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LABEL-FREE ELECTROCHEMICAL IMMUNOSENSOR DEVELOPMENT FOR MYCOTOXINS DETECTION IN GRAIN CORN

(Pembangunan Imunosensor Elektrokimia Tanpa Label untuk Pengesanan Mikotoksin dalam Jagung Bijian)

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Abstract

Mycotoxins have been a huge threat in the agriculture and poultry industries. In Malaysia, the presence of mycotoxins, particularly in grain corn, is not only detrimental to health; it may also have a detrimental effect on the economy, as we rely heavily on this source for animal feed. With this regard, a rapid detection method of mycotoxins on-site lends itself well in reducing the negative economic impact and strengthening the food safety aspect. We report here an electrochemical-based biosensor for mycotoxins detection in grain corn employing differential pulse voltammetry technique. Polyclonal antibodies against Aflatoxin B1 and Ochratoxin A were developed in-house and used in the sensor development. Cross-reactivity study with intra and interspecies proved the selectivity of developed antibodies. The antibodies were then immobilized on a screen-printed carbon electrode functionalized with gold nanoparticles and polyaniline. The developed immunosensor system showed significant performance in both phosphate buffer solution (PBS) and grain corn matrix systems. Excellent R^2 and low limit of detection (LOD) values were achieved in a broad working range for both buffer conditions. Aflatoxin B1 displayed R^2 of 0.9935 and LOD of 0.6 ppb in PBS; and R^2 of 0.978 and LOD of 1.81 ppb in grain corn matrix. Ochratoxin A displayed R^2 of 0.9962 and LOD of 0.87 ppb in PBS; and R^2 of 0.971 and LOD of 3.18 ppb in grain corn matrix. The LODs obtained for both detections are lower than the maximum residues limit (MRLs) permitted for grain corn (5 ppb). Both aflatoxin and ochratoxin biosensors exhibited acceptable recoveries in the 90% to 120% recovery range in grain corn matrix. This successful method could be applied and widened to other cereal grains and their processed food products.

Keywords: aflatoxin B1, ochratoxin A, immunosensor, differential pulse voltammetry, grain corn

Abstrak

Mikotoksin merupakan satu ancaman kepada industri pertanian dan ternakan. Di Malaysia, kehadiran mikotoksin terutamanya dalam jagung bijian bukan sahaja merbahaya kepada kesihatan, tetapi juga membawa kesan yang buruk kepada ekonomi berikutan kebergantungan sepenuhnya kepada jagung bijian sebagai sumber makanan haiwan. Dalam hal ini, kaedah pengesanan pantas mikotoksin di lapangan dapat mengurangkan kesan negatif kepada ekonomi di samping memperkasa aspek keselamatan makanan. Kami melaporkan biosensor berdasarkan elektrokimia untuk pengesanan mikotoksin dalam jagung bijian dengan menggunakan teknik voltametri denyut beza. Antibodi poliklonal terhadap Aflatoksin B1 dan Okratoksin A telah dibangunkan secara dalaman dan digunakan dalam pembangunan sensor. Kajian tindakbalas silang intra dan interspesies membuktikan selektiviti antibodi yang dibangunkan. Antibodi kemudian dipegunkan di atas elektrod skrin-bercetak karbon berfungsi dengan nanozarah emas dan

polianilin. Sistem immunosensor yang dibangunkan menunjukkan prestasi yang signifikan dalam kedua-dua sistem larutan penimbal fosfat (PBS) dan matriks jagung. Nilai R^2 dan had pengesanan rendah (LOD) yang baik dapat dicapai dalam julat bekerja yang luas bagi kedua-dua keadaan penimbal. Aflatoksin B1 menunjukkan R^2 0.9935 dan LOD 0.6 ppb dalam PBS; dan R^2 0.978 dan LOD 1.81 ppb dalam matriks jagung bijian. Ochratoxin A menunjukkan R^2 0.9962 dan LOD 0.87 ppb dalam PBS; dan R^2 0.971 dan LOD 3.18 ppb dalam matriks jagung bijian. LOD yang didapati bagi kedua-dua pengesanan adalah lebih rendah daripada had residu maksimum (MRL) yang dibenarkan untuk jagung bijian (5 ppb). Kedua-dua biosensor aflatoksin dan okratoksin menunjukkan perolehan yang boleh diterima dalam julat perolehan 90% to 120% dalam matriks jagung. Kaedah yang terbukti berjaya ini boleh digunakan dan diperluaskan kepada lain-lain bijian dan produk makanan terproses.

Kata kunci: aflatoksin B1, okratoksin A, imunosensor, voltametri denyut beza, jagung bijian

Introduction

Potent mycotoxins such as aflatoxins, ochratoxins, fumonisins, zearalenones and trichothecenes are produced by microfungi such as *Aspergillus* species, *Penicillium* species and *Fusarium* species, which are specific to certain mycotoxins [1]. The presence of those fungi on crops does not guarantee the production of harmful mycotoxins. However, poor aeration and high humidity during storage can accelerate mycotoxins production and eventually lead to serious crop contamination [2]. Natural weather or environmental changes in certain countries or regions also contribute to mycotoxins' occurrence. For instance, the natural tropical weather of Malaysia has led to manifestation of *Aspergillus* species and *Fusarium* species in soil, resulting in contamination of aflatoxins and *Fusarium* related mycotoxins in agricultural crops [3]. In addition, initial contamination can also take place during harvesting, followed by a lack of good processing, handling and transport practices [4]. Primary corn processing such as cleaning, dry milling, steeping and wet milling can help to reduce the level of mycotoxins in food samples but do not entirely eliminate them [5, 6]. Therefore, most of the food or products are already severely contaminated before being consumed by animals and humans. Figure 1 shows the distribution of mycotoxins from contaminated crops to animals and humans.

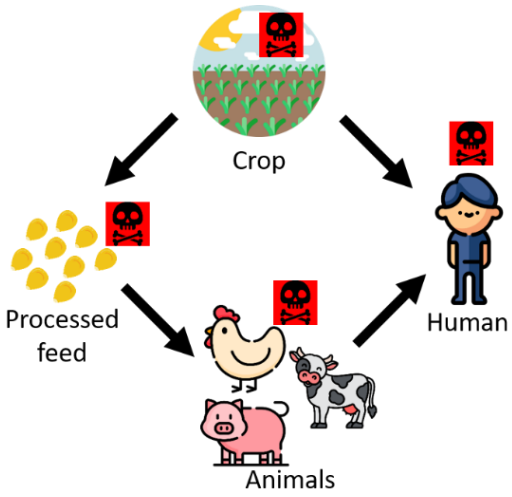

Figure 1. Distribution of mycotoxins from contaminated crops to animals and humans

Mycotoxins are not a minute problem as the occurrence of mycotoxins in food and feed is a global issue and not only subject to certain parts of the world. Even though most places have different natural weather and environment, mycotoxins always have their ways to exist. Ten years of research were conducted by Gruber-Dorninger and team in 2019 [7] to study the occurrence of mycotoxins in 15 regions around the globe. The commodities tested included feed, soybean (grains and meal), corn (silage and grains), wheat, barley and rice.

From the study, deoxynivalenol and fumonisin contaminations were collectively found to be dominating the toxins occurrence in the commodities. The other mycotoxins class also exceeded the maximum limit regulated for each region. Occurrence and contamination of mycotoxins from other studies were also in correspondence with the result obtained [8-12].

The concern for mycotoxins arises due to the alarming consequences of mycotoxin contamination. Crops yield, animal health and productivity, human health, economy and food security are the most affected area, and they are closely related to each other. Low crops productivity due to contamination can cause low income for farmers as no contaminated products are allowed in the market [13]. Consecutively, their well-being can be affected in many ways, such as accessibility to decent food and health care. This situation also applies to animals. Factors such as growth depression, low nutrient absorption, reproductive problems, and low egg production and hatchability result from low mycotoxin consumption of contaminated feed, which is highly associated with declining animal productivity [14]. High ingestion of mycotoxins can lead to acute health problems such as liver, kidney and gastrointestinal damage, haemorrhaging and, in the worst case – death. Since most mycotoxins are carcinogenic, human also shares similar health problems such as oesophageal and liver cancer, alimentary toxic aleukia, kidney disease, cardiac beriberi and death [15]. In terms of economic impact, the final consumer has to pay a high price for the finished products due to the low availability of feed and food in the market, processing and handling cost and regulatory services for monitoring mycotoxins [4]. The United States, Africa and South-East Asia recorded annual losses of around USD 1.68 billion, USD 750 million and USD 1 billion, respectively, as a result of aflatoxins contamination. Eventually, with all these arising problems, food security will be a major concern in the near future.

The most common and conventional mycotoxins detection methods are thin-layer chromatography, gas chromatography, liquid chromatography and real-time polymerase chain reaction [16,17]. Normally, gas and liquid chromatography analysis is often coupled with mass spectrometry to enhance the detection sensitivity [18]. Although these methods can detect multiple analytes in one measurement, the drawbacks (i.e., large sample volume, long sample preparation and analysis, expensive equipment and maintenance, laboratory-based analysis) have urged the development of simpler and easier analytical methods, with similar or better detection. The new approach to the mycotoxin detection method is a biosensor. Biosensor is a device where a reaction between biomolecules is translated into an electrical signal by a transducer, and the data can be recorded and displayed [19]. Biomolecules such as an antibody, aptamer, DNA and molecular imprinted polymer are commonly used as these biorecognition elements are highly sensitive toward their specific target analyte. Electrochemical transducers (potentiometry, voltammetry, amperometry and impedance) are widely used in biosensor development as these techniques offer high sensitivity and a wide working range with a variety of mycotoxins [20]. The combination of biomolecules and electrochemical measurement was proven to detect mycotoxins in the low detection range [21] successfully. Incorporation of nanomaterials (carbon nanotubes, graphene's, gold nanoparticles, quantum dots; to name a few) into the system can enhance the performance of developed biosensors, especially for electrochemical transducers [22, 23]. In addition to simple sample preparation and small sample volume, biosensor carries the potential for in-situ detection that end-users can carry out with moderate training.

Materials and Methods

Materials and instrumentation

Polyclonal antibodies against aflatoxin A (AB1) and ochratoxin A (OA) were produced in-house by the Biotechnology & Nanotechnology Research Centre (Animal Ethics Committee of MARDI approval number 20190215/R/MAEC00045). Phosphate buffer saline (PBS) tablet, dimethyl sulfoxide (DMSO) and OA standard were purchased from Sigma (USA, UK and Israel, respectively). Potassium chloride (KCl), acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade) and glacial acetic acid (GAA) were obtained from Merck (Germany). Potassium hexacyanoferrate (III) (ferricyanide) and potassium hexacyanoferrate (II) trihydrate (ferrocyanide) were

acquired from Sigma-Aldrich (USA and Japan, respectively). Polyaniline emeraldine salt (PANIs) and gold nanoparticle (AuNP) came from Aldrich (USA). AB1 standard, aflatoxin B2 (AB2), aflatoxin G1 (AG1), aflatoxin G2 (AG2), ochratoxin B (OB), fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3) standards were purchased from Romer Labs (Austria). Dry milk powder was obtained from Santa Cruz (USA). All reagents used were of analytical standard or higher, as stated.

Refrigerated centrifuge 5810 R (Eppendorf, Germany) was used during the extraction of corn samples. AUTOLAB Potentiostat (Metrohm, Switzerland) is equipped with Nova 1.10 software for cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurement. Screen-printed carbon electrode (SPCE) with three electrode system comprising working, counter and reference electrodes made from carbon was purchased from BioGenes Technologies (Malaysia). Chromatographic analysis of mycotoxins was conducted using isocratic high-performance liquid chromatography (HPLC, UVE LCTech, Germany) equipped with a fluorescence detector (Waters 2475 Multi λ Fluorescence Detector). RP C18 column (150 x 4.6 mm, LCTech) with guard column 8 x 4 mm and RP C18 column (125 x 3 mm; 3 µm; 120 A, LCTech) were used for aflatoxin and ochratoxin, respectively. Grain corn sample (GWG888) was obtained from a local source and stored at room temperature.

Modification of SPCE

Bare SPCE was modified with AuNP, PANIs (in DMSO) and a mixture of both to improve the surface performance. A 10 µL of each solution was deposited on a carbon working electrode for 30 minutes. Excess liquid was discarded and left dried at room temperature. A 100 µL 5 mM redox electrolyte (ferricyanide/ferrocyanide in 0.1 M KCl) was added to SPCE and an electrochemical measurement was conducted. The stability of the modified electrode was evaluated by measuring CV for 50 cycles.

Standard curve determination

Mycotoxins standard curve was determined by applying direct assay protocol on modified SPCE. A 10 µL AuNP-PANIs mixture was deposited on the working electrode and left for 30 minutes. After discarding the excess liquid, 10 µL 0.1 mg/mL mycotoxin antibody was immobilised for an hour. The SPCE was washed three times with 50 µL PBS followed by deionised water. A 50 µL blocking buffer (0.05% dry milk in 0.01 M PBS) was added and incubated for 30 minutes. The SPCE has washed again. A 10 µL serial dilutions of five different mycotoxins concentrations were immobilised on the working surface and allowed to incubate for 2 hours. The SPCE has washed again, and 100 µL of 5 mM redox electrolyte was added to the SPCE for the electrochemical measurement. The experiment was carried out at room temperature.

Cross-reactivity

Cross-reactivity of each mycotoxin was conducted by introducing mycotoxins of the same and different groups into the assay. Mycotoxins used were AB2, AG1, AG2, OB, FB2 and FB3. The concentration used was 5 ppb for both AB1 and OA.

Real sample analysis

The sensitivity of the developed electrochemical sensor was evaluated with a real extracted corn sample. The direct assay was conducted on the SPCE to construct a standard mycotoxin curve in a grain corn matrix. Mycotoxins were prepared in grain corn matrix instead of PBS during standard preparation. Then, the procedure was repeated with extracted spiked samples for the recovery study.

HPLC analysis

Prior to HPLC, mycotoxins first were extracted from grain corn samples. For aflatoxin analysis, 100 mL extraction solvent (60% MeOH) was added to 25 g ground corn and blended for 3 minutes or shaken for 60 minutes. 4 mL filtered extract was added to 12 mL PBS. For ochratoxin analysis, the extraction method was the same, except that the extraction solvent used was 80% MeOH.

Aflatoxin and ochratoxin analysis were conducted using isocratic HPLC with fluorescence detection in reverse phase analysis. For aflatoxin analysis, water:MeOH:ACN (60:30:15 v/v) was used as eluent.

Injection of sample volume was 10-100 µL at a flow rate of 1.2 mL/min through RP C18 column at a column temperature of 33 °C. Fluorescence detector; Ex.: 365 nm, Em.: 460 nm. For ochratoxin analysis, water:MeOH:ACN/GAA (40:55:5/1 v/v) was used as eluent. Injection of sample volume was 10-100 µL at a flow rate of 0.6 mL/min through RP C18 column at a column temperature of 40 °C. Fluorescence detector; Ex.: 395 nm, Em.: 440 nm.

Results and Discussion

Modification of SPCE

Figure 2 shows the modification and immobilisation of biomolecules on the electrode surface. Both AuNP and PANIs were used to enhance the electrical and electrochemical properties of the electrode. The immobilisation of the nanoparticles and conducting polymer has improved the conductivity of the carbon surface, and this has been proved by a significant increase in current reading in both voltammetric measurements (Figure 3). Besides, the peak separation of modified electrodes was much closer than bare (unmodified) electrodes, as shown in Figure 3(a), which implied that the redox reaction occurred at a fast pace with a stable rate. The AuNP-PANIs modified SPCE recorded the highest DPV current reading of 466 µA (Figure 3(b)) with low peak separation (100 mV) and a current ratio near 1 (1.04) (Table 1). AuNP played an essential role in enhancing the performance of carbon surfaces. In addition to contributing to the conductivity [24], AuNP also provides binding sites for antibodies. Antibodies can bind with reagents such as thiols and polymers, but they can also directly attach to gold [25,26]. Thus, more binding sites are available, and the developed biosensor's sensitivity will increase accordingly.

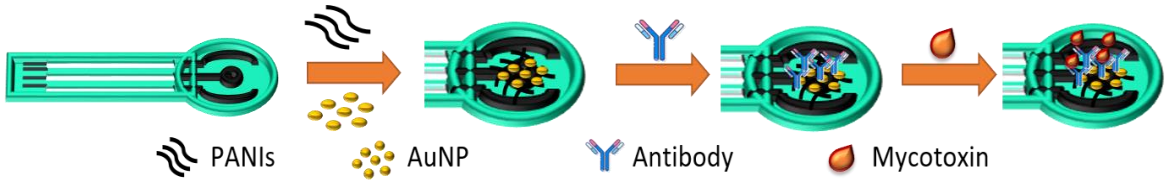

Figure 2. Modification and immobilisation of biomolecules on working SPCE surface

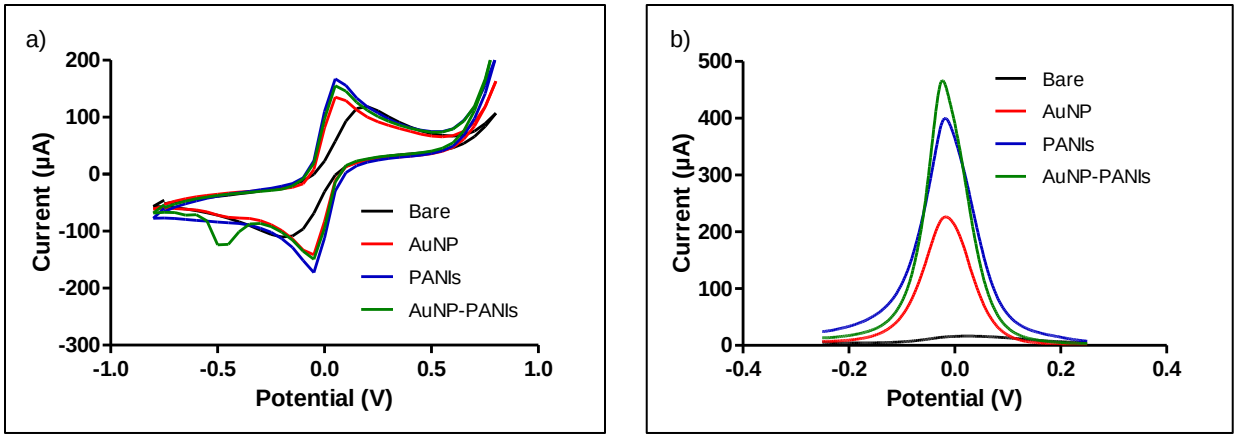

Figure 3. a) CV and b) DPV of bare and modified SPCE in 5 mM ferricyanide/ferrocyanide (in 0.1 M KCl). Scan rate; 50 mV/s (CV) and 2 mV/s (DPV)

Table 1. Electrochemical properties of bare and modified SPCE

	CV		DPV
	Peak Separation (mV)	**Current Ratio (i_a/i_c)**	**Peak Current (µA)**
Bare	400	1.08	16
AuNP	100	0.95	226
PANIs	100	0.97	400
AuNP-PANIs	100	1.04	466

The stability of the AuNP-PANIs-modified electrode has been investigated by allowing the electrode under CV measurement for 50 cycles. Anodic and cathodic peak current values were recorded and presented in Figure 4. A current increase was observed at anodic current starting from cycle 30 onwards. This might suggest that during the measurement, AuNP was fully stabilised by PANIs, resulting in the increased signal current [27]. Overall, the current reading of the modified surface was highly stable throughout the continuous electrochemical cycles, especially the cathodic current. This proved that the immobilised AuNP and PANIs were strongly attached to the carbon surface, and no leaching was detected from the voltammetric measurement.

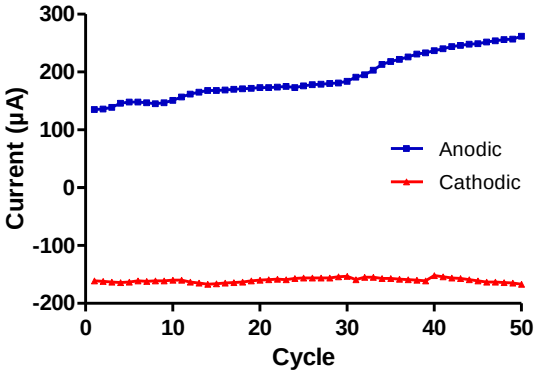

Figure 4. Stability of SPCE modified with 1 mg/mL PANIs and 20 nm AuNP under 50 cycles of CV

Standard curve determination

The sensitivity of developed AB1 and OA sensors were investigated at different concentrations of mycotoxin in PBS. The direct assay was conducted on the modified SPCE (Figure 2), and the sensor's sensitivity was measured using DPV, in terms of the current reading. Both AB1 and OA exhibited good linear correlation (high R^2 values) in a broad working range (Figure 5). Based on the plotted standard curve, the limit of detection (LOD) and limit of quantification (LOQ) were calculated and recorded in Table 2. Both mycotoxins showed lower LOD and LOQ values, which were much lower than the maximum regulation limit (MRL). Aside from contributing to conductivity and large surface area for binding, the combination of AuNP and PANIs also possesses high biocompatibility with antibodies [28]. This allowed more antibodies to be attached to the SPCE surface and maintained the binding stability throughout the assay process. This proved that the developed electrochemical sensor was highly sensitive in detecting the target analytes even in low concentrations.

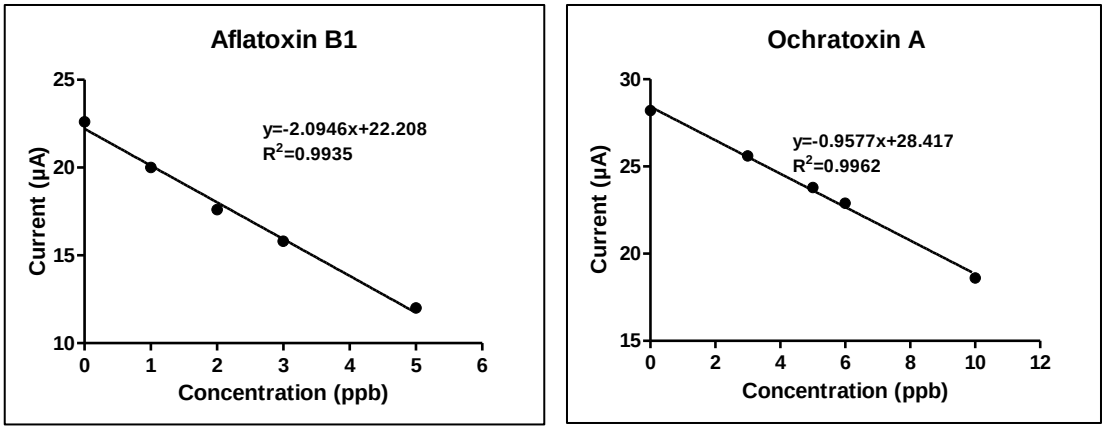

Figure 5. Standard curve of aflatoxin A (AB1) and ochratoxin A (OA) in PBS

Table 2. Calculated LOD and LOQ values for AB1 and OA in PBS

Mycotoxin	LOD (ppb)	LOQ (ppb)	MRL (ppb)
Aflatoxin B1	0.60	1.80	5
Ochratoxin A	0.87	2.63	5

Cross-reactivity

In order to investigate the selectivity of the developed antibody, the assay was tested with other mycotoxins of the same and different groups (Figure 6). AB1 antibody was cross-reacted with AB2, AG1 AG2, OA, OB, FB1, FB2 and FB3, while OA antibody was cross-reacted with OB, AB1, AB2, AG1 AG2, FB1, FB2 and FB3. The results showed that each developed antibody was strongly selective towards its targeted mycotoxin, where no significant electrochemical response was observed when tested against different mycotoxins, even in the same mycotoxin class. This was due to the vast difference in chemical structures between each mycotoxin (Figure 7). AB1 structure depicts stacked oxolane and aromatic rings, while the OA structure exhibits a few aromatic rings containing amine, carboxyl and hydroxyl functional groups. In contrast, the structure of FB1 is a long carbon chain with a number of hydroxyl functional groups, which contributes to its high polarity.

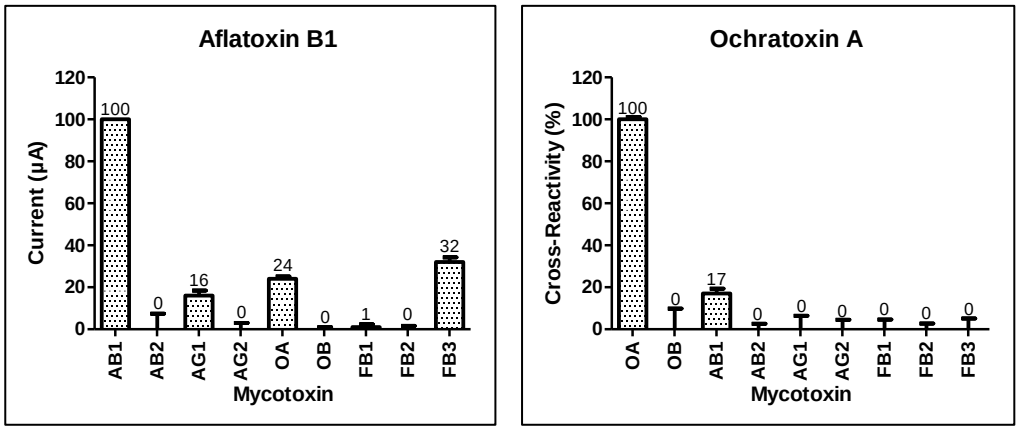

Figure 6. Intra and interspecies cross-reactivity of AB1 and OA

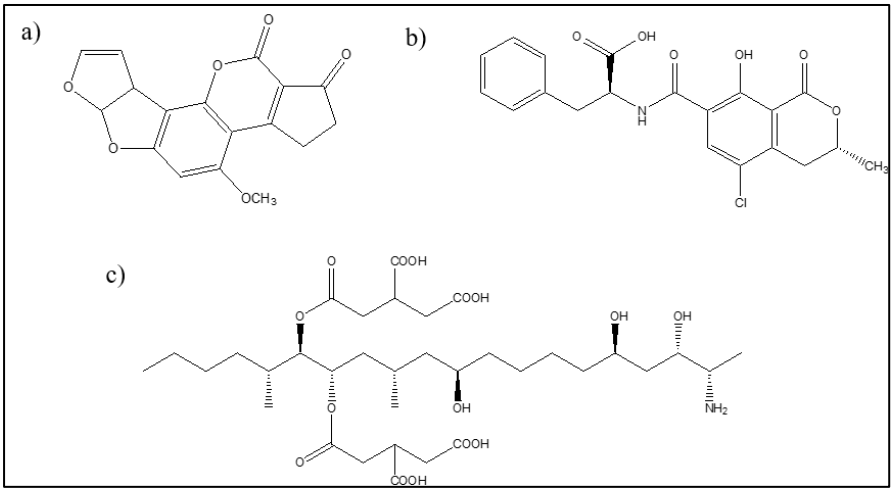

Figure 7. Chemical structure of a) AB1, b) OA, and c) FB1

Real sample analysis

The sensitivity of developed AB1 and OA sensors was also investigated in real grain corn samples. Grain corn is one of the main compositions of animal feed [29], and the inevitable occurrence of mycotoxins in unprocessed and processed grain corn hence gravely affects the animal feed industry. The analysis of samples employed the same direct assay, and the sensor's sensitivity was measured using DPV. Both AB1 and OA exhibited good R^2 values in a wider working range (Figure 8). The calculated LOD and LOQ values were also below the MRL (Table 3). This proved that the developed electrochemical sensor was highly sensitive in detecting the target analytes in the grain corn matrix.

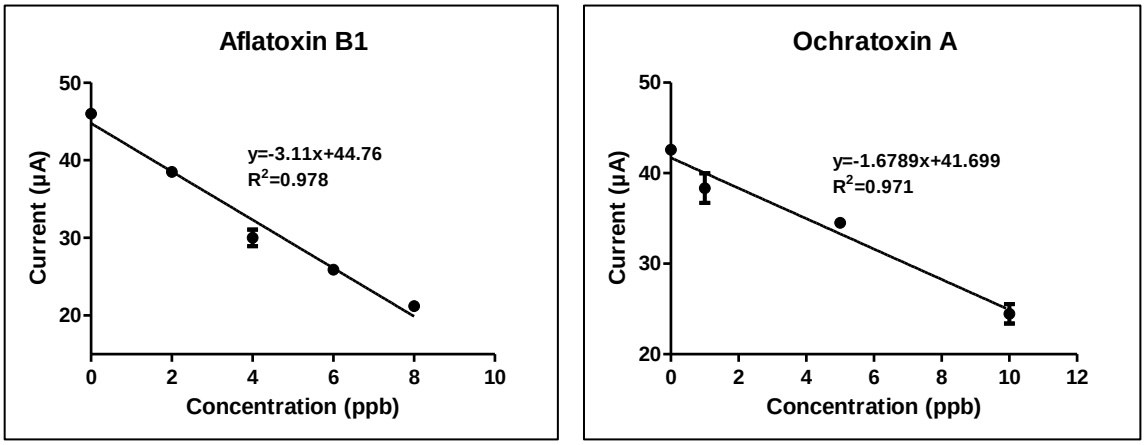

Figure 8. Standard curve of AB1 and OA in grain corn matrix

Table 3. Calculated LOD and LOQ values of AB1 and OA in corn matrix

Mycotoxin	LOD (ppb)	LOQ (ppb)	MRL (ppb)
Aflatoxin B1	1.81	5.48	5
Ochratoxin A	3.18	9.63	5

The recovery study was achieved by spiking several analytes' concentrations into grain corn and electrochemically measuring to detect the presence of mycotoxins in samples (Table 4). Overall, AB1 and OA exhibited good recovery in the range of 90-130%. Polar solvents such as ACN can work with a variety of mycotoxins, thus contributing to the success of mycotoxin extraction in corn samples [30]. The combination of water and the organic solvent was proven to increase polarity and helped in the effective extraction of solid samples [31].

Table 4. Recovery of spiked corn samples with AB1 and OA

Aflatoxin B1			Ochratoxin A		
Spiked (ppb)	**Found (ppb)**	**Recovery (%)**	**Spiked (ppb)**	**Found (ppb)**	**Recovery (%)**
2	1.92	96	5	5.24	105
6	5.52	92	10	12.56	126

Comparison with HPLC analysis

The viability of the developed electrochemical immunosensor for mycotoxins detection in grain corn was compared with chromatographic analysis. Results obtained from both techniques were recorded in Table 5. AB1 was detected lower than the MRL by both methods. AB1 was detected slightly higher from the developed biosensor may be due to the detection date being performed later than HPLC analysis. In contrast, both methods exhibited high OA concentration, which was above the regulated level. The difference in detected concentration between the sensor and HPLC was due to the fact that electrochemical-based detection has a base current limit. For example, the base current for OA was ±15 μA and is able to detect OA concentration in samples up to 15 ppb. The sensor cannot accurately determine the concentration of the target analyte but can determine if the concentration exceeds the MRL. The examined grain corn samples were contaminated by OA and were confirmed by both detection methods.

Table 5. Detected AB1 and OA using HPLC and developed electrochemical sensor

Mycotoxin	Detected Concentration (ppb)		MRL (ppb)
	HPLC	**Sensor**	
Aflatoxin B1	0.34	2.24	5
Ochratoxin A	25.82	15.78	5

Conclusion

The imbuement of AuNP coupled with PANIs onto the carbon working surface has greatly enhanced the properties of the sensor medium in terms of conductivity and as active binding sites. The AuNP-PANIs modified SPCE recorded the highest DPV current reading with low peak separation (100 mV) and a current ratio near 1 (1.04). The performance of the developed antibody-based biosensor has been proven via the sensitivity study in corn matrix and selectivity study with other mycotoxins using the electrochemical technique. Both AB1 and OA displayed high R^2 values in a broad working range with low LOD values. The developed antibodies (AB1 and OA) were exceptionally specific towards their target analyte and showed minimal cross-reactivity toward other mycotoxins. The detection of AB1 and OA in grain corn samples using the developed biosensor was at par with the instrumentation method. When evaluated using both developed electrochemical biosensor and chromatographic techniques, the tested grain corn sample was highly contaminated with OA compared to AB1. In future, AB1 and OA contamination in other food and feeds constituents such as rice, coffee beans, peanut, wheat and barley can be determined using this developed electrochemical biosensor.

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