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Preparation of Electrochemical Immunosensor for Aflatoxin B1 Detection and Normalizing Methods Used for Experiment Parameter Optimization

[1, 2, 3, *] Wenping Zhao, [1] Hui Zhang, [1] Jianfei Sun, [1, 2, 3] Yemin Guo, [1, 2, 3, *] Xia Sun and [1 *] Zhonghe Liu

[1] School of Agriculture Engineering and Food Science, Shandong University of Technology, No. 266 Xincun West Road, Zhangdian District, Zibo City, 255000, Shandong Province, P.R. China
[2] Shandong Provincial Engineering Research Center of Vegetable Safety and Quality Traceability, No. 266 Xincun West Road, Zhangdian District, Zibo City, 255000, Shandong Province, P.R. China
[3] Zibo City Key Laboratory of Agricultural Product Safety Traceability, No. 266 Xincun West Road, Zhangdian District, Zibo City, 255000, Shandong Province, P.R. China
[1] Tel.: +86-533-2786558, fax: +86-533-2786558
E-mail: 187286528@qq.com, sunxia2151@sina.com, 364989694@qq.com

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Abstract: We proposed a sensitive electrochemical immunosensor for rapid aflatoxin B1 (AFB1) detection based on gold nanoparticles (AuNPs) and carbon nanofibers (CNFs) modified electrodes. The modification of nanoparticles and the fixation of biomolecules on the electrodes had an influence on electron transfer on the electrode surface, causing changes in response currents. The response current was tested through cyclic voltammetry (CV) techniques and the current changes were analyzed by normalization method. Experiment parameters were optimized according to the response current changes on electrodes. According to the results of the normalization processing, the best parameters for the biosensor construction were determined and these parameters included the electrode type (Au), electrode-modified materials and its modification sequence (AuNPs/CF), pH of PBS (7.4), BSA concentration (2.5 %) and antibody concentration (10 μg/mL). Under optimized conditions, we constructed an immune sensor for aflatoxin detection and the testing results indicated that the sensor had good performance, which provided a new idea for rapid aflatoxin detection.

Keywords: Immunosensor, Aflatoxin B1, Rapid detection, Parameter optimization, Normalization methods.

1. Introduction

More than twenty types of aflatoxins have been found so far [1-2]. Aflatoxin B1, B2, G1 and G2 were the most common and major varieties [3-4]. Among those various aflatoxins, aflatoxin B1 is the most contaminated substance in the food and it is the most toxic and the most carcinogenic one so that it was recognized as a Group I carcinogen by the International Agency for Research on Cancer by 2002 [5-7]. At present, the methods for isolating and quantifying AFB1 include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS-MS) and enzyme linked immunosorbent assay (ELISA) [8-14]. Most of these

methods require large and expensive detection equipment, and the operation procedures are cumbersome, the detection cycle is long, and it is not conducive to on-site real-time detection. Rapid detection of aflatoxins has been increasing in recent years. Electrochemical biosensors, especially immunochemical biosensors, have been used more and more in the detection of AFB1 [15-18].

Signal amplification is a key factor in biosensor construction and lots of materials such as multi-walled carbon, carbon fiber, enzymes, glutaraldehyde and nanoparticles have been used to amplify immunosensor signals [19-22]. On one hand, various conductive nano-materials improve electron transfer on the electrode surface, and they could amplify the response of the sensor. On the other hand, the large specific surface area and good biological activity of the modified nano-materials could improve adsorption performance of the electrodes and more biological molecules could be fixed on it and maintain the biological activity. The response signals are further amplified. As a result, various nano-materials have been used to develop sensors. Among these targets, AuNPs and CF show excellent performance.

Wang, Xu [23] studied three kinds of prepared AuNPs and evaluated their catalytic activity for the 4-nitrophenol reduction reaction in the presence of sodium borohydride. A fluorescent immunoassay for aflatoxin B1 (AFB1) was established using the catalytic reaction for signal amplification based on target-induced concentration change of AuNPs, where AFB1-BSA-coated magnetic beads andanti-AFB1 antibody-conjugated AuNPs were employed as capture and signal probes, respectively.

Rizwan, Mohammad [24] used a nanocomposite of AuNPs/CNOs/SWCNTs/CS to develop a highly sensitive electrochemical immunosensor for the detection of carcinoembryonic antigen (CEA), the AuNP nanocomposite showed a 200 percent increase in the effective surface area and electronic conductivity. The CEA-immunosensor demonstrated a wide linear detection range of 100 fg mL^{-1}-400 ng mL^{-1} with a low detection limit of 100 fg m^{-1}.

Signal amplification materials such as enzymes, antibodies, glutaraldehyde, and nanoparticles need to be used in specific pH solutions and the concentrations and methods of these materials also need to be determined according to different application environments.

The fabrication procedure of the immunosensor and standard AFB1 sample detection were characterized by CV. The influences of the experiment parameters were investigated. We used the normalization method to process the sensor's current according to the voltages before and after incubation by different pH values of the test solutions, and different concentrations of BSA and antibodies. Under optimized conditions, an excellent biosensor was fabricated. It provided a new idea for rapid aflatoxin detection.

2. Materials and Methods

2.1. Apparatus and Reagents

Cyclic voltammetry was carried out with CHI660 electrochemical workstation (Shanghai, Chenhua Co., China). The test system consisted of conventional three-electrode. The working electrode was a gold electrode or glassy carbon electrode (Au, d = 3 mm, GCE, d = 3 mm), Ag/AgCL was a reference electrode, and the platinum electrode was used as an auxiliary electrode. If not mentioned, all potentials given below were relative to the Ag/AgCl (saturated KCL) electrode.

Monoclonal antibodies against AFB1 were made according to the literature [25]. Bovine serum albumin (BSA) was obtained from Bio-Dev-Tech. Co. (Beijing, China). Carbon fiber was purchased from Shanghai Jingan Biological Co., Ltd. (Shanghai, China). AFB1 antigen was purchased from Sigma. AuNPs were prepared based on the literature [26]. The other reagents were of analytical grade or better. AFB1 antibodies were diluted with the PBS solution according to the instructions printed on the package. Then, it was absorbed into a centrifuge tube and diluted with PBS to a certain concentration as the mother liquid. The mother liquor was stored at 4°C for further dilution.

2.2. Preparation of the Immunosensor

In order to achieve a clean and smooth surface, the GCE and Au electrodes were prepared as described in the literature [27-28]. CF or AuNP solution (5 μL) was dipped on the GCE and Au electrodes firstly and incubated under room temperature for 90 min and then thoroughly flushed by ultra-pure water at their electrode work areas to remove unbonded CF or AuNP residues and blow dried with nitrogen, AuNP or CF solution (5 μL) was dipped on the GCE and Au electrodes and then the same procedure and method were repeated. Four types of different modification electrodes were obtained, and they were CF/AuNPs/GCE, CF/AuNPs/Au, AuNPs/CF/Au and AuNPs/CF/ GCE, respectively. Fig. 1 showed the four different modification methods and fabrication processing of the electrodes.

Four kinds of modified electrodes were added with 5 μL of AFB1 antibodies and incubated at 37 °C for 1 h, rinsed with ultra-pure water thoroughly to eliminate unbonded antibodies, and then dried with nitrogen. Finally, 2.5 % BSA (w/v) was added on the electrodes, which were also incubated at 37°C for 1 h, the electrode surfaces were completely washed with ultra-pure water to discard excess BSA, and then dried with nitrogen. Four kinds of different immunosensors were prepared, and they were
BSA/AFB1/CF/AuNPs/GCE,
BSA/AFB1/CF/AuNPs/Au,
BSA/AFB1/AuNPs/CF/Au
and BSA/AFB1/ AuNPs/CF/GCE, respectively.

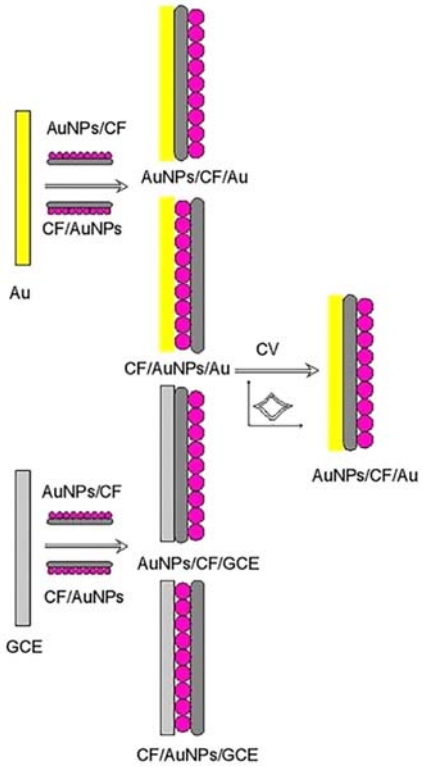

Fig. 1. Four different modification methods and fabrication processing of the electrodes.

The prepared immunosensors were incubated in the PBS solution containing different concentrations of incubated aflatoxins for 30 min at 37 °C, and AFB1 detection was performed according to the above methods by CV (–0.4-0.6 v, 0.05 v/s). The test solution was PBS containing 5 mM of $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCL.

3. Results and Discussion

In order to construct the immunosensor under the best circumstances and provide a fixed platform with the maximum sensitivity and the minimum limit of detection for AFB1, the experiment parameters were optimized. The influencing factors mainly include the type of electrode for modification electrodes, pH value of the test solution, concentrations of the antibodies and BSA.

3.1. Optimization of Modification Electrodes

Six different modification electrodes (bare GCE, AuNPs/CF/GCE, CF/AuNPs/GCE, bare Au electrode, AuNPs/CF/Au, and CF/AuNPs/Au) were investigated by CV. The amplification signals on different modification electrodes were caused by different modification sequences on these electrodes. The influence of the electrode type on current responses was determined by the same concentration of antibodies on different types of electrodes in the test solution of 50 μL $[Fe(CN)_6]^{3-/4-}$. Fig. 2 and Fig. 3 showed the relationships between the sequences of the modified materials and the response of the immune sensor current.

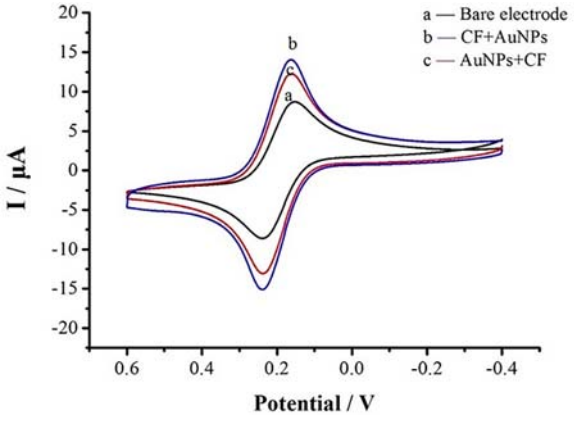

Fig. 2. The variations of the current responses on the surface of GCE with different modification sequences. (a) GCE; (b) AuNPs/CF/GCE; (c) CF/AuNPs/GCE.

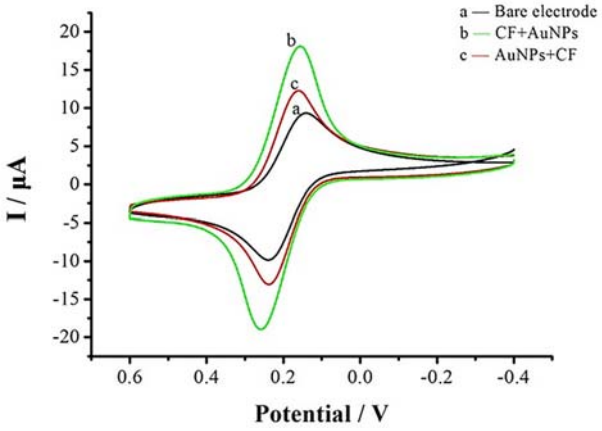

Fig. 3. The variations of the current responses on the surface of Au with different modification sequences. (a) Au; (b) AuNPs/CF/Au; (c) CF/AuNPs/Au.

Fig. 2 was the current change of GCE before and after the modification. Fig. 2 showed CV of the bare GCE electrode (a), and different modified sequences of the electrodes AuNPs/CF/GCE (b) and CF/AuNPs/GCE (c). The peak currents of the bare electrode, AuNPs/CF/GCE and CF/AuNPs/GCE were 8.84 μA, 14.43 μA and 12.18 μA, respectively. We could see that the peak currents of these three kinds of electrodes were different, AuNPs and CF were all nano-materials, and they both had good conductivity and biocompatibility. In order to compare the performance of the two materials in modifying the electrodes, different modification sequences were proposed. The results indicated that AuNPs/CF/GCE modified sequences were better. The peak current of the modified sequences were stronger than bare the

GCE electrode (a) and CF/AuNPs/GCE (c) and could reach up to 14.43 μA. CF exhibited good biocompatibility and adsorbed more AuNP particles attached on the GCE electrode while the high conductivity of AuNPs resulted in a sharp increase of the current peak compared with the other two methods.

A similar situation happened on the gold electrode in Fig. 3. Fig. 3 was the current change of the Au electrode before and after the modification. Fig. 3 showed CV of the bare Au electrode (a) and different modified sequences of the electrodes, AuNPs/CF/Au (b) and CF/AuNPs/Au (c). We could see that peak currents of these three kinds of electrodes were different. The results indicated that AuNPs/CF/Au modified sequences were the better ones. The peak current of the modified sequences was stronger than the bare Au electrode (a) and CF/AuNPs/Au (c) and could reach up to 18 μA. AuNPs/CF/Au modified sequences were proven to be better than the others.

These two figures both showed that the best modification sequences of the two electrodes were AuNPs/CF. We could still find out that the Au electrode was better than the GCE electrode when they were modified by the same materials (both CF and AuNPs) and the same sequences (AuNPs/CF). Fig. 4 showed that the modified Au electrode (AuNPs/CF/Au) was more sensitive than the modified GCE (AuNPs/CF/GCE) in the same manner. In a word, AuNPs/CF/Au was the best modified electrode and the next experiment proceeded with this kind of modified electrode.

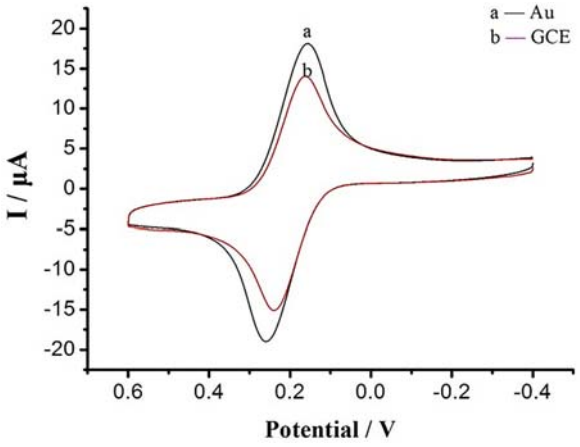

Fig. 4. The current responses of Au and GCE modified by CF and AuNPs. (a) AuNPs/CF/Au; (b) AuNPs/CF/GCE.

3.2. Optimization of the Testing Solution pH

Many biochemical reactions needed to be carried out within an appropriate pH range, and they generally had an optimal pH value that affected the activity and biochemical reactions of many biological substances.

In this experiment, the test solution pH was from 7.0 to 8.0, including 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0. Immune tests were performed in a 50 μL $[Fe(CN)_6]^{3-/4-}$ test solution with different pH values, and the differential value (ΔI) of the current in the test was obtained before and after antigen incubation. For each pH of the testing solutions, measurements were conducted three times and the average was obtained, and then their standard deviations were calculated and analyzed.

Fig. 5 showed CV of the immunosensor and the response current (I) was different before and after incubation by the test solutions with different pH values.

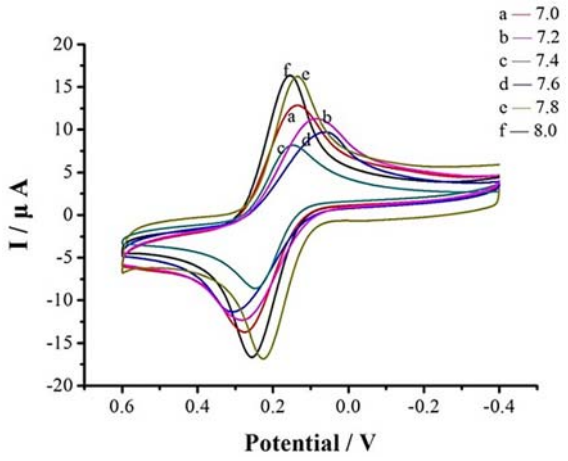

Fig. 5. The influence of the test solution pH on the immunosensor current before and after incubation.

Fig. 6 showed the current differences (ΔI) of the immunosensors before and after incubation by the test solutions of different pH values. The effects of the current differences on immunosensors before and after incubation treated by testing solutions with different pH values were displayed. When pH was 7.4, the best current difference (ΔI) was nearly 9.3 μA and NSPV (ΔI/I0) was almost 57 % in Fig. 6.

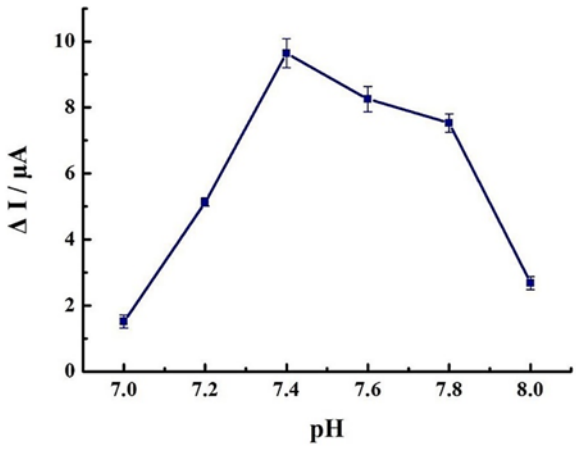

Fig. 6. The influence of the test solution pH on the current difference of the immunosensors before and after incubation.

But the basic current of different electrodes was different. This difference had a sufficient effect on the weak electrochemical immune current. To eliminate the difference of these electrodes, a normalization method needs to be applied to process the response currents.

Using the normalization method (NSPV) to treat the detection current in different PH solution environments, the formula is as follows:

$$NSPV = (I1 - I0)/I0 \times 100\% \quad (1)$$

In this equation, I1 was the response currents of the BSA/antibody/AuNPs/CF/Au biosensor in testing solutions when pH was different, I0 was the AuNPs/CF/Au biosensor in testing solutions when pH was different, $\Delta I = (I1 - I0)$.

The variation trend of the current change rate ($\Delta I/I0$) was shown in Fig. 7. According to Fig. 6 and 7, the electrode current continuously decreased after aflatoxin incubation, whereas ΔI continued to increase in the pH range from 7.0 to 7.4. Beyond this range, the response current started to increase and the current difference decreased. We found ΔI and $\Delta I/I0$ were both the biggest in the testing solution with the pH value 7.4. When the response current of the immune electrode was the largest, the antigen-antibody production was high and stable. When pH was inappropriate, the aflatoxin-antibody compounds were easily decomposed to reduce the impedance response. In the CV diagram in Fig. 5, we could also clearly see that when pH was 7.4, the value of the response current was the largest. As a result, the optimization pH of the testing solution was 7.4.

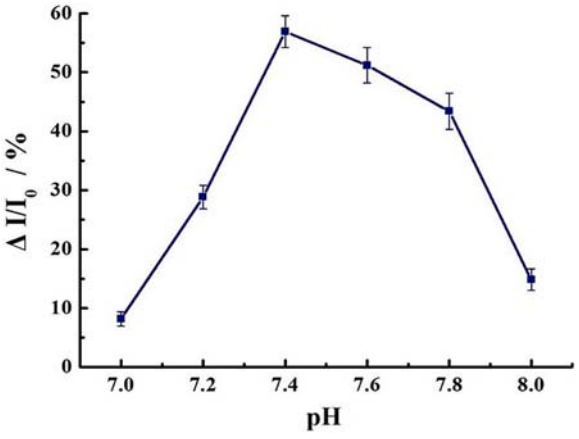

Fig. 7. The effect of the pH value on the immunosensors before and after incub.

3.3. Optimization of the Antibody Concentration

In the experiment, the AFB1 antibody was fixed on the electrode to recognize target antigen AFB1 in the sample. On the electrode, the antibody amounts directly indicated the performance of the sensor. If the concentration of the antibody solution was too high, molecular competition between antibodies existed and it would be difficult to increase the fixed antibody amounts so that antibodies were wasted.

Therefore, we should optimize the AFB1 antibody concentration. Different concentrations of antibodies (0.1, 1, 10, 40, 70 and 100 μg/mL) were used to fix on the electrodes. The influence of the antibody concentration was determined by current responses on the electrode, and the current was measured in the test solution of 50 μL $[Fe(CN)_6]^{3-/4-}$. The concentration gradients of the antibodies were 0.1-100 μg/mL and the average of each concentration gradient was measured three times.

Fig. 8 was the electrode response current graph under different antibody concentrations, and it demonstrated that the peak response current was the maximum when the antibody concentration was 10 μg/mL. Fig. 9 showed the influence of the antibody concentration on the immune electrode current difference (ΔI). ΔI was current difference on electrode before (I0) and after (I1) antibody immobilization with different concentrations.

In the same way, using the normalization method (NSPV) to treat the detection current when the antibody concentration was different, the formula is as (1).

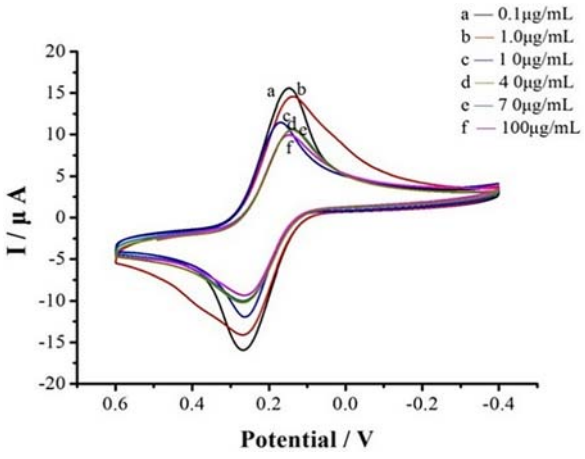

Fig. 8. Influence of the antibody concentration on the immune electrode current.

In this equation, I1 was the response currents of the BSA/antibody/AuNPs/CF/Au biosensor in the testing solutions when it was fixed with different concentrations of antibodies, I0 was the AuNPs/CF/Au biosensor current response in testing solutions, and $\Delta I = (I1 - I0)$.

Fig. 10 revealed the change of $\Delta I/I0$ along with the change of the antibody concentration. Obviously, under the antibody concentrations from 0.1 to 10 μg/mL, ΔI increased in response to the antibody concentration increase. Within the range of 10 μg/mL to 100 μg/mL, ΔI did not change much.

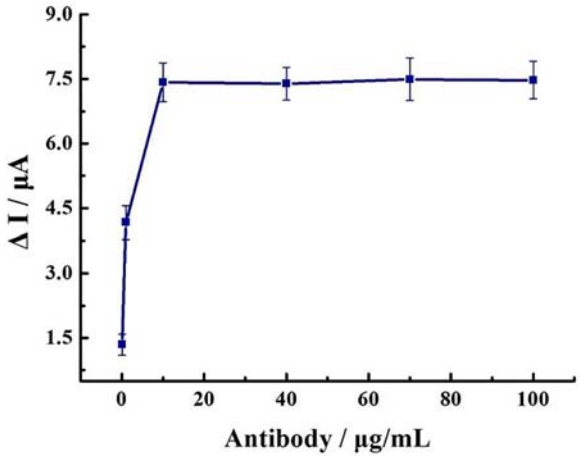

Fig. 9. The influence of the antibody concentration on the immune electrode current difference (ΔI).

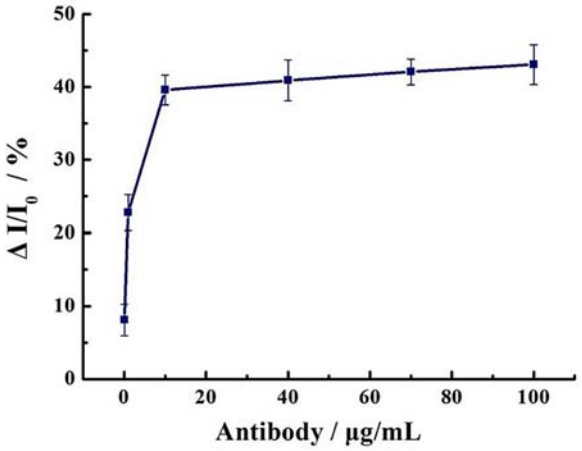

Fig. 10. The influence of the antibody concentration on the current of the immune electrode treated by NSPV (change rate of the current, ΔI/I0).

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. Excessive antibodies were not conducive to the subsequent capture of antigenic toxin. Therefore, the optimal concentration of the antibody load was 10 μg/mL, which was consistent throughout the experiment.

3.4. Optimization of the BSA Concentration

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

Fig. 11 showed the change trend of the current of the electrode response current of the sensor under different BSA concentrations, and it demonstrated that the peak response current was different when the BSA concentration was different.

Fig. 12 revealed the variation trend of current differences (ΔI) on the immune electrode at different BSA concentrations. It showed that when the changes in the BSA concentration ranged from 1 % to 2.5 %, the response current decreased as a result of BSA concentration increase, whereas ΔI increased as a response to BSA concentration increase. However, when the concentration was beyond 5 %, ΔI 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 ΔI before and after closure was not significant.

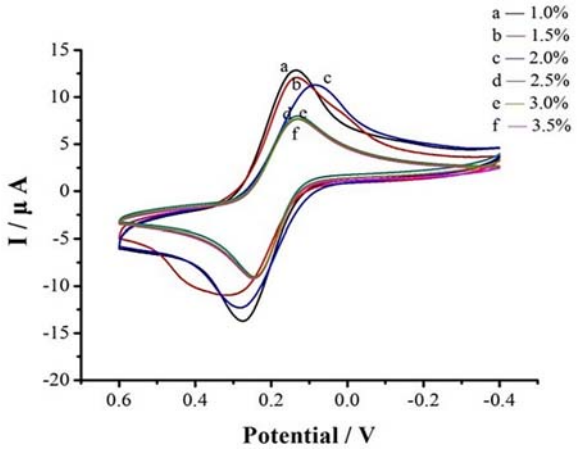

Fig. 11. The influence of the BSA concentration on the current of the immune electrode.

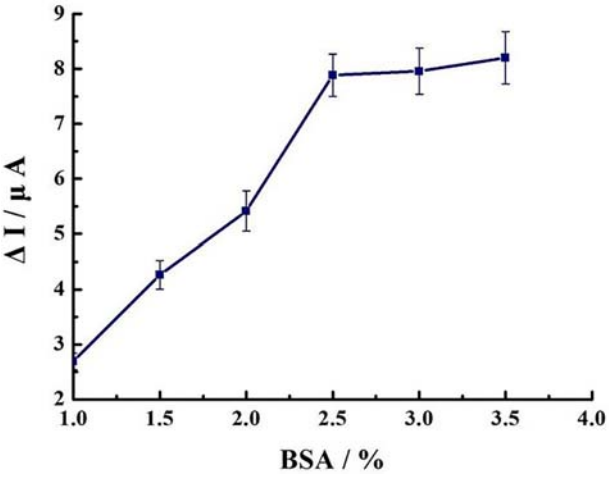

Fig. 12. The influence of the BSA concentration on the variation of current differences on the immune electrode.

In similar circumstances, using the normalization method (NSPV) to treat the detection current when the BSA concentration was different, the formula is as (1).

In this equation, I1 was the response currents of the BSA/antibody/AuNPs/CF/Au biosensor in testing

solutions when blocked with different concentrations of BSA, I0 was the AuNPs/CF/Au biosensor current response in testing solutions, and ΔI=(I1 – I0).

Fig. 13 showed the trend of ΔI/I0. It showed a similar situation with Fig. 12. When the changes in the BSA concentration ranged from 1 % to 2.5 %, the response current (I0) decreased as a result of BSA concentration increase, whereas the rate of response current change (ΔI/I0) increased with BSA concentration increase. However, when the concentration was beyond 5 %, ΔI/I0 did not change much.

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 optimum concentration of BSA was 2.5 %, and the concentration was used throughout the experiment.

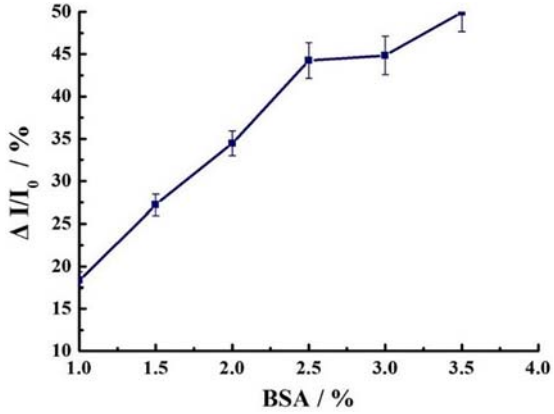

Fig. 13. The influence of the BSA concentration on the current of the immune electrode treated by NSPV

3.5. Immunosensor used for Standard AFB1 Sample Testing

Fig. 14 showed the changes in response currents generated by the electrode when AFB1 of different concentrations was tested. When the AFB1 concentration became higher, the antigen-antibody combination increased the impedance of the electrode surface, and the current response value decreased. The experimental results showed that the electrochemical biosensor had great potential in aflatoxin tests. Sample testing and performance testing of the sensor will be discussed in another article of ours.

4. Conclusion

In this article, we proposed a kind of immunosensor used for rapid and direct AFB1 detection based on gold nanoparticles (AuNPs) and carbon nanofibers (CNFs) modified Au electrode. In order to obtain the best construction method of the biosensor, experiment parameters were optimized according to the response current changes on the electrodes. The response current was tested through cyclic voltammetry (CV) techniques and the current changes were analyzed by the normalization method. The result showed these parameters, including the electrode type (Au), electrode-modified materials and its modification sequence (AuNPs/CF), pH of PBS (7.4), BSA concentration (2.5 %), and antibody concentration (10 μg/mL). With these optimized parameters, the immunosensor exhibited good performance and it provided a quantitative AFB1 detection method in rapid detection.

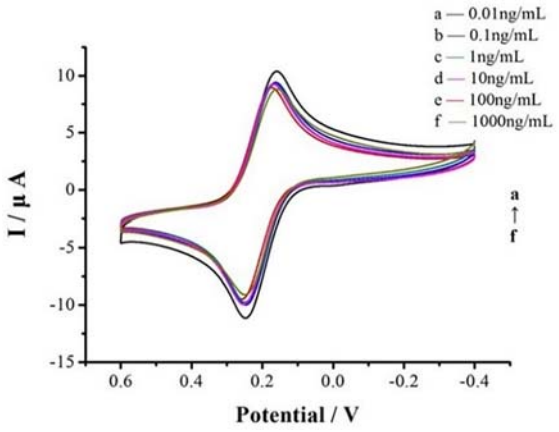

Fig. 14. Current changes on the electrode caused by aflatoxin at different concentrations.

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