120%, indicating that the sensor was good and this method was proper for detecting practical samples.

CONCLUSION

Recently, because of its superiority, the immunosensor was extensively used in the field of the residue detection (pesticide and veterinary residues) in the food. In this study, a NAA electrochemical immunosensor was introduced, by using self-assembled monolayer membrane and polyclonal antibody. In this method, the range of NAA concentration from 0.05 to 2 μg/mL could be detected. And the detection limit reached as low as 0.035 μg/mL. In addition, this method was proved to be easy, rapid, specific, reproducible, with high recovery rate. Although there was no legislation about the residue limit of NAA in China, it is necessary to strengthen the supervision of NAA residues in food. Due to sensitive, convenient, time-saving, the method of NAA electrochemical immunosensor was a rapid detection method and it was proper for sample selection and on-site test, which would provide a fast method for the supervision of NAA residue. It was almost a soundly based conclusion that the sensor was a good detection method with prosperous application.

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