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Ultrasensitive Immunosensor for Aflatoxin B_1 Detection Based on Screen-Printed Carbon Electrode Modified by Ferrocene @Multi-Walled Carbon Nanotubes

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An electrochemical immunosensor for the ultrasensitive detection of aflatoxin B_1 (AFB_1) in agricultural products was developed. Ferrocene (Fc), with favourable oxidation-reduction properties, is widely used in chemically modified electrodes. Multi-walled carbon nanotubes (MWNTs) have good catalytic activity, chemical stability and electronic transport properties. Chitosan (CS) can promote membrane formation and improve adhesion. Fc was fixed on the surface of a screen-printed carbon electrode (SPCE) via MWNTs and CS, and then the Fc@MWNT nanostructure was formed. The increased specific surface area of SPCE modified by Fc/MWNT/CS facilitated the binding of AFB_1-BSA, while the excellent electrical conductivity of Fc/MWNT/CS promoted electron transfer. These advantages not only amplified the immunosensor signal but also made the prepared immunosensor have high sensitivity, selectivity and stability. Under optimal conditions, the proposed immunosensor obtained a wide linear range from 10^{-3} ~2×10^{4} ng/mL and a low AFB_1 detection limit of 0.159 pg/mL via cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Its stability and effect on actual sample detection were also proven in this paper. This new immunosensor achieved favourable stability, reproducibility and selectivity and had potential application in real sample analysis.

Keywords: ferrocene, multi-walled carbon nanotubes, immunosensor, AFB_1

1. INTRODUCTION

Aflatoxin (AFT) is a secondary toxic metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus*. AFT can commonly be found in cereals, feed and other foods, which can enter the human

body through the diet. Among the AFT variants, AFB_1 is the most common, and its carcinogenicity and toxicity are the strongest. AFB_1 is also used as a common pollution index for food testing. Universal data also show that there is an urgent need for an efficient and sensitive method to detect widespread AFB_1 pollution in China. In recent years, the current detection methods mainly include stratography [1], chromatography [2], and biosensors [3,4]. According to basic principles, the electrochemical enzyme sensor for the detection of AFT can be divided into two kinds [5]: one is based on the inhibition of an antibody by AFT, and the other is the aflatoxin-oxidase (AFO), which catalyses a reaction at unsaturated carbon bonds. Daling Liu et al. [6] constructed an AFB_1 electrochemical enzyme biosensor for the first time in 2008. In two years, this group [7] had improved the original method. AFO was embedded in a sol-gel, and a platinum electrode modified by MWNTs was combined with it, which provided a good environment for enzyme fixation and maintaining the activity of the enzyme. Despite the fact that the enzyme sensor is simple to prepare and easy to commercialize, its selectivity and sensitivity are deficient compared with those of the immunosensor. Cancan Gu et al. [8] proposed a label-free homogeneous electrochemical method for AFB_1 detection in 2017. On the basis of that aptamer, the toxin could be identified specifically. The characteristic short chain DNA would form after the DNA chain labelled by ferrocene on the ITO electrode was cut by an enzyme, and its increased diffusivity enhanced the electrical signal to complete the identification and conversion of signal. Chao Mi et al. [9] made a new preparation of an electrochemical DNA biosensor in 2018: a glassy carbon electrode was modified by N-G/AuNP composites, methylene blue (MB) was used as an indicator, and a connector was produced by Au-S bonds formed between AuNPs and sulfhydryl DNA probes. Immunosensors were a new concept proposed by Henry et al. [10] in 1990, and the combination of sensor technology and immunology can not only improve the specificity of detection but also detect the target more accurately [11] because of the great sensitivity. An immunosensor is relatively portable and easy to operate; furthermore, its operation is highly automated [12]. However, the generation of antigen antibodies is not mature, and precision and sensitivity remain to be improved. Furthermore, mass production and multivariate detection are still difficult to achieve [13]. Electrochemical immunosensors are based on antigen-antibody reactions [14] and can carry out specific quantitative analyses. With the antigen and antibody on electrochemical sensing elements for molecular recognition, electrical signals can be converted by a series of concentration signals [15]. This device has the advantages of simplicity, good selectivity, efficient detection and being easily realizable online [16]. Qing Sun et al. [17] developed a monoclonal antibody specifically for AFB_1 and AFM_1 and specifically established a rapid AFT detection method for edibles and dairy products. Furthermore, a matching AFT ELISA Kit and immune detection strip were developed, which provided a relatively fast and effective method for AFT pollution detection. Y. Uludag et al. [18] reported a biochip and automated MiSens electrochemical biosensor for the detection of AFB_1 in wheat and figs, with a recovery rate of 80%~98% and a detection limit of 0.33 ppb. Due to the strong toxicity and carcinogenicity of AFT, which seriously affect human health, it is necessary to develop a reliable and sensitive technology for AFT detection.

In this paper, the electrode modification method of an immunosensor based on an ordered Fc/MWNT composite nanomaterial was developed and used for AFB_1 detection. Fc with favourable oxidation-reduction properties is widely used in chemically modified electrodes [19]. However, Fc is difficult to adsorb to the surface of the electrode, so other materials need to be used to improve the

fixation, such as MWCNTs and CS [20,21]. The catalytic activity, chemical stability and electron transfer of MWNTs are excellent. CS contains many active amine and hydroxyl groups and is non-toxic and inexpensive. In addition, CS has biocompatibility, great film formation and adhesion ability [22], which makes it widely used in the fixation of biomolecules and preparation of modified electrodes.

2. EXPERIMENTAL

2.1 Instruments and Reagents

The test in this study was an electrochemical operation on the CHI660C electrochemical workstation produced from the Shanghai Chenhua Co., China. All screen-printed carbon electrodes were purchased from Chan Pu Polytron Technologies, Inc.

An electronic analytical balance (AL-104) was produced by Metle TOLI Instrument Co., Ltd. An ultra-sonic cleaner (SK3300H) was produced by Shanghai Kedao Ultrasonic Instrument Co., Ltd. A pH meter (FE20K) was produced by Shanghai Zhiguang Instrument Co., Ltd. A digital display constant temperature magnetic stirrer (GL-3250A) was produced by Metle TOLI Instrument Co., Ltd. A high-speed centrifuge was produced by Shanghai Anting Scientific Instrument Factory, and the usable range of pipettes (Eppendorf) was 1~5000 μL. The PALL ultra-pure water system (LS MK2) was produced by the United States Pall Corporation, and a programmed mixer (PRT-35) was produced by the British Grant Company.

Aflatoxin- and AFB_1-specific antibodies (Ab-AFB_1) were purchased from the Chinese Academy of Agricultural Sciences Oil Crop Research Institute. CS was purchased from Shanghai Chemical Reagent Co., Ltd. Potassium chloride was purchased from Tianjin Yongsheng Fine Chemical Co., Ltd. Potassium ferricyanide/potassium ferrocyanide was purchased from the Fine Chemical Plant of Laiyang Economic and Technological Development Zone, and sodium dihydrogen phosphate/disodium hydrogen phosphate was purchased from Tianjin Northern Tian Yi Chemical Reagent Factory. Bovine serum albumin (BSA) was purchased from Sigma. Ferrocene was purchased from Tianjin Red Rock Chemical Reagents Factory, and carboxylated MWNTs with length of 10~30 mm and outer diameter of 10~20 nm were purchased from Nanjing Xianfeng Nano Co., Ltd. All of the reagents were analytically pure, and water was obtained from an ultra-pure water machine (18.2 MΩ·cm) produced by the PALL company.

2.2 Preparation of Nanocomposite Materials

The methods of Dongxiang Xie et al. [23,24] were used to prepare Fc composite materials: first, 0.1 g CS powder was added into 50 mL acetic acid solution (concentration 1.0%) to obtain CS solution (0.2%). The CS was dissolved fully after more than 8 h of magnetic stirring. Then, NaOH was added to adjust the prepared CS solution pH to 5. Excessive Fc was added to the CS solution (more than 10 mL) and then stirred fully to ensure that the inclusion occurred. After standing completely, 25 mg MWNTs was added to 5 mL supernatant liquor, and ferrocene composite materials (uniform, black, turbid liquid)

were obtained after 60 min of ultrasonic dispersion. The Fc/MWNT/CS injectable suspension was kept at 4℃.

2.3 Pretreatment of the SPCE

The working area of the SPCE (TE100) was 0.071 cm^2, and the diameter was only 3 mm. Referring to the literature [25,26], the working part was immersed completely in PBS (0.05 M, pH 7.4), then characterized by a voltammeter (1.7 V) for 180 sec to observe its electrochemical stability. Later, cyclic voltammetry was performed for electrodes that were not pretreated and electrodes that were pretreated in $K_3[Fe(CN)_6]$ solution. The peak potential difference (ΔEp) was reduced to 100 mV with pretreated electrodes, and the charge moved faster with them, which indicated that $K_3[Fe(CN)_6]$ was effectively activated and the reversible process could be realized. The surface of the electrodes became cleaner due to the oxidation of impurities caused by high potentials.

2.4 The Preparation of Immunosensors

Eight microliters of Fc/MWNT was added to the surface of SPCE and dried naturally to obtain Fc/MWNT/SPCE. Then, AFB_1 monoclonal antibody (Ab-AFB_1) that was diluted with PBS (pH 7.4) to 10 ng/mL was added to this electrode, and the Ab/Fc/MWNT/SPCE electrode was desiccated at room temperature. Eight μL of 0.5% BSA solution was added to the above electrode, and the BSA/Fc/MWNT/Anti/SPCE electrode could be obtained by natural drying in air. The prepared electrode should be kept at 4°C.

2.5 Electrochemical Test Methods and Principles

This experiment was carried out through indirect competition with the immune response. Free AFB_1 and AFB_1-BSA coupling in the mixture competed for a limited amount of Ab-AFB_1 on the electrode.

Then, the electrode performance was tested in 0.1 M PBS (pH 7.0, including 0.1 M KCl and 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in a 1:1 ratio). Cyclic voltammetry (CV, scanning range -0.6~1.0 V, scanning speed 0.1 V/s, static time 2 s) and differential pulse voltammetry (DPV, pulse amplitude 50 mV, static time 2 s, pulse period 0.5 s, scanning range -0.1~0.4 V) were used to measure at room temperature. All the data points were obtained in triplicate, and each value was the average of three kinds of data. The oxidation peak steady current produced by the specific identification by the immunosensor before the reaction was recorded as I_0, and the oxidation peak steady current produced after the full reaction (immunosensor and AFB_1 standard solution) was recorded as I, so the response current (ΔI) was:

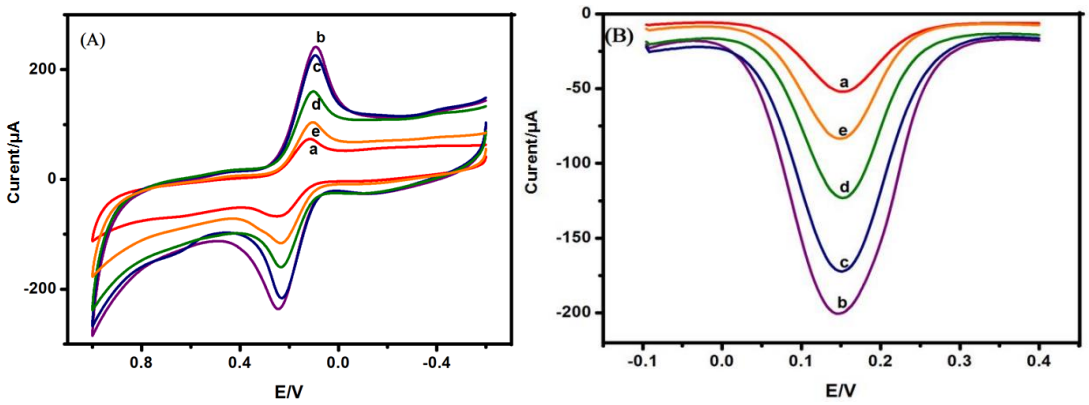
$$\Delta I = I_0 - I$$

2.6 The pretreatment of practical samples

Mashed and ground the peanuts, rice and corn were purchased from the supermarket and weighed to 5 g by electronic analytical balance (AL-104). Afterward, moderate AFB_1 buffer and 100 mL phosphate buffer was added to them, and they were whirlpool blended for 1 min. Then 25 mL extracting agent (+1% DMF+29% PBS+70% methanol) was added. The supernatant was removed after 15 min of manual uniform stirring and 5 min of centrifugation (4000 r/min). Moderate phosphate buffer was used to dilute the supernatant concentration to 1 tenth of the original solution concentration. The method for detecting AFB_1 was the same as that of the previous test.

3. RESULTS AND DISCUSSION

3.1 Electrochemical Characterization of Assembly of Immunosensors



(A) CV (B) DPV

Figure 1. CV response and DPV of different substances assembled on the screen-printing carbon electrode modified with Fc/MWNT/CS: (a) bare electrode, (b) Fc/MWNT/SPCE, (c) Ab/Fc/MWNT/SPCE, (d) BSA/Ab/Fc/MWNT/SPCE, and (e) AFB_1/BSA/Ab/Fc/MWNT/SPCE

CV curve characterization was performed in PBS (pH 7.0) containing 5 mM $[Fe(CN)6]^{3-/4-}$ and 0.1 mol/L KCl. The symmetric trend of the redox peak (curve a) of the bare electrode is obvious in Fig 3.1 (A); the peak current (curve b) increased significantly when the bare electrode was modified by Fc/MWNT/CS, indicating that MWNT/CS can promote the transfer of $[Fe(CN)6]^{3-/4-}$ electrons in solution on the surface of the electrode effectively and increase the specific surface area. Additionally, the amount of AFB_1-BSA fixed on the electrode increased significantly. The result is in accordance with other reports [27]. The peak current decreased (curve d) with the increase of resistance after the modified immunosensor fully integrated anti-AFB_1, which indicated that the construction of the sensor for anti-AFB_1 had been completed and recognized anti-AFB_1. When anti-AFB_1 was added to the surface of activated electrodes, electron transfer was hindered by the insulation and steric hindrance produced by anti-AFB_1, which clearly decreased the peak current (curve c). As shown in Fig 3.1 (B), the DPV response was consistent with CV, and the peak current increased significantly after MWNT/CS covered

the surface of the bare electrode. In contrast, the peak current decreased gradually after the modified electrode was incubated at the temperature shown for AFB_1-BSA (curve c) and anti-AFB_1 (curve d). The results showed that the electrode was modified effectively.

3.2 Parameter optimization of immunosensors

The performance of the immunosensor was affected by many factors, including the pH of PBS, incubation time of specific binding to target material, and antibody concentration. The test parameters that have the greatest effects need to be optimized.

The current response of the immunosensor was affected easily by the pH of the test buffer solution. To this end, a series of PBS solutions (pH 5.0~9.0) was produced for the detection of immunosensors. After much data analysis, the current value was determined to be proportional to the pH when the pH of the test solution was between 5.5 and 8.0; when the pH was below 5.5 or above 8.0, the current value was inversely proportional to the pH (Fig 3.2 A). The change in the current value was most obvious when the pH of the test base solution was 8.0, so 8.0 was the optimal pH of the experiment. This selection of optimal pH occurred similarly to that in the previous literature [28].

Since a certain amount of BSA on the electrode surface would compete with AFB_1 to bind the antibody, the test amount of the target substance was determined by the concentration of the antibody, which meant that the effective binding amount of the antibody was limited. Having excess antibody can waste antibody, but the detection of the target object cannot be completely effective if the concentration of antibody is low. Therefore, the concentration of antibody as a critical parameter in the incubation liquid that would directly affect the performance of the sensor. To obtain the optimal concentration of antibody, the modified Fc/MWNT/CS/SPCE was incubated in antibody solution at different dilution ratios. After CV and series comparisons, the optimal concentration of antibody was obtained. As shown in Fig 3.2 (B), the maximum redox peak was reached when the concentration was 600 ng/mL. When the concentration was above or below 600 ng/mL, the redox peak value was lower, so the optimal antibody concentration was 600 ng/mL.

The response signal of the sensor was also strongly affected by incubation time, which directly affected the degree of immune response that was sufficient. At room temperature, AFB_1 was added to the surface of the electrodes, and then multiple independent electrodes reacted in turn for 10~120 min (the mean interval was 10 min). The results showed that the current response reinforced constantly with increasing slack time during 10~30 min. The current response remained essentially the same after 30 min, and there was a downward trend with increasing time (Fig 3.2 C). The best time for antibody incubation was 30 min when the concentration of antibody was defined, and the result is mostly consistent with our previous studies [29].

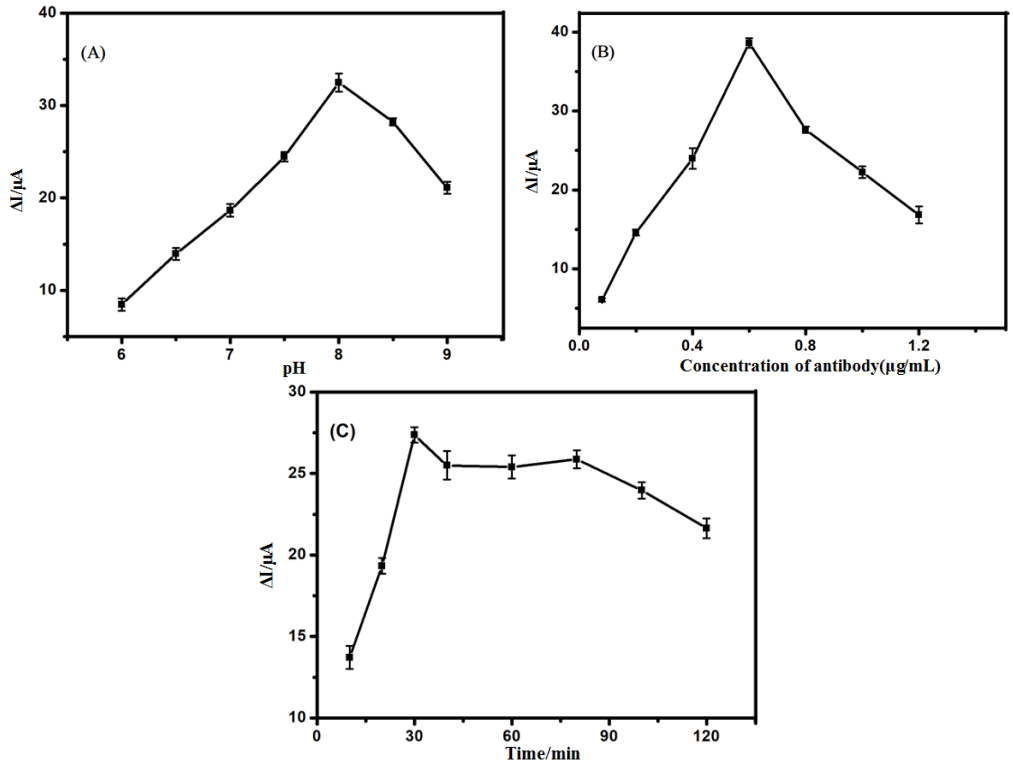


Figure 2. (A) Optimum pH of PBS: 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; and 9.0. (B) Optimum concentration of the antibody: 100 ng/mL; 200 ng/mL; 400 ng/mL; 600 ng/mL; 800 ng/mL; 1000 ng/mL; and 1200 ng/mL. (C) Optimum incubation time: 10 min; 20 min; 30 min; 40 min; 50 min; 60 min; 70 min; 80 min; 90 min; 100 min; 110 min; and 120 min.

3.3 The current response characteristics of the immunosensor

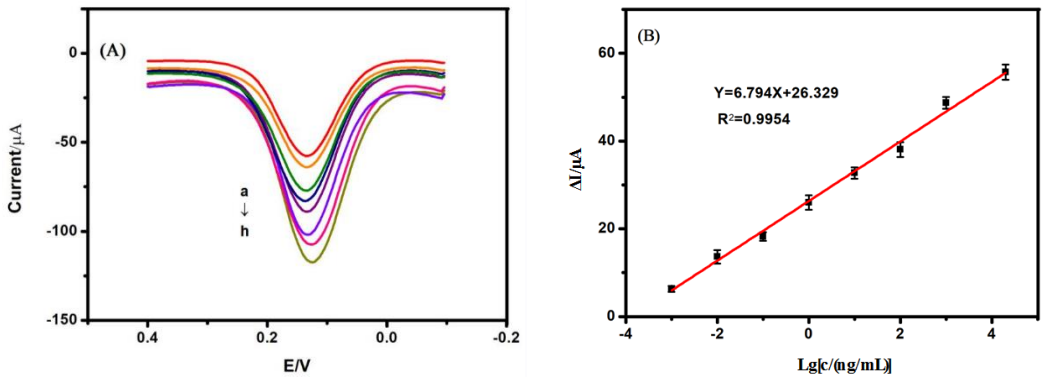


Figure 3. (A) Effect of different concentrations of AFB_1 standard liquid on immunosensor current under optimal experimental conditions: (a to h) 2×10^4, 10^3, 10^2, 10, 1, 10^{-1}, 10^{-2}, 10^{-3} ng/mL; (B) Working curve of the immunosensor: current variation and logarithm of AFB_1 concentration.

The performance of the immunosensor was verified by analysis of AFB_1 standards at different concentrations in PBS (pH 7.4). The current response detected by DPV is shown in Fig 3.3 (A). The current peak decreased with increasing AFB_1 concentration. The current peak had a linear correlation with the logarithm of AFB_1 concentration between 10^{-3} ~2×10^4 ng/mL. As shown in Fig 3.3 (B), the linear equation was Y=6.794X+26.329, and the correlation coefficient was 0.9954. Three times the

standard deviation (SD) was used in this linear equation to estimate its detection limit, and the detection limit was 0.159 pg/mL.

Table 1. The performance comparison of the present device with typical reported methods for AFB_1.

Method	Linear range (ng/mL)	Detection limit (ng/mL)	Reference
Chemiluminescence	0.1-10	0.11	[30]
Fluorescence	0.5-20	0.16	[31]
Quartz crystal microbalance	0.3-8.5	0.3	[32]
Electrochemistry	0.1-10	0.09	[33]
Electrochemiluminescence	0.01-100	0.0039	[34]
Photoelectrochemistry	0.01-20	0.0021	[35]
ELISA	0.5-20	0.16	[36]
Immunosensor	10^{-3} -2×10^{4}	0.159×10^{-3}	This work

3.4 Performance analysis of immunosensors

The specificity of the immunosensor was tested by incubating α-ZEN, OTA, FB_1, ZEN, AFM_1 (all above were 5 μg/mL) and AFB_1 (100 ng•mL^{-1}) for 30 min in the prepared immunosensor. As shown in Fig 3.5, the response current of DPV to five kinds of mycotoxins was close to that of the blank sample, but the response current of DPV to AFB_1 was still significant. An error bar chart shows the standard deviation of three tests. The immunosensor was sufficient for identification of different types of mycotoxins and had a good specificity.

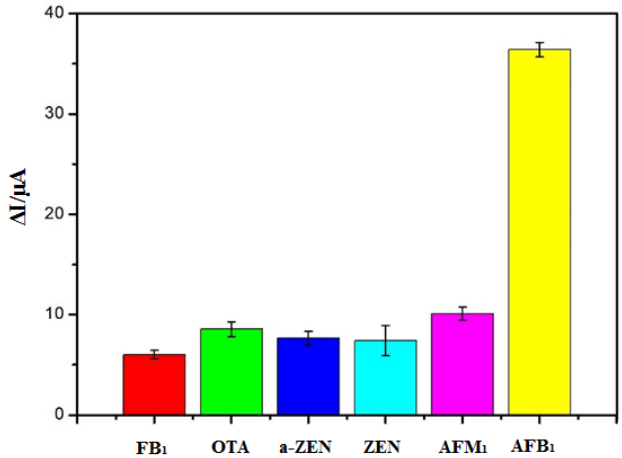


Figure 4. Specific analysis of immunosensors

The prepared immunosensor was kept at 4°C and tested after three, five, seven, ten, and fourteen days. As shown in Fig 3.5, the response current of the working electrodes remained 97.22% of the

original response current after 3 days, 93.87% of the original after 7 days, and 89.3% of the original after 14 days. The results showed that the sensor was stable.

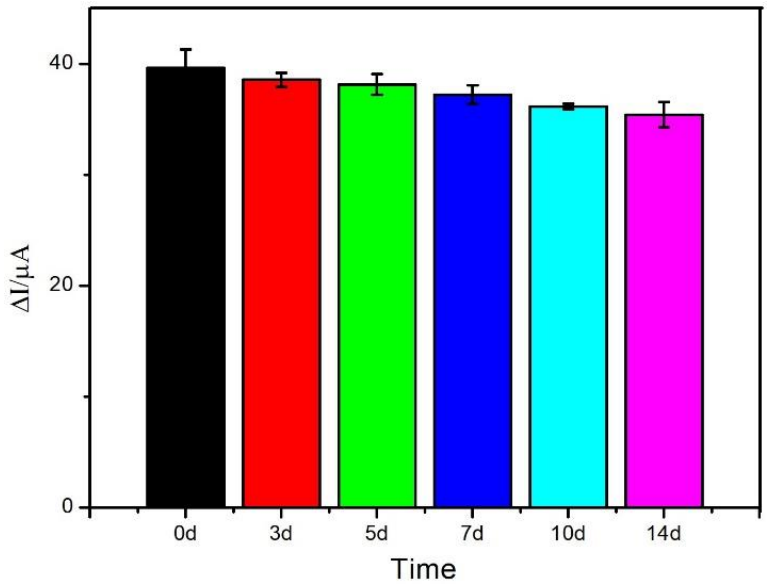


Figure 5. Stability analysis of immunosensors

3.5 Detection of practical samples

Table 2. Actual recovery rate of AFB_1 detection

Sample	Addition of AFB_1 (μg/L)	Test quantity of AFB_1 (μg/L)	Recovery rate (%)
rice	1.0	0.935	93.5
peanut	1.0	1.023	102.3
corn	1.0	0.957	95.7

To verify the feasibility and reliability of the prepared immunosensor for the detection of practical samples, 1 μg/L AFB_1 was added separately to practical samples (rice, peanuts and corn) and the recovery rate was detected. As shown in Table 2, the recovery rate was between 93.5% and 102.3%, indicating that the detection by practical samples of the sensor was excellent, and that they can be used for the construction of a detector.

4. CONCLUSIONS

The electrode modification method for immunosensors based on ordered Fc/MWNT composite nanomaterials was developed and used for the detection of AFB_1. The successfully constructed MWNT/CS can improve the sensitivity of immunosensors. Because Fc has good conductivity and the MWNT/CS composite material has an increased biocompatibility and specific surface area, the

immunosensor had increased sensitivity and stability. Moreover, the preparation process for the electrode was simple and easy to learn. The test results were satisfying in the detection in practical samples, including corn flour, peanut powder and rice flour. In addition, the pretreatment of samples was not complicated, which provided the method with high sensitivity, simplicity and efficiency for the detection of AFB_1 in practical samples. In this study, an electrochemical method was used to characterize an immunosensor during assembly by DPV and CV. The optimal pH of the test liquid was 8.0, the optimal antibody concentration was 600 ng/mL, and the optimum incubation time was 30 min. All of these values were determined by optimizing the experimental conditions. The linear equation of the sensor was Y=6.794X+26.329, and the correlation coefficient was 0.9954 over the concentration range was 1×10^{-3}~2×10^{4} ng/mL. This range was obtained by detecting AFB_1 samples at a series of concentrations. These results indicate that the sensor has good sensitivity and stability and can be used to detect AFB_1 in food.

ACKNOWLEDGMENTS
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