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Detection of Aflatoxin B1 Using an Immunosensor Based on CF/AuNPs Composite Membrane and Normalized Sweep Potential Voltammetry

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Abstract: A novel electrochemical immune sensor was prepared for rapid detection of aflatoxin B1 (AFB1) using experimental data processed by normalized sweep potential voltammetry (NSPV).Carbon fiber (CF) and gold nanoparticles (AuNPs) had been coated on gold (Au) electrodes as sensitive composite membrane to prepare sensor for AFB1 detection. The immunoreaction monitoring was achieved by cyclic voltammetry (CV) techniques and the immunoreaction currents were analyzed by NSPV. According to different normalized values, a better electrode type was selected (Au), and the optimized types of electrode-modified materials (CF and AuNPs), modification sequence (AuNPs/CF), pH of PBS (7.4), BSA concentration (2.5%), and antibody concentration (10 μg/mL) were determined. The best detection voltage (0.11 V) was obtained by normalized processing. With the optimal conditions met, the linearity between peak currents of the incubated electrochemical biosensor and AFB1 concentrations was investigated, and the results indicated a standard curve, which showed an excellent linear relationship within the concentration range of 0.01 to 1000 ng/mL. The linear regression formula for the concentrations of AFB1 versus the currents was $y = 0.12967 + 0.03774x$ ($R^2 = 0.98858$), while the limit of detection (LOD) for this method was 0.01 ng/mL. The recovery results showed that the sensor had high specificity and could be used for rapid detection of AFB1 residues in rice.

Keywords: Electrochemical immune sensor, AFB1, Gold nanoparticles, Carbon fiber, Normalized sweep potential voltammetry (NSPV).

1. Introduction

According to a survey conducted by the Food and Agriculture Organization (FAO) of the United Nations, about 25% of the world's food is contaminated each year with mycotoxin [1-2]. Aflatoxins are proven to be carcinogens, mutagens, and teratogens [3]. Severe human health disorders were all caused by aflatoxins, especially AFB1 [4-5]. Therefore, a rapid detection system is urgently required for quantitative AFB1 detection and reliable quantification at the trace level.

Currently, plenty of novel detection techniques have been developed, such as chemical analysis, instrumental analysis, biological identification, and immunoassay [6]. The mainstream method is to use electrochemical immune sensors, which are based on different immobilization methods and various transducers, and have garnered considerable attention [7-8]. The high specificity between antibodies and antigens helps identify events with appropriate transducers. Their potential advantages include specific, label-free, easy signal amplification, and simple operations, and the rapid detection approaches have the advantages such as a reduction in the size, cost and analysis time [9-11]. A lot of materials, for example, enzymes, multi-walled carbon, glutaraldehyde and nanoparticles, have been employed to amplify immune systems' signals in immune sensors and therefore have been considered in aflatoxin analysis [12-15]. Carbon fiber (CF) and gold nanoparticles (AuNPs) have some advantages. CF has the characteristics of corrosion resistance, abrasion resistance, high temperature resistance, high strength and light weight [16]. AuNPs have a large surface area, good biocompatibility, in addition to a strong adsorption capacity, which can be combined with a variety of biological macromolecules without affecting its biological activities [17]. Yang et al. [18] selected a sandwich electrochemical immunosensor, with nitrogen-supported AuNPs as the substrate material in their study. Zhang et al. [19] used single-wall covalent functional nanotube/chitosan and carbon nanotubes/chitosan for signal amplification. Electrochemical biosensors combined with biological analysis and electrochemical sensor techniques have broad applications and prospects in biological engineering, environmental monitoring, pharmaceutical industry, food safety, agricultural analysis and other fields [20]. Meanwhile, there are still many key technical problems to be resolved in order to facilitate their applications in practice as soon as possible [21]. Therefore, further research is particularly important.

Normalization is often used to process data, which simplifies calculation and changes dimensional expression into dimensionless expression [22]. In a multi-index evaluation system, different dimensions and orders of magnitude are produced because of different properties of each evaluation index. The normalization method can improve the accuracy and make the experimental results more accurate [23-24].

In this study, an immune sensor was introduced to detect AFB1 based on antigen-antibody specificity and Au sensitivity. The immune sensor was developed, and CF and AuNPs were used to modify the electrode followed by immobilization of antibodies on BSA. They could be strongly adsorbed on the gold electrode and provide a good biocompatibility environment for antibody modification, which would adsorb more antigens in the test solution and did not affect the activity of immune response [25]. AFB1 in different concentrations was arrested by antibodies that were fixed on the electrode, leading to current changes on the electrode surface. Immunoreaction monitoring was achieved by cyclic voltammetry (CV) techniques and the measured data were processed by NSPV.

The important experimental conditions were optimized and the AFB1 calibration curve was established, which was prepared by considering the differences in the current before and after aflatoxin B1 binding. AFB1 was considered a good example for future extension to other antigens or antibodies. It provided theoretical support for subsequent biosensor preparation and aflatoxin detection.

2. Experiments

2.1. Apparatus

CV was performed for measurements based on CHI 660D. This study used a traditional three-electrode system: a 3-mm-diameter working electrode, Ag/AgCL saturated with KCL as the reference, and platinum wire as an auxiliary electrode. All experiments were done at room temperature. Experimental water was all produced by milli-Q Academic A10. The specific instruments were shown in Table 1.

Table 1. Main instruments and equipment.

Name	Manufacturer
CHI 660D electrochemical workstation	Shanghai Chenhua Instruments Co., Ltd.
Gold and glassy carbon electrode	Mettler Tory Instrument Co., Ltd.
DHG-9070A electrothermal blowing dry box	Shanghai Yiheng Technology Co., Ltd.
FE20K pH meter	The United States Aolilong Co., Ltd.
SK3300H ultrasonic cleaner	Shanghai Branch Ultrasonic Instrument Co., Ltd.
AL 104 electronic balance	Mettler-Toledo Instruments (Shanghai) Co., Ltd.
Microoscillator	Jiangsu Jintan Instrument Factory

2.2. Reagents and Materials

Monoclonal antibodies against AFB1 were provided by Oil Crops Research Institute, which is an institute of Chinese Academy of Agricultural Sciences. The production method was according to the literature [26]. AFB1 antigen was purchased from Sigma. CF and BSA were bought from Shanghai Jing An Biological Co., Ltd. (Shanghai, China). Phosphate buffered saline (PBS, 10 mM, pH 7.2-7.4) was used for dissolving antibodies. Different pH values (7.0, 7.2, 7.4, 7.6, 7.8 and 8.0) were to be prepared for the experiment. H_2O_2 (30%, AR) and H_2SO_4 (70 %, AR) were mixed (V/V, 3:7) to prepare a piranha solution in a fume hood. AuNPs were prepared based on previous descriptions in the literature [27]. A variety of nanoparticles of different sizes can be prepared by the reduction method [28]. The other reagents were of analytical grade or better.

2.3. Preparation of Antibody and Other Biochemical Reagents

The defrosted antibodies were diluted with the PBS solution according to the instructions printed on the package. Then, a small amount of diluted antibodies was absorbed into a centrifuge tube and diluted with PBS to a certain concentration as the mother liquid. The mother liquor was also stored in a frozen state.

Before use, the mother solution was dissociated and then diluted by a small amount to avoid unnecessary errors.

2.4. Procedure of AFB1 Detection by an Electrochemical Immune Sensor

The whole experimental procedure was carried out as follows:

(1) Electrode cleaning: The pretreatment of electrodes was essential and the main methods referred to the literatures [29-30], in order to achieve a clean and smooth surface similar to a mirror.

(2) Modification of the electrode: In this experiment, amplification signals resulted from disparate modification sequences on different electrodes were compared. For AuNPs/CF/Au, the clean and dry surface of the electrode was first added with 5 μL of CF, incubated under room temperature for 90 min, and then thoroughly flushed at their electrode work areas to remove unbonded CF residues using ultra-pure water and blow dried with nitrogen. Then, using the same procedure and method, 5 μL AuNPs were fixed on the modified electrode to obtain a gold electrode modified with CF and AuNP self-assembly membrane. The others also followed these steps.

(3) Surface immobilization: The modified electrodes were added with 5 μL of antibodies and incubated under room temperature for 1 h, and then their surfaces were thoroughly rinsed with ultra-pure water in order to eliminate unbonded antibodies, and blow dried with nitrogen. Finally, 2.5 % BSA (w/v) was added to the electrodes, which were also incubated at the same temperature for 1 h, the electrode surfaces were completely washed with ultra-pure water to discard excess BSA, and then dried with nitrogen. Since then, the biosensor had been prepared for aflatoxin detection. This situation of the electrodes could be directly used in the detection of pesticide residues of aflatoxin. They could also be hung in PBS (pH = 7.4), in a 4 °C refrigerator for possible later use. The fabrication process of the immunosensor was shown in Fig. 1.

(4) AFB1 detection: During a trial, in the absence of specific instructions, the prepared BSA/antibody/AuNPs/CF/Au immunosensors were incubated in the PBS solution containing different concentrations of incubated aflatoxin for 30 min at 37 °C, and then the electrochemical test could be performed. The assembly step of the electrodes and electrochemical properties for immune reaction were tested by CV (–0.4-0.6 v, 0.05 v/s). The base liquid for the test was PBS (pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCL. CF and AuNPs had the effect of amplifying the signals of the electrodes, and the combination of antibodies and antigens would increase the resistance and reduce the current. Therefore, the residual concentration of AFB1 could be measured by the change in the current ($I = I_0 - I_1$) before and after immunization. I_0 represented the current in the test substrate prior to electrode modification and antigen reaction. I_1 represented the current in the test substrate after the electrode modification and antigenic reaction.

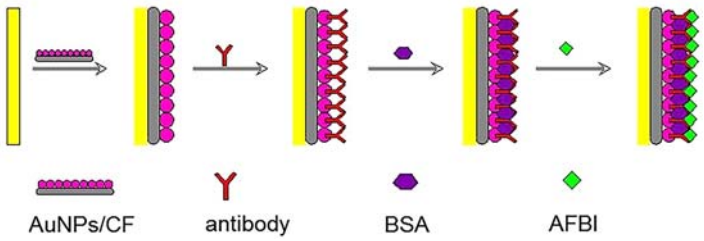

Fig 1. The process of assembling electrochemical immune sensors.

3. Results and Discussion

3.1. AFB1 Immunoassay Construction

In order to determine the best method used as a fixed platform for AFB1, this experiment optimized the parameters. In experimental processes, the influencing factors mainly include the type of electrode for immunosensors and modified materials, modification sequence of the materials, pH value of the test solution and concentrations of BSA and antibodies. Fig.1 showed the process of assembling electrochemical immunosensor.

The support electrode was one of the key technical issues affecting the development of immunosensors [31-32]. Therefore, we selected the glassy carbon electrode (GCE) and gold electrode (Au) in this experiment to construct the immune sensors, respectively.

During the construction of an immune sensor, the current response in a CV test would also change with the modification of the surface material on the electrode [33]. Therefore, the best CF and AuNP modification sequence was determined in this experiment. CF and AuNPs were combined in different sequences to modify the cleaned electrode, and then antibodies and BSA of the same concentration were added.

According to our experiments results, the best modification sequences of the two electrodes were both AuNPs/CFand the modified Au electrode (AuNPs/CF/Au) was more sensitive than the modified GCE (AuNPs/CF/GCE) in the same manner. Similar practice and results could be found in the literature [34]. Therefore, Au was continually used throughout the experiment.

Many biochemical reactions needed to be carried out within an appropriate pH range. The test solution pH influenced the stability of antigen-antibody complexes, impacting the immunosensor sensitivity and stability [35]. Therefore, the optimum acidity of the test solution was selected. The gradient value of the pH selected in this optimization process was 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0. The biosensors were measured in a 50 μL [Fe(CN)6]3-/4- test solution, and the differential value (ΔI) of the current in the test was obtained before and after the antigen incubation. The average pH gradient was measured three times. According to our experiment results, at different pH levels, the trend of the current change rate ($\Delta I/I_0$) was different. , When pH was 7.4, the $\Delta I/I_0$ of the immune electrode was the largest, and the antigen-antibody production was high and stable. When pH was inappropriate, the aflatoxin-antibody compounds were easily decomposed to reduce the impedance response. As a result, pH of the base solution (PBS) for the tests was determined as 7.4. Similar practice and results could be found in the literature [36].

In the experiment, the antibody was fixed on the electrode. If the concentration of the antibody solution was too high, there was a molecular competition between antibody molecules, and it would be difficult for the antibodies of the fixed amount to increase. On the electrode, the antibody directly indicated the performance of the shadow electrode [37]. Therefore, the best concentration of the antibody was determined. The influence of the antibody concentration on current responses was determined by response current on the electrode, and then the current was measured in the test solution of 50 μL $[Fe(CN)_6]^{3-/4-}$. The current responses before and after antibody treatment were measured while the antibody concentration range from 0.1μg/mL to 100 μg/mL, and the average of each concentration gradient was measured three times. The concentration gradients of the antibodies were 0.1, 1, 10, 40, 70, and 100 μg/mL.

Experiments results revealed the change of $\Delta I/I_0$ along with the change of the antibody concentration and it demonstrated that the response current at the peak was the maximum when the antibody concentration was 10 μg/mL. This was due to the fact that the bio-specific antibodies, which were fixed to the surfaces of the electrodes, were competitive. There was competition between the antibodies that were bound to the surface of the sensor, leading to fewer antibody-binding sites that could capture aflatoxin. The excessive antibodies were not conducive to the subsequent capture of antigenic toxin. Therefore, the determined concentration of the antibody load was 10 μg/mL, which was consistent throughout the experiment. Similar practice and results could be found in the literature [38].

The bovine serum protein concentrations selected in this experiment ranged from 1.0 % to 3.5 % (1.0 %, 1.5 %, 2 %, 2.5 %, 3.0 %, and 3.5 %).

The results showed the trend of $\Delta I/I_0$ at different levels of BSA concentration was different. When the concentration was 5 %, the response current at the peak was maximum. When the concentration was beyond 5 %, $\Delta I/I_0$ did not change much. This was because excess BSA was close to the non-specific binding site on the antibody, and there was no additional binding site for BSA attachment. In the following cleaning process, excess BSA was washed out, which did not cause a larger impedance change, so that $\Delta I/I_0$ before and after closure was not significant. However, the application of a large concentration of biochemical reagents did not achieve the desired effect, which would surely cause a waste of reagents. Therefore, the optimization of the BSA concentration was indispensable. The determined concentration of BSA was 2.5 %, and the concentration was used throughout the experiment. Similar practice and results could be found in the literature [39].

3.2. Current Measurement and Standard Curve Extracting

3.2.1. Normalized Current Change

Normalization is a simplified calculation method, meaning that there is a dimension expression, which is

transformed into an infinitesimal expression and becomes a scalar. In this article, we use the normalization method to process the response current before and after AFB1 incubation.

BSA/antibody/AuNPs/CF/Au without AFB1 was used in all the experiments in this study as a control. To investigate the influence of the current measurement of AFB1, the magnitude of the current with and without AFB1 was compared. A calibration curve for NSPV was plotted based on the difference from the control in terms of the magnitude of current. The value of NSPV was calculated as follows:

$$NSPV = (I_{sample} - I_{control})/I_{control} \times 100\% \quad (1)$$

In this equation, $I_{control}$ represents the magnitude of the current for the control, and I_{sample} represents the magnitude of the current for an AFB1-containing sample. For each concentration of AFB1, measurements were conducted three times and the average was obtained, and then their standard deviations were calculated and analyzed.

The current values at several different concentrations of aflatoxins (0.01, 0.1, 1, 10, 100, and 1000 μg/mL) were selected as the basis for the calculation. Fig. 2 clearly showed that the maximum difference in NSPV values was near 0.11 V. Therefore, the optimal voltage for AFB1 detection was 0.11 V, and the current value near 0.11 V was selected throughout the experiment.

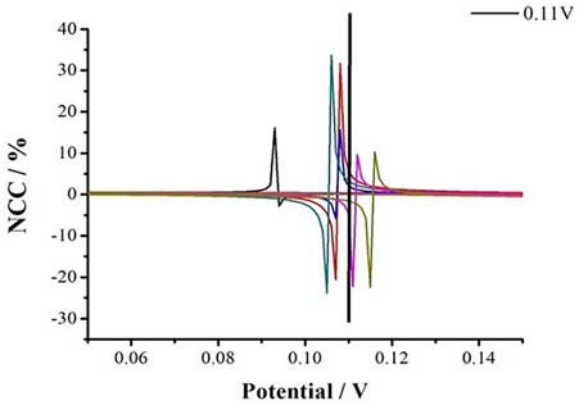

Fig. 2. NSPV values and voltage curves of different concentrations of aflatoxins.

3.2.2. Establishment of Standard Curves for Immune Sensors

Electrochemical immune sensors were prepared according to all of the above optimized conditions (Au, CF+AuNPs, pH 7.4, antibody 10 μg/mL, BSA 2.5 %, and voltage 0.11 V). The biosensors were incubated for 15 min in standard solutions with varied aflatoxin concentrations. The current difference before and after electrode incubation was measured at different AFB1 concentrations in 50 μL of pH 7.4 solution (5 mM $[Fe(CN)_6]^{3-/4-}$, 0.1 M KCL and PBS). We could see from Fig. 3 that the linearity between the aflatoxin concentration and current difference change rate for the immune sensors. The standard curve was established in the range from 0.01 to 1000 ng/mL and the equation was:

$$y = 0.12967 + 0.03774x \ (R2 = 0.98858) \quad (2)$$

In this formula, y was the current difference of the electrode surface measured in the test solution before and after incubation of aflatoxins at different concentrations, and x was the value LgC (ng/mL), where C was the actual concentration of aflatoxin B1 for incubation of the electrode. R^2 was a linear correlation coefficient, which was applicable to actual aflatoxin detection. The limit of detection (LOD) was 0.01 ng/mL in this method. The EU stipulated that the content of AFB1 in human consumer goods should not exceed 0.05 μg/kg [40-41]. The sensor in this experiment has met this requirement.

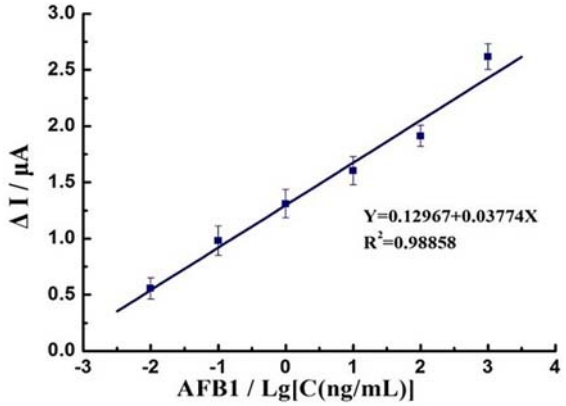

Fig. 3. The linearity of the aflatoxin concentration versus the electrode current.

According to Table 2, AFB1 standard solutions of three concentrations (10, 50 and 100 ng/mL) were evenly sprayed on rice samples. The samples were extracted and concentrated by the ultrasonic centrifuge extraction method and detected by the prepared sensor. According to the recovery results, the recovery rates of the samples in the rice samples were 91.2-104.7 %, falling into the allowable recovery range. The results implied that the immune sensor could be applied to rapid detection of aflatoxin B1 in rice samples.

Additionally, as shown in Table 3, Y. Liu proposed an immunoassay tool to determine AFB1 based on bio-electrocatalytic reaction that took place on micro-comb electrodes. The LOD for the proposed immune-biosensor was 0.1 ng/mL [42]. A. Beheshti-Marnani studied the behavior of modified electrodes in presence of reduced graphene, and the LOD was 0.07 ng/mL [43]. K. Gouda et al. used methylene blue labeled aptamers as signal fragments, and the minimum LOD for the electrochemical aptamer sensor was 0.05 ng/mL [44]. L. L. Yu used electrochemical impedance spectroscopy (EIS) to prepare MWCNTs/RTIL composite film-based immuno-

sensors for the purpose of determining AFB1, which was sensitive and convenient, and the LOD for this method was 0.03 ng/mL [45]. Compared with other reported sensors, the sensors in our work had a lower LOD (0.01 ng/mL) and excellent sensitivity. Therefore, the electrochemical biosensor had great potential in aflatoxin tests.

Table 2. The recovery results for determination of AFB1 in rice.

Rice sample	Found [ng/mL]	Recovery [%]	RSD [%, n = 5]
10	9.12	91.2	5.2
50	52.35	104.7	3.4
100	103.2	103.2	4.1

Table 3. Comparison of the sensor with other similar sensors used for aflatoxin B1 determination.

Nanoparticles in detection	LOD[ng/mL]	Detection technique	References
Gold nanoparticles	0.1	CV and DPV	42
Graphene oxide	0.07	CV and DPV	43
Graphene oxide	0.05	DPV	44
Carbon nanotube	0.03	EIS	45
Carbon fiber/gold nanoparticles	0.01	CV	Our work

4. Conclusions

A novel electrochemical immunosensor for direct detection of AFB1 was proposed in the present study. First, the order of the electrode modification materials and the type of electrode were selected by cyclic voltammetry (CV). Second, several experimental parameters were determined, including pH of PBS, concentrations of BSA and antibodies, and detection voltages. They were identified by NSPV. Finally, the standard curve of AFB1 was plotted under the optimal conditions, indicating that the linear relationship was acceptable, the limit of detection (0.01 ng/mL) was low enough, and the performance of the sensor was good. The experimental results showed a stable, reliable and inexpensive method for detection of AFB1, which featured high sensitivity and selectivity.

Moreover, this method provided a new perspective for immune analysis of other poisonous and harmful residues in food control.

Acknowledgments

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