

Immobilization of anti-aflatoxin B1 antibody by UV polymerization of aniline and aflatoxin B1 detection via electrochemical impedance spectroscopy

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Abstract

In the study, we investigated the practicality of the UV polymerization of aniline for anti-aflatoxin B1 antibody immobilization, and utilization of the resulting biosensor in the impedimetric determination of aflatoxin B1. The anti-aflatoxin B1 antibody was physically immobilized on gold electrodes by UV polymerization of aniline at a fixed wavelength. The biosensor was based on specific interaction anti-aflatoxin B1 – aflatoxin B1 recognition and investigation of this recognition event by electrochemical impedance spectroscopy. A calibration curve was obtained in a linear detection range 1–20 ng/mL aflatoxin B1. Finally, the biosensor was applied to analysis of a real food sample.

Keywords: Aflatoxin B1, anti-aflatoxin B1 antibody, electrochemical impedance spectroscopy, immunosensors, biosensor

Introduction

It is clearly known that aflatoxins are very toxic contaminants that can cause serious disorders for humans and animals (Williams et al. 2004). Aflatoxins are a very important problem throughout the world, but in developing countries aflatoxin contamination is a particularly serious issue (Gürbay et al. 2010, Cano-Sancho et al. 2010, Fallah 2010, Hussain et al. 2010, Corcuera et al. 2010, Jossé et al. 2010). Aflatoxins have the potential to cause mutagenesis, carcinogenesis, and also impair important metabolic functions in humans (Johnson et al. 2010, Illic et al. 2010). In the literature, current studies explaining the mechanism of the toxicological effects of aflatoxin B1 have been reported. First, aflatoxin B1 is activated and then the active form, aflatoxin B1-8,9-epoxide, can bind to DNA. The combination of aflatoxin B1 and DNA can induce carcinogenesis (Sharma and Farmer 2004, Klein et al. 2002, Preston and Williams 2005). Consequently, it is enormously important to detect aflatoxin B1

sensitively and accurately. Historically, as the first time, thin layer chromatography was developed for specific determination of different kinds of aflatoxins in some foods (Chu et al. 1977, Chu et al. 1987). Today, there are several analysis methods for aflatoxin B1, including different measurement principles. Good examples based on thin layer chromatography (TLC), high performance liquid chromatography (HPLC), as well as ELISA-based aflatoxin screening kits, have been developed and used for detecting aflatoxins Gilbert and Vargas 2003, O'Riordan and Wilkinson 2009, Herzallah 2009, Nonaka et al. 2009, Alcaide-Molina et al. 2009). Although many methods have been developed for the accurate detection of aflatoxin B1, these methods have shown serious disadvantages. For example, most of these methods are sophisticated. Moreover, a few are time-consuming and not economical in their preparation and use (Melikhova et al. 2006, Tan et al. 2009). Certainly, this does not mean that the methods based on immune-biosensors are perfect. Immune-biosensors need a label molecule which biospecifically binds to the substance concerned. The other possible limitation is its cross-reactivity with the secondary antibody of the label antigen, which is immobilized onto the transducer surfaces of the biosensors. Of course, the cost of developing relatively new antibodies or antigens should definitely be taken into account. In our study, we report on a biosensor based on an immunological component specifically for aflatoxin B1. Anti-aflatoxin B1, which recognizes aflatoxin B1, was immobilized onto gold electrodes by the help of a very simple immobilization procedure. In the study, some important parameters were investigated and systematically optimized.

Experimental

Materials and methods

All the electrochemical measurements, including Electrochemical Impedance Spectroscopy, were carried out by a Potentiostat/Galvanostat [(AutoLAB, PGSTAT 302

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(FRA 2-220, ECD-220 Modules)]. The measurements were controlled with a personal computer running the electrochemical software package of AutoLAB (GPES and FRA) for parameter set-up, data acquisition, and processing. A three-electrode configuration was employed in all experiments, with potentials referring to an Ag/AgCl reference electrode. Gold electrodes (Metrohm, Switzerland) and a platinum electrode (Metrohm, Switzerland) were employed as a working and an auxiliary electrode (2.0 ± 0.1 mm in diameter), respectively. All measurements were carried out at a constant temperature with the help of a cryostat (Lauda RE106, Germany). Before every experiment, the gold electrode tips were polished using alumina polishing kits (Metrohm, Switzerland).

Cleaning procedure for the Au electrode surface

The surfaces of the Au electrodes were first polished with $0.05 \mu\text{m}$ Al_2O_3 powder and then washed ultrasonically in deionized water for five minutes to remove alumina particles. Then the electrodes were immersed into Piranha ($\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 30/70, v/v) solution for 10 seconds. Following that, the electrodes were washed with ultra pure water by immersion into water 20 times. For the next step, the surfaces of the electrodes were dried by a pure nitrogen stream. This polishing and cleaning procedure was repeated before every electrode preparation step.

Immobilization of anti-aflatoxin B1 antibody onto Au electrode surface

20 μL of aniline solution (0.4 M) were mixed with 20 μL anti-aflatoxin B1 solution (0.01 M phosphate buffer, pH 7.4, containing 15 mM azide, with 6 $\mu\text{g}/\mu\text{L}$ protein concentration) in an Eppendorf tube. This mixture was dispersed over the clean Au electrode by a micro-pipette. Anilin was polymerized under UV light with a wavelength of 319 nm for 30 minutes. At the end of this period, the electrode was immersed five times into an ethanol solution to remove unpolymerized anilin monomers.

Electrochemical measurements

Faradaic impedance and cyclic voltammetry measurements were carried out in the presence of 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ (1:1) solution which served as a redox probe. The redox probe also contained 0.1 M KCl. For all impedance measurements, a DC potential of 0.25 V was used as a bias potential in the frequency range of 100000 to 0.1 Hz. In the experiments, the alternating voltage was 0.01 V. The impedance data were shown as Nyquist plots. This is the common representation method of such spectra.

Measurement procedure

The biosensor based on anti-aflatoxin B1 antibody was put into the thermostatic reaction cell containing working buffer (20 mL, 50 mM, pH 7 phosphate buffer). Following that, aflatoxin B1 standard solution was injected into the thermostatic reaction cell and the magnetic stirrer was fixed at a constant speed of 100 rpm. Thirty minutes later, after binding of aflatoxin B1 to its antibody, anti-aflatoxin B1, the biosensor was removed from the reaction cell. Then

the biosensor was gently immersed into ultra pure water 10 times to remove physically adsorbed aflatoxin B1 molecules. Finally, the biosensor was again put into the cell containing $\text{Fe}(\text{CN})_6^{4-/3-}$ redox probe solution and the electrochemical measurements were taken as described in the previous section. The difference in charge transfer resistance between the biosensor unbounded and bounded aflatoxin B1 was used to prepare aflatoxin B1 calibration curves.

Sample preparation procedure

To understand the performance of the biosensor in a real sample analysis, a peanut sample purchased from a local market was used. First of all, 10 grams of sample were homogenized using a mortar. Then, 5 grams of the homogenized sample were weighed out into a centrifuge tube. A 15 mL extraction solution containing methanol and NaCl (15 % solution) (7:3) was added to the tube. Following that, the tube was centrifuged at 4000 r.p.m. for one minute. The supernatant was then transferred into a clean tube. Then 10 mL of methanol was added to the tube carrying the precipitate. It was vortexed for two minutes. Then it was centrifuged at 4000 r.p.m. one more time for one minute. Lastly, the supernatants were collected in the same tube and used as the sample solution.

Results and Discussion

Anti-aflatoxin B1 immobilization

We used a very simple and useful method for the immobilization of anti-aflatoxin B1 antibody. UV light was used to perform two important duties. One was to bring the aniline to an excited state and reduce its reduction potential. The other was that, of course, UV light can accelerate the polymerization process of aniline (Li et al. 2008). As a general rule, bioreceptors which are immobilized onto any transducers should conserve their initial activities after the immobilization process. As shown in the Nyquist diagrams in Figure 1, there was an increase in the diameter of the semicircle after immobilization of anti-aflatoxin B1 antibody onto the Au electrode surface. The diameter of the semicircle represented an increase in the charge transfer resistance, which indicated that the redox probe experienced a blocking effect when approaching the electrode surface. The result was caused by formation of an immobilized bio-molecular layer on the electrode surface.

The cyclic voltammogram of the redox probe, $\text{Fe}(\text{CN})_6^{4-/3-}$, showed a reversible manner on the bare working electrode, as expected (Figure 2). The formation of a bioactive layer on the electrode surface passivated it and efficiently inhibited the charge transfer between the redox probe in solution and the gold electrode surface. As a result, the cyclic voltammogram with reversible behavior turned into a capacitive shape.

Optimization studies for anti-aflatoxin B1 antibody biosensor

Optimization studies of current working conditions led to identification of the most suitable circumstances for using the biosensor developed. For this purpose, the incubation period for aflatoxin B1, the effect on the response of stirring

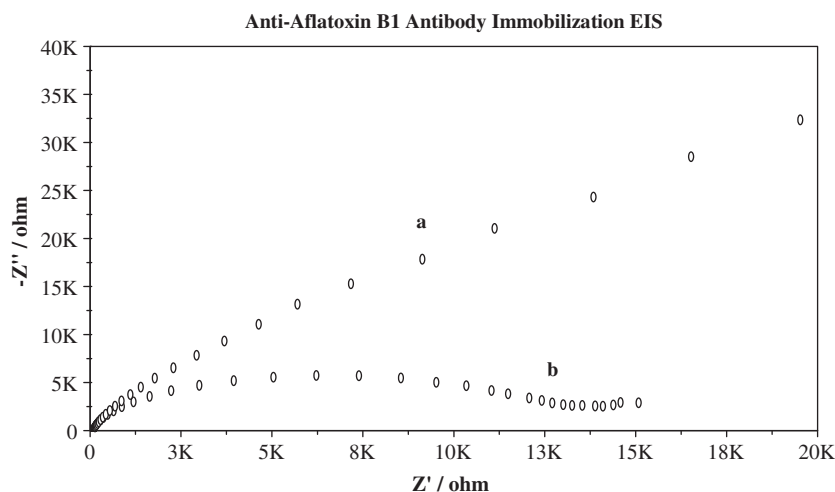


Figure 1. EIS spectra of the immobilization steps of the biosensor [a: Bare gold electrode, b: after UV polymerization. Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential: 0.025 V].

the incubation buffer, and the effect of DC potential on electrochemical impedance measurements were investigated.

Aflatoxin B1 standards were incubated for 10, 20, and 30 minutes after injection into the reaction cell. The results are given in Figure 3.

The incubation period was important for precision of the results. The highest response was observed when a 30-minute incubation period was used. With shorter incubation periods, charge transfer resistances were decreased considerably. The impedance differences between two consecutive aflatoxin B1 standards were decreased to about 50% for 10- and 20-minute incubation periods, respectively. Moreover, it was seen from the R^2 values that the linearity of the standard curves was impaired by a decrease in the incubation period. The best results were obtained when an incubation

period of 30 minutes was used. In order to minimize the total measurement period of the biosensor, incubation periods higher than 30 minutes were not tested.

The effect of magnetic stirring at intervals from 100–900 r.p.m. on the biosensor response time was investigated. The calibration curves obtained in these stirring conditions are shown in Figure 4.

The experiments showed that the stirring procedure was very important. It was observed that an increase in the rate of stirring resulted in a decrease in charge transfer resistance. Moreover, increasing the stirring rate to over 600 r.p.m. caused the results to be converted into a meaningless shape. It is likely that, at 600 and 900 r.p.m., the bioactive layer of the biosensor was physically destroyed because of the high stirring rate. The impedance variations between the two

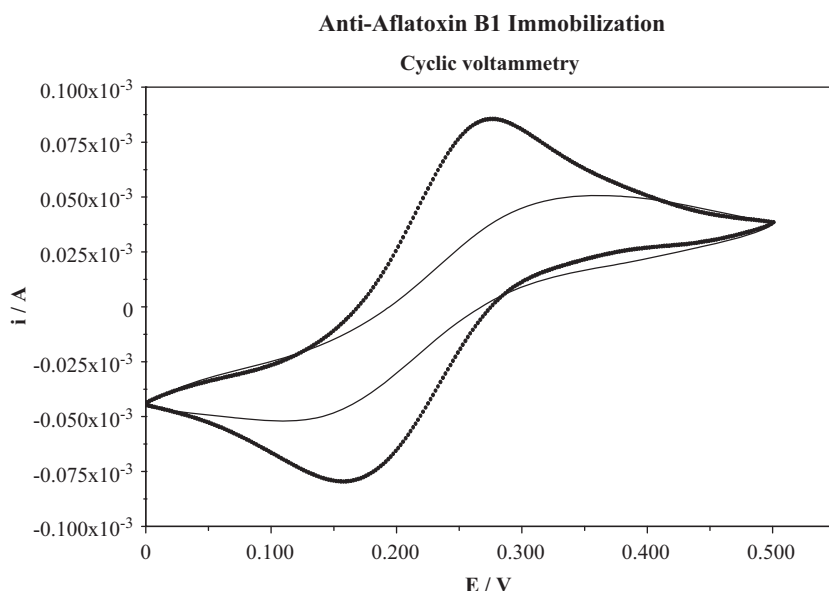


Figure 2. Cyclic voltammograms for the immobilization steps of the biosensor [Thicker line: bare gold electrode; thinner line: after UV polymerization. Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential: 0.025 V].

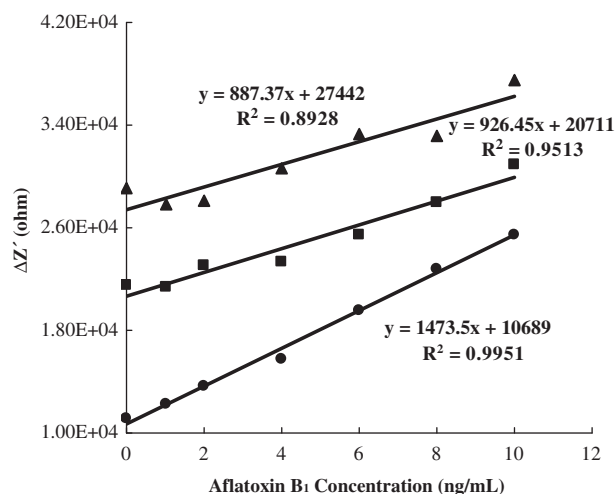


Figure 3. The effect of aflatoxin B1 incubation period on the biosensor response [Incubation periods: $\circ$ - $\circ$ - $\circ$: 30 min. $\triangle$ - $\triangle$ - $\triangle$: 20 min. $\square$ - $\square$ - $\square$: 10 min. Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential: 0.025 V].

standard concentrations were very low when we worked at the speed of 300 r.p.m. Moreover, a different kind of stirring procedure was also tested. During the first two minutes of the incubation period the incubation buffer was stirred at a constant speed of 300 r.p.m. After two minutes, the magnetic stirrer was shut down. The results were still not ideal. Finally, the best results were obtained by stirring the incubation buffer at a rate of 100 r.p.m.

The other important parameter optimized was the DC potential applied to the system. This DC potential value is also referred to as the bias potential. The calibration curves drawn by using the values of $\Delta Z'$ obtained with varying bias potentials are given in Figure 5.

Nyquist ($Z' - Z''$) diagrams obtained with different bias potentials indicated that an increase in the potential resulted in a decrease in the Z' value in terms of aflatoxin B1 standards.

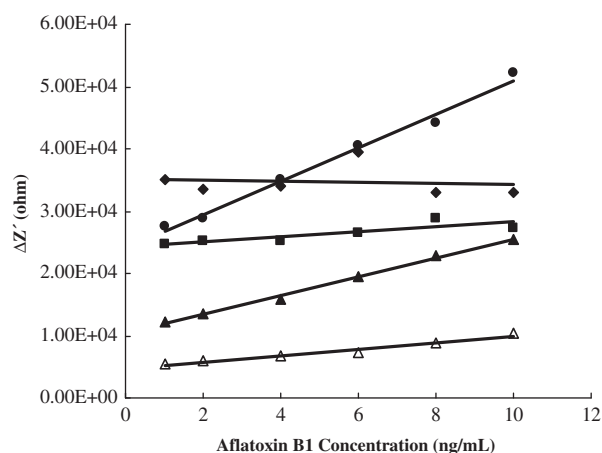


Figure 4. The effect of stirring rate on the biosensor response and bioactive layer [Stirring rates(r.p.m.): $\circ$ - $\circ$ - $\circ$: 100, $\triangle$ - $\triangle$ - $\triangle$: 300, $\square$ - $\square$ - $\square$: 600, $\diamond$ - $\diamond$ - $\diamond$: 900, $\times$ - $\times$ - $\times$: to stir for the first 2 min at 300 r.p.m. Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential: 0.025 V].

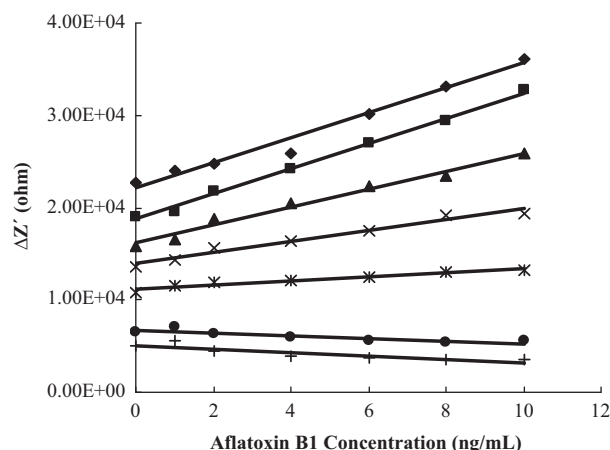


Figure 5. The optimization of bias potential applied to the system [Bias potentials tested: $\circ$ - $\circ$ - $\circ$: 10 mV, $\square$ - $\square$ - $\square$: 25 mV, $\triangle$ - $\triangle$ - $\triangle$: 50 mV, $\times$ - $\times$ - $\times$: 75 mV, $*$ - $*$ - $*$: 100 mV, $+$ - $+$ - $+$: 200 mV. Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V].

High bias potentials probably induced over-oxidation of the polyaniline film on the electrode surface. Hence, this improved the conductivity of the surface. Moreover, charge transfer resistance was decreased with the increase in bias potential. As a result, 25 mV DC potential was chosen as the bias potential of the biosensor system.

Characterization of anti-aflatoxin B1 antibody biosensor

Figure 6 shows the Nyquist plot diagrams for the biosensor for different aflatoxin B1 concentrations. It was observed that the semi-circle diameter in the Nyquist plot increased with increasing aflatoxin B1 concentration. The low frequency ranges were particularly suitable for concentration-dependent impedance measurements.

The charge-transfer resistance differences between before and after aflatoxin B1 injection were used for preparation of the aflatoxin B1 calibration curve. This can be seen in Figure 7.

The standard graph showed saturation of the antibodies on the sensor surface with increasing aflatoxin B1 concentration up to 20 ng/mL. A linear relationship between the charge transfer resistance values and the concentration of aflatoxin B1 existed over the range from 1 to 20 ng/ml aflatoxin B1. The detection limit of the biosensor was also determined to be 1 ng/ml.

Assay variances were evaluated by the repeatability study. A standard sample was analyzed five times for aflatoxin B1 standard with a concentration of 6 ng/mL. The average value, standard deviation, and variation coefficient were 6 ng/mL, ± 0.25 ng/mL, and 4.2%, respectively. The results indicated that the anti-aflatoxin B1 antibody-based biosensor was very reliable with regard to repeatability.

The reproducibility of the biosensor preparation process was evaluated by preparing the calibration graphs and linear detection ranges for aflatoxin B1 consecutively under the same experimental conditions. The results are summarized in Table I below.

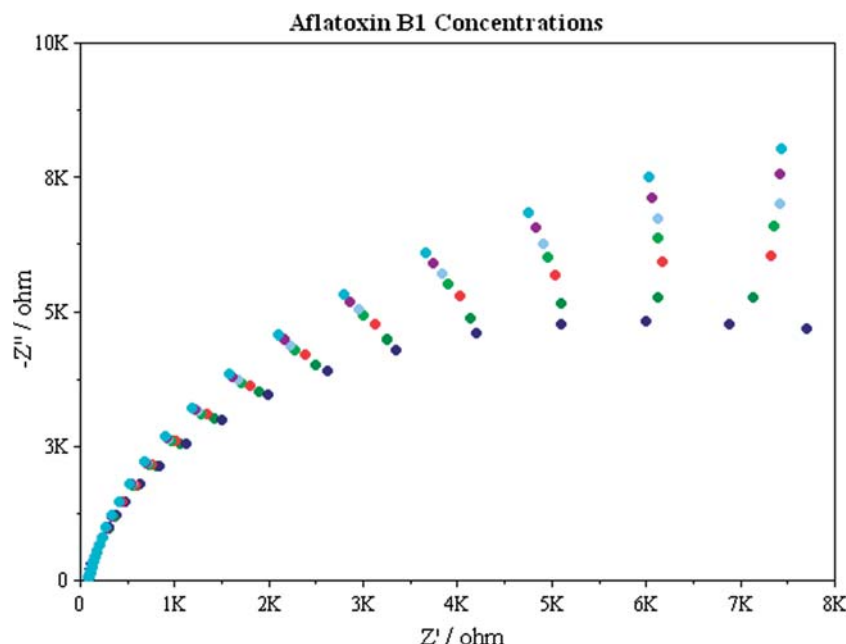


Figure 6. Nyquist plots for increasing concentrations of aflatoxin B1 [Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential 0.025 V].

A determined unit of aflatoxin B1-spiked peanut sample was analyzed with the help of the biosensor. The results are shown in Table II.

The experimental results indicated a good recovery. These results showed that the proposed biosensor can be applied successfully for the determination of aflatoxin B1 impedimetrically.

Conclusion

In this study, it was shown that in order to detect Aflatoxin B1, anti-aflatoxin B1 antibody immobilized by UV polymerization of anilin was used. In the literature no samples of these kinds of biosensor systems by UV polymerization of

anilin have been reported. In this sense, the biosensor submitted here could contribute a new aspect of immobilization of bio-receptors for biosensor applications. The results from cyclic voltammetry and electrochemical impedance spectroscopy measurements indicated that anti-aflatoxin B1 antibodies were successfully immobilized onto the solid Au electrode with the help of UV polymerization of aniline. Impedance measurements were used to characterize the electrochemical properties of the surface of the biosensor and to reveal the binding of aflatoxin B1 to the biosensor surface. The studies showed that a stable bioactive layer of anti-aflatoxin B1 antibodies was formed. The antibody-antigen binding in the bioactive layer resulted in a considerable impedance response that was successfully detected even at a concentration of 1 ng/mL aflatoxin B1. Finally, we can conclude that as the limitations of the biosensor, detection limit and incubation periods should be pointed out. This is because in some circumstances it can be necessary to analyze Aflatoxin B1 in the level of picograms. Consequently, a pre-step is needed to concentrate the sample in such a situation. Also, the 30-minutes incubation period for interaction of Aflatoxin B1 and biosensor should be relatively long for an analysis. However, in general, immunosensors, especially impedimetric immunosensors, have relatively long incubation periods.

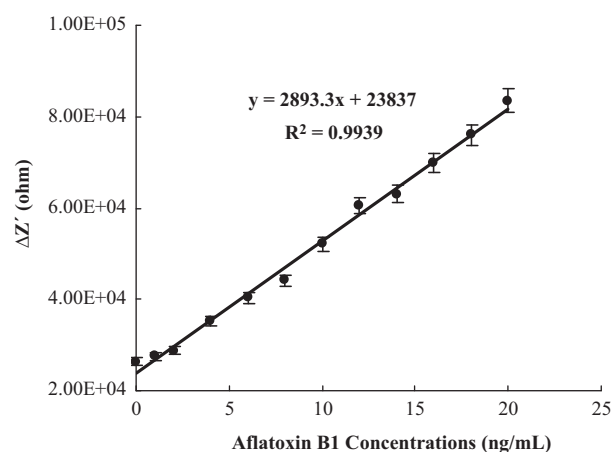


Figure 7. Calibration curve for aflatoxin B1 [Working conditions: Incubation period for anti-aflatoxin B1 antibody: 30 min., stirring rate: 100 r.p.m., electrochemical redox prob solution: $\text{Fe}(\text{CN})_6^{3-/4-}$, 0.005 M + 0.1 M KCl, frequency range: 0.1–100000 Hz, AC potential: 0.01 V, bias potential 0.025 V].

Table I. The reproducibility of the biosensor based on anti-aflatoxin B1 antibody.

Biosensors	R ²	Linear ranges (ng/mL)
1	0.919	20-Jan
2	0.99	20-Jan
3	0.994	20-Jan
4	0.996	20-Jan
5	0.893	20-Jan

Table II. Aflatoxin B1 detection in peanut sample spiked with aflatoxin B1 by the biosensor.

Added	Aflatoxin B1 (ng/mL)*		
	Found by the biosensor	% Recovery after extraction	% Relative difference
0.2	0.21	105	5

*The average of six measurements.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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