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Analytical Methods

A novel electrochemical immunosensor for highly sensitive detection of aflatoxin B_1 in corn using single-walled carbon nanotubes/chitosan

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ABSTRACT

A sensitive electrochemical immunosensor for aflatoxin B_1 (AFB_1) detection based on single-walled carbon nanotubes/chitosan was presented. The immunosensor was based on an indirect competitive binding to a fixed amount of anti-AFB_1 between free AFB_1 and AFB_1-bovine serum albumin, which conjugate immobilized on covalently functionalized nanotubes/chitosan laid on the glass carbon electrode. Then, the anti-mouse immunoglobulin G secondary antibody labeled with alkaline phosphatase was bound to the electrode surface through reacting with primary antibody. Finally, alkaline phosphatase catalyzes the hydrolysis of the substrate α-naphthyl phosphate, which produced electrochemical signal. Compared with conventional methods, the established immunosensor was more sensitive and simple. Under optimal conditions, this method could quantitatively detect AFB_1 from 0.01 to 100 ng mL^{-1} with a detection limit of 3.5 pg mL^{-1}. Moreover, the immunosensor was successfully applied to assay AFB_1 in corn powder, which showed good correlation with the results obtained from high performance liquid chromatography.
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1. Introduction

Aflatoxins, a group of structurally related toxic secondary metabolites of mycotoxins, are primarily produced by Aspergillus flavus and Aspergillus parasiticus groups of fungi that could be found in corn, nuts, peanuts, coconut, dried fruits and beer (Frenich, Vidal, Romero-Gonzalez, & Aguilera-Luiz, 2009; Li, Zhang, & Zhang, 2009; Li, Zhang, Zhang, Chen, et al., 2009; Williams et al., 2004). There are four major aflatoxins named as B_1, B_2, G_1 and G_2. Among them, aflatoxin B_1 (AFB_1) is classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC) as it may interfere the inductive of specific enzymes and forbid the synthesis of RNA (IARC., 2002; Merrick et al., 2013; Wild & Montesano, 2009). Owing to the highly resistant to degradation to survive during food processing, AFB_1 could enter the food chain and provide a threat to human health (Castells, Ramos, Sanchis, & Marin, 2007). Therefore, the regulatory limits for aflatoxins ($B_1 + B_2 + G_1 + G_2$), even for AFB_1, have been established in several countries. The European Commission has set strict limits for the maximum allowed levels (MAL) of AFB_1 in ground-nuts, dried fruits and their products, in which the MAL of AFB_1 could not be greater than 2 μg kg^{-1} for Retail Ready Foods (van Egmond, 1995). It has been proved that the intake of AFB_1 over a long time may be dangerous even at a very low concentration. Thus, the assays with high sensitivity and specificity are required to determine aflatoxins at trace level in foods and agricultural products.

During the past few years, many analytical techniques for quantitative determination of mycotoxin have been studied, including thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), which are sensitive but typically require skilled operators, extensive sample pretreatments, expensive instruments and time-consuming, thus impairing their applications in food products detection (Jaimez et al., 2000; Stroka & Anklam, 2002). Enzyme-linked immunosorbent assays (ELISA) relying on the specific binding between toxin target antigen and its antibody are widely used for mycotoxin detection as well. However, the main disadvantages of the ELISA are the high volumes, the time necessary for the assays and the frequent negative or positive false screening results (Kolosova, Shim, Yang, Eremin, & Chung, 2006; Li, Zhang, & Zhang, 2009; Li, Zhang, Zhang, Chen, et al., 2009; Liu, Tsao, Wang, & Yu, 2008). Therefore, it is urgent to develop a novel strategy for mycotoxin detection.

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With the development of nanotechnology, the immunosensor with high specificity and sensitivity is currently one of the most active areas of analytical research for mycotoxin assay (Das, Aziz, & Yang, 2006; Ricci, Volpe, Micheli, & Palleschi, 2007; Wang, 2007). In recent years, single-walled carbon nanotubes (SWNTs) have been widely applied in the immunosensor fabrication to improve the sensitivity. SWNTs have unique electronic and mechanical properties (Singh et al., 2009). Owing to its physical and chemical characters, SWNTs had demonstrated a strong predominance in the manufacture of electrochemical immunosensors (Guo, 2013). Its considerable specific superficial area could increase the quantities of enzymes immobilized, enlarge the reacting areas between enzyme and substrate, improve electrical conductivity, and enhance the biosensors responsibility (Babaei, Afrasiabi, & Babazadeh, 2010; Singh et al., 2009). On the other hand, with the strong attractive interaction among the nanotubes, SWNTs could not disperse stably in aqueous solution. To overcome the stable insolubility, chitosan, a low acetyl substituted form of chitin and having a unique property of biocompatibility, has been adopted. When composited with SWNTs, chitosan could enhance the dispersive property of SWNTs (Guo, Zheng, Mo, & Ye, 2009; Najeeb, Chang, Lee, Lee, & Kim, 2011). Herein, we designed a novel electrochemical immunosensor based on an indirect competitive format to a fixed amount of anti-AFB_1 between free AFB_1 and AFB_1-bovine serum albumin, which conjugate immobilized on covalently functionalized nanotubes/chitosan using single-walled carbon nanotubes/chitosan (SWNTs/CS) for signal amplification. The aim of this work is to provide a sensitive and specific method for quantitative determination of AFB_1 in foods and agricultural products.

2. Materials and methods

2.1. Reagents

AFB_1-BSA and anti-AFB_1 were provided by Huaan Magnech Bio-Tech Ltd. Co. (Beijing, China). AP-anti-antibody, AFB_1, Chitosan, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA) and α-naphthyl phosphate (α-NP) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Carboxylic group-functionalized SWNTs (<5 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China). The corn flour samples were purchased from Trilogy Analytical Laboratory, Inc. (Washington, Mo.). Other reagents were of analytical grade and all aqueous solutions were prepared using Millipore-Q water ($\geqslant$18 MΩ). 0.01 M phosphate-buffered saline (PBS), as washing buffer, contained 2.7 mM KCl, 14 mM KH_2PO_4, 87 mM Na_2HPO_4 and 136.7 mM NaCl (pH 7.4). Diethanolamine (DEA) buffer contained 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl (pH 9.6).

2.2. Apparatus

All the electrochemical measurements including differential pulse voltammetry (DPV), cyclic voltammetry (CV) and square wave voltammetry (SWV) were carried out using a model CHI 660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China). All the measurements were performed with a conventional three-electrode system that composed of an Ag/AgCl saturated with KCl as reference electrode, a platinum wire as auxiliary electrode and a 3-mm-diameter GCE modified with the SWNTs/CS as working electrode at room temperature. Scanning electron microscopic (SEM) image was taken with the S-3000N scanning electron microscopic (Hitachi, Japan).

2.3. Design of electrochemical biosensor

As illustrated in Scheme 1, a sensitive electrochemical immunosensor for AFB_1 detection in corn using single-walled carbon nanotubes/chitosan (SWNTs/CS) was designed. Firstly, a glass carbon electrode (GCE) with AFB_1-bovine serum albumin (AFB_1-BSA) conjugated covalently to functionalized single-walled carbon nanotubes/chitosan (AFB_1-BSA-SWNTs/CS/GCE) was prepared. Because of the excellent electrical conductivity and relatively large ratio surface of SWNTs/CS, there were more antigens for competitive recognition to anti-AFB_1 and the up-limit of the detectable range was extended. Then plenty of alkaline phosphatase (AP)-anti-antibody selectively bound to the electrode surface via the primary antibody. The AFB_1 concentration in the sample could be quantitated exactly. AP was introduced into electrode surface to catalyze the hydrolysis of α-naphthyl phosphate (α-NP), which produced electrochemical signal (Wang et al., 2013). A large surface area was furnished by the SWNTs/CS film assembled on the GCE and resulted in greatly augment the quantity of antigens fabricated on the surface. Otherwise, the SWNTs/CS film could likewise improve the electron transport between the GCE and the sample liquid (Li et al., 2013).

2.4. Preparation of SWNTs/CS nanocomposite

5 mg carboxylic group-functionalized SWNTs fine powders was dispersed into 5 mL 1 mg mL^{-1} CS aqueous solutions. Then the mixed liquor was sonicated for 2 h to obtain uniform mixture of SWNTs/CS nanocomposite. Before use, the SWNTs/CS dispersion liquid was stored at 4 °C (Luo et al., 2013).

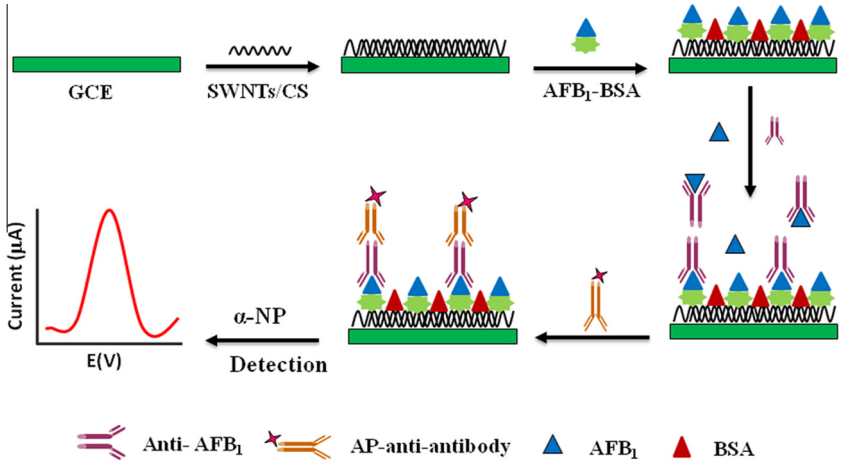
2.5. Preparation of electrochemical immunosensor

The unmodified GCE was firstly polished to a mirror with alumina slurry (particle diameters 0.05 μm) and then ultrasonically rinsed in ultrapure water for a few minutes. After sonicated in 1:1 (v/v) acetone and nitric acid, the electrode was ultrasonically rinsed once more in ultrapure water for a few minutes and followed by rinsing with deionized water. After dried at room temperature, 2 μL of the SWNTs/CS nanocomposite dispersion liquid (1 mg mL^{-1}) was dropped carefully onto the surface of the cleaned GCE. Finally, the modified GCE was dried at room temperature overnight in order to obtain the SWNTs/CS/GCE.

After rinsed with PBS, the SWNTs/CS/GCE was immersed into 50 μL freshly prepared activate fluid containing EDC and NHS (5 mM:2 mM) and incubated in a chamber at 37 °C for 1 h to activate the carboxylic group functionalized SWNTs. After the activation, SWNTs/CS/GCE was thoroughly washed with PBS. Following, 10 μL of 10 μg mL^{-1} AFB_1-BSA was immediately dropped on the electrode surface and then incubated at 37 °C for 1 h. Subsequently, the fabricated electrode was rinsed with PBS and further treated in 10 μL 5 mg mL^{-1} BSA solution at room temperature for 30 min to block the unreacted active sites on the surface. Finally, the electrode was rinsed with PBS thoroughly again, and the AFB_1-BSA electrochemical immunosensor based on SWNTs/CS/GCE was successfully obtained and stored at 4 °C prior to use.

2.6. Electrochemical measurements

To perform the competition assay, 5 μL of diluted anti-AFB_1 (1:150, v/v) was firstly blended with 5 μL of AFB_1 standard solution, with concentrations ranging from 0 to 500 ng mL^{-1} in PBS. The miscible liquids were added onto the surface of the modified AFB_1-BSA electrochemical immunosensor and then incubated at 37 °C for 90 min. During the incubation, a competition between



Scheme 1. Schematic illustration of the strategy for AFB_1 assay using GCE modified with a single-walled carbon nanotubes/chitosan film.

the immobilized AFB_1-BSA and free AFB_1 in the mixture to a rationed anti-AFB_1 in the mixture was carried out. After rinsed with PBS, the prepared immunosensor was incubated at 37 °C for 90 min with 10 μL of diluted AP-anti-antibody (1:200, v/v). Washed with PBS, the resulting electrode was finally immersed into the freshly DEA solution with 0.75 mg mL^{-1} α-NP. The differential pulse voltammetry (DPV) measurement was performed with modulation time of 0.05 s, potential scan from 0 to 0.5 V, pulse amplitude of 70 mV, a pulse period of 0.2 s and interval time of 0.017 s at room temperature.

2.7. Sample preparation

The corn powder contained AFB_1 was prepared by Trilogy Analytical Laboratory, Inc. (Washington, Mo.) using AOAC method 994.08 with modifications. A 25 g finely pulverized corn powder was extracted with 125 mL methanol–water (60:40, v/v) contained 5 g NaCl for 30 min on a high speed vortex. 5 mL extract was diluted with 10 mL PBS (pH 7.4), filtered on a microfiber glass filter (Whatman 934-AH), and applied to an immunoaffinity column at a flow rate of approximately 1 drop/s. The column was washed with 10 mL deionized water to remove interfering compounds, and the adsorbed AFB_1 was eluted with 1 mL methanol and collected in a clean vial. After that, the AFB_1 of the solution was measured by HPLC to be 7.3 ppb. Then the obtained sample solution with AFB_1 was diluted into different concentration (121.5, 40.5, 13.5 pg mL^{-1}) using PBS. Before use, the samples were stored at −20 °C.

3. Results and discussion

3.1. Characterization of the SWNTs/CS composite

Fig. 1 showed the scanning electron microscopic (SEM) images of the pure SWNTs (Fig. 1A) and SWNTs/CS composite (Fig. 1B). As seen from the photographs, the pure SWNTs powders still exhibited nanometer rod crystals due to the aggregations of SWNTs. After mixed with chitosan, SWNTs powders had a good dispersity, which displayed uniform morphology and offered a large biocompatible film surface area.

3.2. Characterization of the immunosensor

To monitor the interface properties of the electrochemical immunosensor, square wave voltammetry (SWV) and cyclic voltammetry (CV) measurements were taken after each step. Fig. 2A showed the cyclic voltammograms of different fabricated electrodes taken in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.5 M KCl: the bare GCE (curve a), SWNTs/CS/GCE (curve b), AFB_1-BSA-SWNTs/CS/GCE (curve c), anti-AFB_1/AFB_1-BSA-SWNTs/CS/GCE (curve d) and AP-anti-antibody/anti-AFB_1/AFB_1-BSA-SWNTs/CS/GCE (curve e). The peak current increased obviously after the SWNTs/CS film was dropped on GCE surface (curve b), the results illustrated that the coated SWNTs/CS film could facilitate the electron transfer of the $[Fe(CN)_6]^{3-/4-}$ couple and had a larger specific surface area to enlarge the load amount of AFB_1-BSA. Subsequently, when AFB_1-BSA dropped onto the activated electrode, the peak current apparently decreased for the steric hindrance and a barrier of insulated AFB_1-BSA for the electron transfer, illustrating that AFB_1-BSA had been immobilized on the surface of SWNTs/CS/GCE successfully (curve c). After the fabricated immunosensor incubated with anti-AFB_1, an increasing electron transfer resistance led to a further decrease in peak current (curve d), indicating that the developed electrochemical biosensor for recognition and binding of anti-AFB_1 was successful obtained. When the diluted AP-anti-antibody was added onto the interface of the fabricated electrode, the peak current further decreased indicating that the AP-anti-antibody was successfully immobilized at the immunosensor. The results above were in good corresponding with those obtained from square wave voltammograms (Fig. 2B), in which the peak current remarkably increased after the SWNTs/CS film was coated on bare GCE surface. On the contrary, the peak current decreased gradually when incubated with AFB_1-BSA (curve c), anti-AFB_1 (curve d) and AP-anti-antibody (curve e) at 37 °C, respectively.

3.3. Optimization of experimental conditions

In order to achieve excellent analytical capability, some testing key parameters were optimized. As seen in Fig. 3A, the peak current increased to be a steady value while the concentration of immobilized AFB_1-BSA increased up to 10 μg mL^{-1}. Thus 10 μg mL^{-1} AFB_1-BSA was adopted as the optimal AFB_1-BSA concentration. The dilution ratio of anti-AFB_1 in the incubation solution was also an important parameter in the competitive format because of the competitive immunoreaction between the fixed amounts of AFB_1-BSA domains on the electrode interface and the free AFB_1 in solution for binding to anti-AFB_1. To obtain the optimal dilution ratio of anti-AFB_1, the fabricated AFB_1-BSA-SWNTs/CS/GCE was incubated with different dilution ratios of anti-AFB_1 solutions. As shown in Fig. 3B, the DPV response increased gradually as the

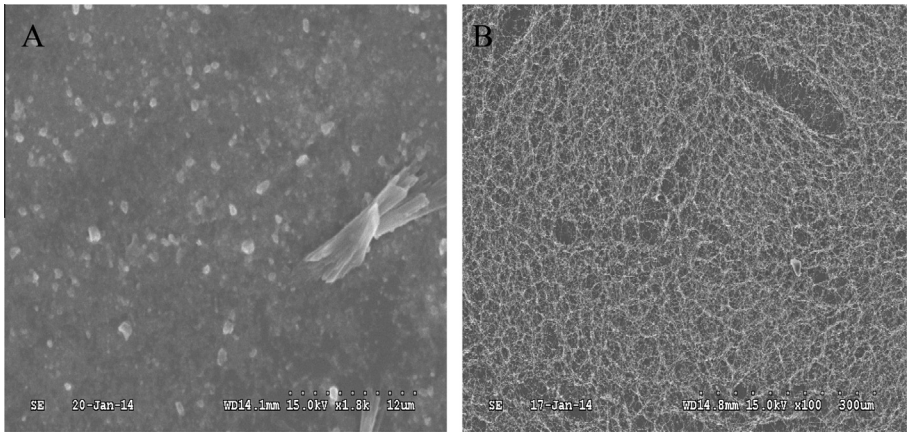


Fig. 1. SEM images of the (A) pure SWNTs and (B) SWNTs/CS composite.

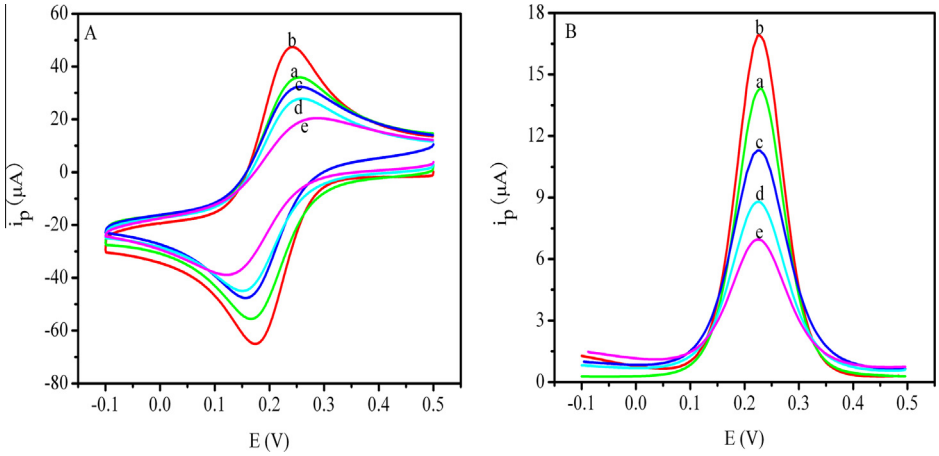


Fig. 2. (A) Cyclic voltammograms and (B) square wave voltammograms of (a) bare GCE, (b) SWNTs/CS/GCE, (c) AFB_1-BSA-SWNTs/CS/GCE, (d) anti-AFB_1/AFB_1-BSA-SWNTs/CS/GCE, (e) AP-anti-antibody/anti-AFB_1/AFB_1-BSA-SWNTs/CS/GCE in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.5 M KCl.

dilution ratio of anti-AFB_1 decreased and trended to level off at 1:300, which was chosen as the optimal anti-AFB_1 dilution ratio. In addition, the enzymatic reaction rate was greatly affected by the dilution ratio of AP-anti-antibody binding on the immunosensor. In Fig. 3C, with the decreasing dilution ratio of AP-anti-antibody from 1:400 to 1:50, the DPV response increased and trended to level off at 1:200. Thus 1:200 was chosen to be the optimal AP-anti-antibody. Finally, when the AP-anti-antibody dilution ratio was constant, the concentration of substrate α-NP would be another critical parameter that influenced the enzyme-catalyzed reaction. With the increasing concentration of α-NP in DEA solution, the signal increased gradually. When the concentration of α-NP was greater than 0.75 μg mL^{-1}, further increasing of α-NP concentration would lead to unobvious signal increase (Fig. 3D). Therefore, 0.75 μg mL^{-1} was chosen as the optimal α-NP concentration.

3.4. Analytical performance of designed biosensor

To evaluate the performance of the electrochemical immunosensor, different concentrations of AFB_1 prepared with PBS (pH 7.4) were assayed, and the DPV responses could be seen in Fig. 4A. The peak currents showed a linearly decrease with the logarithm of target AFB_1 concentrations ranging from 10 pg mL^{-1} to 100 ng mL^{-1} with a correlation coefficient of 0.9979 (Fig. 4B), and the error bars represented standard deviation for three measurements. The limit of detection (LOD) of this electrochemical immunosensor was estimated as threefold the standard deviation of the blank sample measurements and was calculated to be 3.5 pg mL^{-1}. To evaluate reproducibility of the developed immunosensor, five reduplicative measurements of AFB_1 at 100 pg mL^{-1} and 100 ng mL^{-1} with the same batch of immunosensors were carried out. The coefficients of variation were calculated to be 3.69% and 4.75%, respectively. As shown in Table S1, compared with other reported methods (Kolosova et al., 2006; Shim et al., 2014; Yun, Xia, GuoLi, & RuQin, 2009; Zhang et al., 2014) for AFB_1 detection, the results indicated that the designed electrochemical immunosensor using SWNTs/CS as a signal amplification strategy was more sensitive while maintaining a good reproducibility.

In order to investigate the specificity of the immunosensors, 1 μg mL^{-1} of other three mycotoxins including Ochratoxin A (OTA), Zearalenone (ZEN), Fumonisins B_1 (FB_1) and 100 ng mL^{-1} AFB_1 were examined respectively. As shown in Fig. S1, the DPV responses of the three different mycotoxins were close to that of the blank (without AFB_1) while the DPV signal of AFB_1 turned out to have a distinct reduction. The error bars represented standard deviation for three measurements. These consequences illuminated that the designed electrochemical immunosensors could distinguish different mycotoxins effectively.

3.5. Detection of AFB_1 in actual samples

In order to validate the feasibility of the developed immunosensors to detect AFB_1 specifically and sensitively, the corn powder samples naturally contaminated with AFB_1 at concentrations of

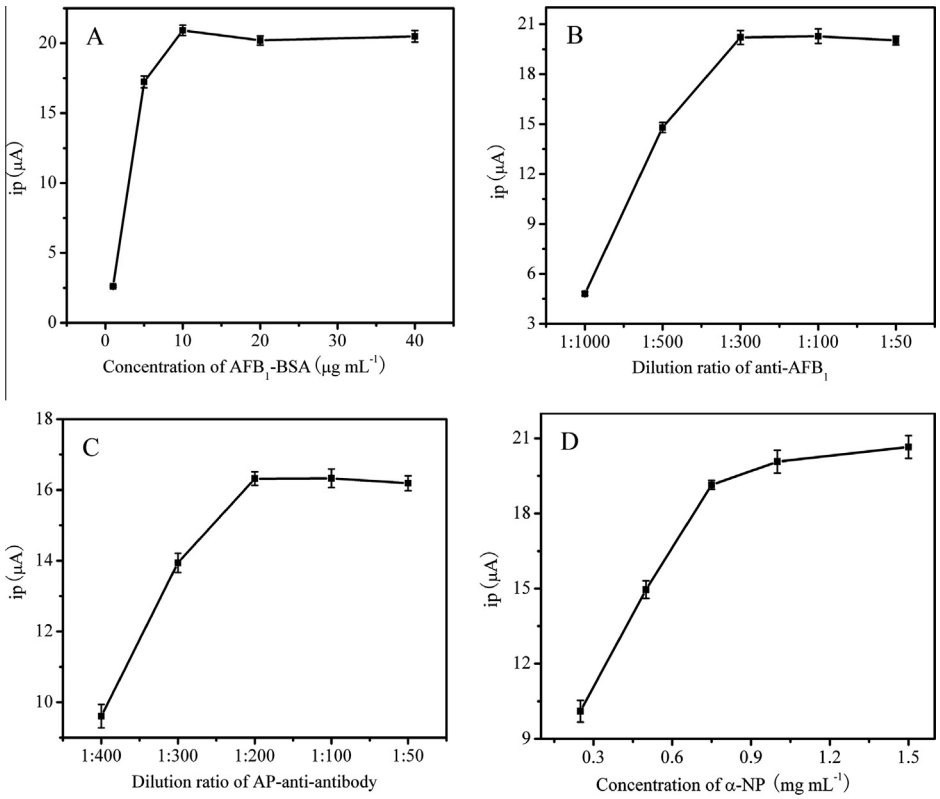


Fig. 3. Dependences of DPV peak currents on (A) AFB_1-BSA concentration, (B) dilution ratio of anti-AFB_1, (C) dilution ratio of AP-anti-antibody, and (D) α-NP concentration, when one parameter changes while the others are unaltered. The error bars represented standard deviation for three measurements.

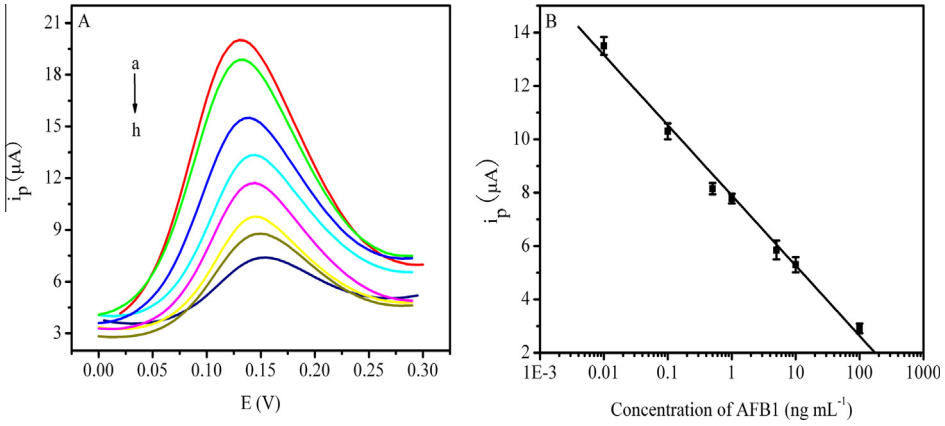


Fig. 4. (A) DPV peak currents of (a) 0, (b) 10 pg mL^{-1}, (c) 100 pg mL^{-1}, (d) 500 pg mL^{-1}, (e) 1 ng mL^{-1}, (f) 5 ng mL^{-1}, (g) 10 ng mL^{-1} and (h) 100 ng mL^{-1} AFB_1. (B) Plot of DPV peak currents vs. different AFB_1 concentrations. The error bars represented standard deviation for three measurements.

121.5, 40.5, 13.5 pg mL^{-1} were assessed by using the designed method. As listed in Table 1, the results obtained using proposed immunosensor were consistent with those obtained by HPLC using AOAC method 994.08 with modifications by Trilogy Analytical Laboratory, the relative errors between the two results ranged from −4.4% to 8.4%, indicating a high level of accuracy of the constructed immunoassay. The results demonstrated the developed method could be regarded as a potential alternative for quantitative determination of AFB_1.

Table 1
Assay results of practical specimens using the constructed and HPLC methods.

Sample no.	1	2	3
Developed immunosensor (pg mL^{-1})	119.0	41.3	12.9
HPLC* (pg mL^{-1})	121.5	40.5	13.5
Relative error (%)	−2.1	8.4	−4.4

* The results were obtained from Trilogy Analytical Laboratory Inc. (Washington, Mo.) by HPLC using AOAC method 994.08 with modifications.

4. Conclusions

This report described a sensitive, specific and simple electrochemical immunosensor for detection of AFB_1 by integrating SWNTs/CS with enzymatic signal readout. The developed approach exhibited an acceptable fabrication reproducibility and a high level of specificity and sensitivity for detection of AFB_1. It also showed a wide liner range from 0.01 to 100 ng mL^{-1} with the limit of

detection calculated to be 3.5 pg mL^{-1}. In addition, this method could detect AFB_1 as low as 13.5 pg mL^{-1} in actual corn powder samples which is much lower than that of acceptance criteria set by most countries. In consequence, the suggested technique could be a powerful immunosensor for AFB_1 detection in the future. It might further furnish a versatile and forceful tool to guarantee food safety.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.06.044.

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