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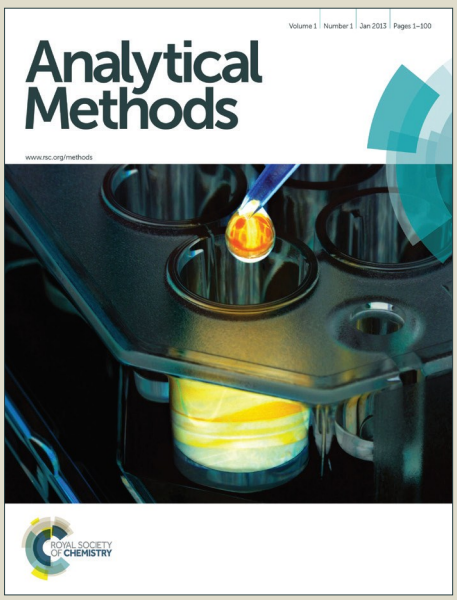

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ARTICLE

A portable electrochemical immunosensor for rapid detection of trace aflatoxin B_1 in rice

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To explore the possibility of rapid and in-situ detection of aflatoxin B_1 (AFB_1), a portable biosensing instrument consisting of a impedance detector and a 3D-printed USB-compatible sensor chip was developed. In this study, we proposed an electrochemical biosensor based on screen-printed interdigitated microelectrodes (SPIMs) and a portable detector to achieve low-cost, highly selective and sensitive detection of AFB_1 in rice. Under optimized conditions, the immunosensor provided a detection limit of 5 ng mL^{-1}, which was below the allowable concentration. The total detection time including incubation was less than 1 h. The results obtained from this developed portable detection system were comparable to those from the commercial electrochemical station in the laboratory. Hence, the portable detector offers new tools for detection of a wide variety of analytes in clinical and environmental samples.

1. Introduction

Among all the mycotoxins, aflatoxin brought the greatest losses (such as human and animal health) and management costs because of its high toxicity [1]. It is estimated that about 4.5 billion people are chronically exposed to aflatoxins [2]. Since it is impossible to reverse the carcinogenic effects, the identification and prevention of human exposure to aflatoxins become a major research topic in food safety area [3, 4]. As one of the most toxic aflatoxins, aflatoxin B_1 (AFB_1) was repeatedly found to form DNA adducts and cause hepatocellular or cholangiocellular liver tumors [5-7]. Therefore, the analysis of AFB_1 in food products was indispensable for ensuring that the products offered meet the regulatory and market requirements.

The detection was arduous due to that there are trace or ultratrace amounts of aflatoxins in food samples [8, 9]. Traditional analytical techniques, such as GC-MS, LC-MS and HPLC, are standard methods with high sensitivity and accuracy. However, they are either time consuming, expensive, or require complicated instruments and trained technicians. It seems impractical to frequently monitoring the frequent, rapid and in-field

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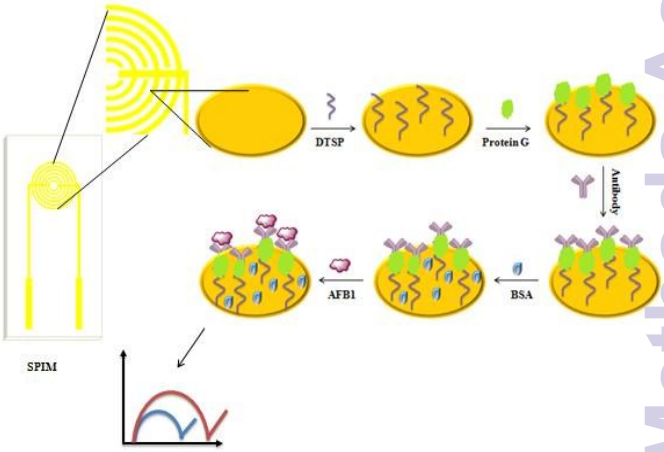
monitoring of aflatoxins in food products by using those conventional methods. Biosensing methods are alternatives for aflatoxins determination with the main advantages of high sensitivity and specificity, cost-effective, fast and portable detection [10, 11]. Electrochemical biosensing methods allow real time monitoring to be free of extensive sample preparation. An electrode is a vital element in an electrochemical biosensor, which is designed to transform the recognition of a biological molecule into an easily quantifiable electrical signal [12, 13]. Screen printing technology is a well-developed method and widely used to fabricate disposable and economical electrochemical sensors. Screen printed electrodes (SPEs) satisfy the highly reproducible and sensitive methods of detection with cost effectiveness [14, 15]. The adaptability and ease of modification are of great importance and allow for specific targets, such as organophosphate pesticides [16], drug residue [17], heavy metal ions [18, 19], organic compounds [20], gunshot residue [21], lactate [22], uric acid [23], glucose [24], hydrogen peroxide [25], and so on. Different dimensions of SPEs, from centimeter to micrometer [18, 25, 26], have been developed for application. Screen printed microelectrodes have been studied for the detection of mycotoxins [27]. In this paper, screen-printed interdigitated microelectrodes (SPIMs), which work in a two-electrode system, integrate the merits of screen-printed microelectrodes and interdigitated microelectrodes to develop highly sensitive, rapid-responding and cost-effective biosensors.

The commercial electrochemical stations, such as ZAHNER electrochemical station, Parstat 4000 station, HP 4194A station and SI1260-1287 station, have drew great attentions and widely used due to their high sensitivity and the combination of multi-techniques. However, these large and expensive instrumentations were unfavorable for in-field applications. As alternatives, portable electrochemical detectors with advantages of miniaturization, low-cost, rapid response and no complicated data analysis required, are widely researched [28, 29]. In this research, screen-printed interdigitated microelectrodes (SPIMs) were used to develop highly sensitive, rapid-responding and cost-effective biosensors for lab-free detection of AFB_1 in rice. An electrochemical biosensor with the combination of SPIMs and self-assembly monolayers were proposed for the detection of AFB_1 with lower cost, higher sensitivity and selectivity (**Schematic 1**). A portable impedance detector was introduced and its detection performance was evaluated for the comparison with that of the ZAHNER station. The constructed detector coupling with a 3D-printed USB-compatible sensor chip has the potential in the development of portable detection system.



Schematic 1 The concept of an electrochemical impedance immunosensor based on SPIMs and SAMs for the detection of AFB_1.

2. Experimental

2.1. Apparatus and reagents

Electrochemical impedance measurements were performed using ZAHNER electrochemical station (Kronach, Germany). SPIMs were purchased from AIBIT

Biotech Instrument (Jiangyin, China). The width of a finger and the gap between two fingers for SPIM were 200 μm, respectively. The rubber ring confined area was used for the detection area and the sample was dropped on the detection area for incubation. A figure shows the details of the SPIM (Fig. 1A). We also characterized the surface of the bare SPIM and the SPIM after protein incubation using scanning electron microscopy (SEM, Fig.1B).

The portable impedance detector was developed in our lab and provided five frequencies (0.1, 1, 10, 20 and 100 kHz) for impedance measurement at different alternating current potential (0.2, 0.4, 1.0 and 2.0 V). A printer and bluetooth modules can be connected with the detector for data transmission. Moreover, this detector can be powered by build-in battery or direct current supply (12 V).

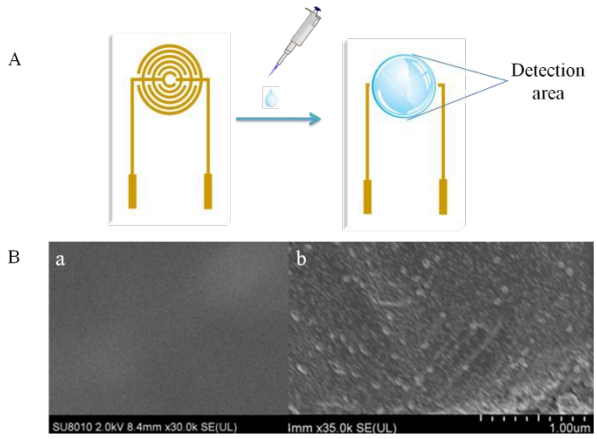


Fig. 1 A) SPIM and its detection area where the solution was added for incubation. B)SEM images. a) the surface of the bare SPIM and b) the surface of the SPIM after protein incubation.

3-Dithiobis-(sulfosuccinimidyl-propionate) (DTSP), AFB_1, and phosphate buffer solutions (PBS, pH 7.4) were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) and protein G were purchased from Sangon Biotech (Shanghai, China). Anti-AFB_1 monoclonal antibodies were obtained from Jiangxi Zodolabs Biotech Corp. (Jiangxi, China). PBS solution containing 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Sangon Biotech., Shanghai, China) was used for electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements. Ultrapure water (18.2 MΩ cm) was obtained from a Millipore Milli-Q purification system (Merck Millipore, MA). All chemicals were of analytical grade.

2.2. Preparation of rice samples

The fresh rice samples purchased from a supermarket were finely ground using pulverizerand 5 g of the powder sample was placed in a centrifuge tube (50 mL). Then, 15 mL of methanol–water solution (80:20, v/v) containing 4% NaCl was added [30, 31], and the sample was mixed with a vortex for 5 min. After centrifugation at 4,000 rpm for 5 min at room temperature, 0.5 mL of the supernatant was transferred to a 2 mL tube and diluted with 1.0 mL of ultrapure water. The resulting solution was used as the blank sample. Different concentration of AFB_1 were added into the solution for preparing the spiked samples.

2.3. Immunosensor fabrication

In order to clean the bare SPIMs, NaOH solution (50 μL, 1 M) was dropped on the detection area of the electrode. It was incubated for 5 min and then washed with water, followed by a similar treatment with HCl solution (50 μL, 1 M) for 2 min. EIS methods using $K_3Fe(CN)_6/K_4Fe(CN)_6$ probe were adopted to investigate the cleaning efficiency (1 Hz–1 MHz, 10 mV). CV technique was also used for this purpose. Self-assembly was carried out by immersing the electrodes in 2 mM DTSP (dissolved in acetone). The effect of different incubation times on sensor performance were studied. After incubation, the SPIMs were washed immediately with acetone to remove free DTSP.

After DTSP immobilization, the SPIMs were incubated in protein G solution (50 μL, 1.0 mg mL^{-1}, 45 min) and then washed with ultrapure water. Then the SPIMs were incubated with antibody solution (50 μL) for another 45 min and washed with ultrapure water again. The concentration of the antibody used in this study were optimized (0.1, 0.2 and 0.4 mg mL^{-1}). Finally, the BSA solution (50 μL, 10 mg mL^{-1}) was applied to block non-specific adsorption sites on the surface of SPIMs.

2.4. AFB_1 detection

Different dilutions of AFB_1 were dropped on the detection area of SPIMs and incubated for 45 min. Impedance measurements (1 Hz–1 MHz, 10 mV) were conducted using a 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture in PBS. All the tests were repeated more than three times. Several non-AFB_1 solutions, including ochratoxins A (OTA) and zearalenone (ZEN), were used to evaluate the specificity of this immunosensor.

2.5. Portable impedance detector

A portable impedance detector was designed and constructed for the detection of AFB_1 and there are five different frequencies (0.1, 1, 10, 20 and 100 kHz) for choosing. Impedance measurements were conducted using a 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture in PBS (100 Hz, 200 mV). **Fig. 2A** shows the photo of this detector. The electrode was held with an electrode clamp during detection. The samples were dropped on the detection area of the electrode. The impedance was measured by the portable detector and the results were shown on the LED screen of the detector. The detection performance between the portable impedance detector and the ZAHNER station were compared.

Furthermore, a 3D-printed USB-comparable sensor (**Fig. 2B and C**) was designed and fabricated. **Fig. 2D** shows the fabrication process. An SPIM was fabricated into the suitable groove. And then a ring was confined on the detection area before another part coated. Finally, an USB connector was inserted to complete the fabrication. The rubber ring with a diameter of 5.5 mm was used to confine the detection area of 22.89 mm^2 on the SPIM. An open area could be used for dropping the solution of a sample without use of any pump and tubing, which eliminates the cleaning process and reduces the fabrication cost. The SPIMs can be fabricated in to the USB sensor which was designed and printed by a 3D printer. This sensor could be linked to the portable detector through USB interface to make a completed portable biosensing system.

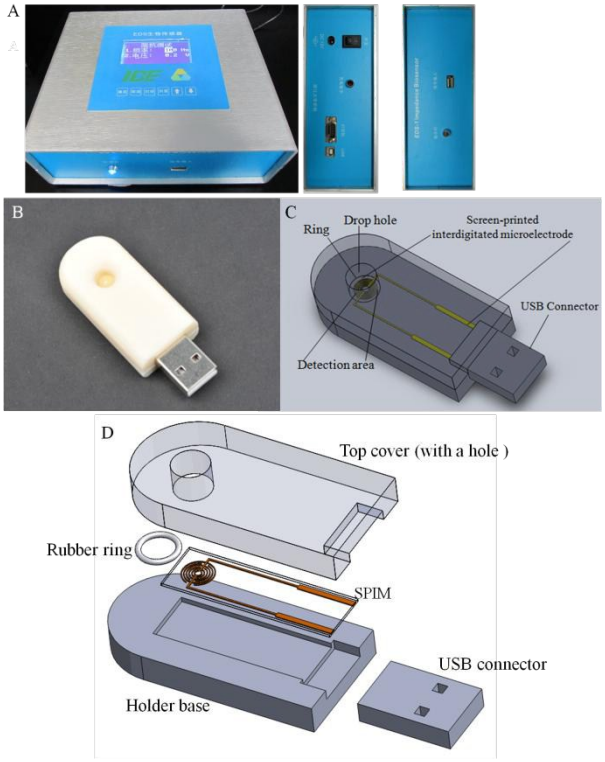


Fig. 2 A) The portable impedance detector; B) The photo and C) drawing of a 3D-printed USB-compatible sensor chip; D) Fabrication process of the USB-compatible sensor chip.

2.6. Regeneration of SPIMs

After each test, the used SPIMs were immobilized with biomaterials (such as antibody and aflatoxin). The SPIMs were treated with NaOH solution (1 M, 50 μL) to be

regenerated. All the materials immobilized onto the surface of SPIMs were disassociated and the signal was evaluated.

2.7. Statistical Analysis

Three replications for each diluted concentration of AFB1 were tested and statistical analysis was conducted using SPSS 17.0. For the purpose of this study, the biosensor responses were considered statistically different when the P-value was less than 0.05 (95% confidence interval). The lowest detection limit of the biosensor was determined as the lowest AFB1 concentration with a mean impedance value significantly different from the blank.

3. Results and discussion

3.1. Characterization of SPIMs

The SPIMs could be properly cleaned with NaOH and HCl solution. The surface of the SPIM was apt to transfer electrons because of the dissociation of the non-electroactive species. The results showed that the peak currents of the electrochemical probe increased and the electron transfer resistance (R_{et}) decreased, respectively, after the cleaning (**Fig. 3**). This treatment was much more convenient and time-saving compared with treatments commonly used for rod electrodes or plate electrodes, which generally need complicated polishing procedures [32, 33].

The self-assembling of DTSP was monitored through measuring the impedance value. The incubation time of 4 h was chosen as the optimized time because of the self-assembling efficiency and signal response. Furthermore, the concentration of DTSP was also investigated and optimized to be 2 mM because of the highest signal response. Besides the self-assembling time and the concentration of DTSP, we also optimized the concentration of the antibody (**Fig. 4A**). There was no significant difference between the concentrations of 0.2 and 0.4 mg mL^{-1} ($p > 0.05$). The test with a concentration of 0.2 mg mL^{-1} possessed the highest signal response and

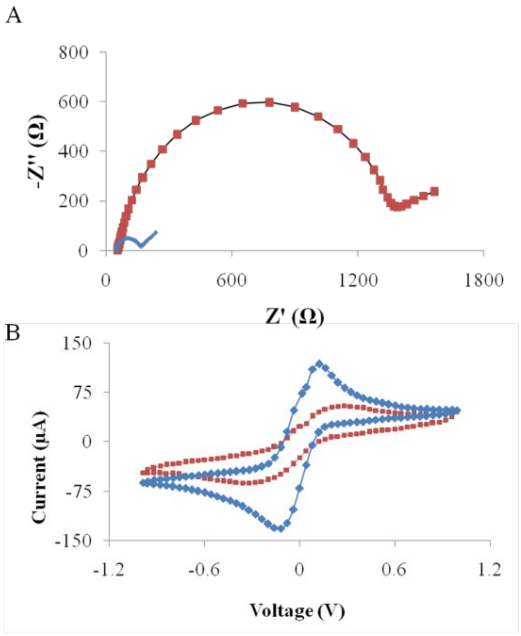


Fig. 3 Characterization of the bare SPIMs before (red) and after (blue) the cleaning process. A) EIS and B) CV.

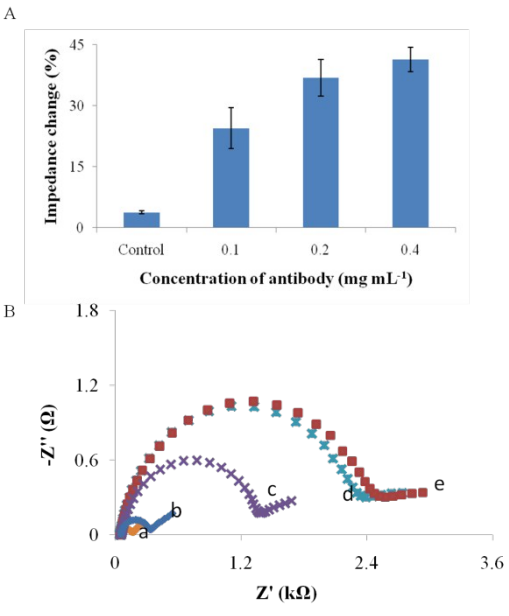


Fig. 4 A) Optimization of the concentration of antibodies. B) Impedance signal changes after the immobilization of different materials. Curve a-bare SPIM; b-SAMs; c-protein G; d-antibody; e-BSA.

the signal did not change any more when higher concentrations were used. Therefore, 0.2 mg mL^{-1} was chosen as the optimized concentration. Each step of the surface modification was tested and the result is shown in **Fig. 4B**. The bare microelectrode and self-assembled process showed lower R_{et}. When Protein G, antibody and BSA were immobilized onto the microelectrode surface, the signal increased, which confirmed the successful surface immobilization.

3.2. Detection performance

In the impedance measurement, increased R_{et} was observed at the lower applied frequency. This demonstrated that binding of the AFB_1 molecules led to the signal change. It was observed that the signal remained constant in the higher frequency region. Meantime, a significant change was observed in the low frequency region with highest signal change observed. For better understanding of the detection mechanism, a modified Randles' equivalent circuit obtained using Zpswin 3.10 software was used to fit adequately the measurement data over the whole frequency range (**Fig. 5A**). Wherein, R_s stands for the resistance of the electrolyte solution, R_{et} for the charge transfer resistance, C for the capacitance of the double layer and the biomaterial absorption, and W for the Warburge impedance. The R_{et} value is the most important electrical parameter in analyzing the impedance signal change and can be used to evaluate the detection performance [34, 35]. When AFB_1 was introduced to the fabricated immunosensor, there was a signal change in R_{et}, indicating that the microelectrode surface had been attached with a large number of targets. According to the previous research, the signal change depends on the detection frequency which can be used to compare the detection performance [36, 37]. There were obvious signal changes in the frequency range of 10–100 Hz in the detection of AFB_1. Triplicates were performed for each concentration in the range of 0.1–20 ng mL^{-1}. The impedance magnitude measured at the characteristic frequency of 10 Hz was plotted for each step in the procedure of AFB_1 detection. As shown in **Fig. 5B**, a linear relationship between the impedance signal change

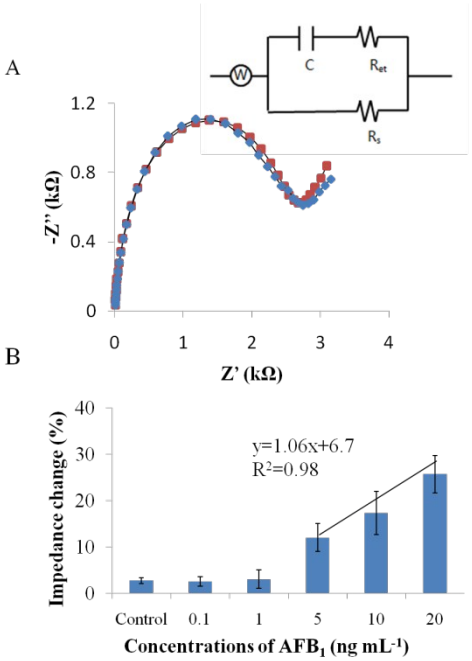


Fig. 5 A) A modified Randles' equivalent circuit obtained using Zpswin 3.10 software. Red-calculated data; blue-measured data B) Performance of the proposed immunosensor for detection of AFB_1 in rice.

and the value of AFB_1 concentration was found in the concentration range of 5–20 ng mL^{-1} and could be described as $y=1.06x+6.7$ ($R^2=0.98$). Under optimized conditions, the immunosensor provided a detection limit of 5 ng mL^{-1}, which was lower than the allowable concentration (20ppb) [38]. The total detection time including incubation was less than 1 h. There was no significant attachment when non-target mycotoxins were incubated, which clearly evidenced the sensing specificity to AFB_1 (**Fig. 6A**).

SPIMs can be regenerated from cleaning solutions and the results suggested that the electrodes could be

continuously regenerated (**Fig. 6B**) and the signal only decreased by 5%. Moreover, the cost of one SPIM was estimated to be less than $1 which is much cheaper for the practical application than the expensive non-screen printed microelectrodes.

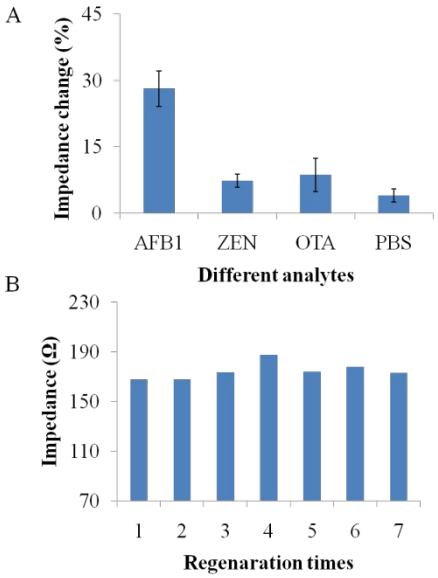


Fig. 6 A) The specificity of the immunosensor. B) Regeneration of the used SPIMs.

3.3. Performance of the portable impedance detector

The portable impedance detector was used to detect AFB_1 in rice. Impedance measurements were conducted with similar electrochemical probes (100 Hz, 200 mV). The signal changes were different between the spike samples and blank samples with a detection limit of 10 ng $mL^{-1,}$ which was lower than the allowable concentration (20 ppb) (**Fig. 7A**). The discriminant rate was about 90%, which was suitable for the practical application. Although the detection limit of the proposed immunosensor was not as low as some related reports, free of complicated signal generation and amplification process broaden the application of portable detector and open a door of development advanced portable system for mycotoxins research [27, 39, 40]. The detection performance between the portable impedance detector and commercial equipment is shown in **Figure 7B** (100 Hz, 200 mV). When protein G was immobilized on the surface of a bare microelectrode, the result showed that there was no significant difference between these two instruments at five different frequencies, indicating feasibility of application of the portable impedance detector. However, the difference of signal change was significant between these two instruments after the modification of the biomaterials. The detection frequency of the portable impedance detector (100 Hz) was higher than the one of ZANHER station (10 Hz). The different frequency may be a reason for the performance difference between the two instruments. The immobilization of biomaterials cause different signal changes and the commercial instruments propose advanced calibration modules which are useful to keep the detection stability. The performance of the portable detector is comparable, although lack of these calibration modules.

Our immunosensor has also been compared to other typical immunosensors previously reported for detection of mycotoxins (Table S1). The detection performance was comparable and the fabrication process was more simple in comparison with previous research. Free of label non-enzymatic label amplified electrochemical strategy would be more advantageous than the enzyme-based amplification strategy due to stability problems and the large size of the enzymes. The proposed immunosensor could provide satisfying sensitivity in comparison with the enzyme-based amplification immunosensor. The USB-compatible sensor chip can be used with a portable detector which avoids the complexity of amplification process and nanoparticles synthesis. Moreover, the proposed immunosensor can be regenerated simply by using NaOH solution to remove the immunocomplex. The detection cost can be greatly reduced because of the regeneration. All the results

indicated that the proposed portable immunosensor combined with the printed USB-compatible sensor chip was simple, rapid, flexible and practical.

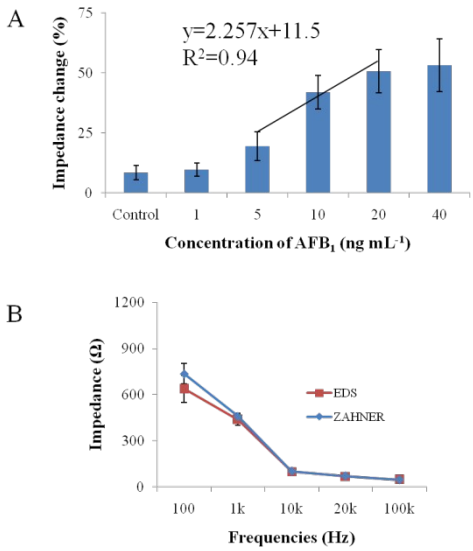


Fig. 7 A) Performance of the portable impedance detector; B) Comparison of the detection performance between the portable impedance detector and ZAHNER station at five frequencies (0.1, 1, 10, 20, and 100 kHz) after the immobilization of protein G.

4. Conclusions and perspectives

In this research, we proposed an electrochemical biosensor based on SPIMs and portable detector to achieve low-cost, highly selective and sensitive detection of AFB_1 in rice. Under optimized conditions, the immunosensor provided a detection limit lower than the allowable concentration. The total detection time including incubation was less than 1 h and the cost of one SPIM can be further reduced by the good reusability. More importantly, the results obtained from the developed portable detection system and the commercial electrochemical station in the laboratory were comparable. A USB compatible sensor was also designed and printed by a 3D printer, which could be plugged into the portable detector through USB interface to make a completed portable biosensing system. All the results indicated that the proposed portable immunosensor is simple, flexible and practical, which opens the door for rapid detection of mycotoxins and other toxins.

Acknowledgments

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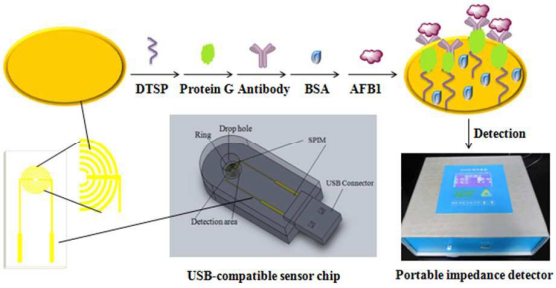

To explore the possibility of rapid and in-situ detection of aflatoxin B_1 (AFB_1), a portable biosensing instrument consisting of a impedance detector and a 3D-printed USB-compatible sensor chip was developed.