Chapter

Immunosensing of Aflatoxin B1 and Ochratoxin A on a Portable Device as Point-of-Care

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Abstract

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are potent mycotoxins produced by the fungal genus *Aspergillus*. Their occurrence in grain corn is alarming hence the need for rapid on-site detection. An immuno-based biosensor technique for detection of the aforementioned toxins is described here. Highly specific in-house polyclonal antibodies against AFB1 and OTA were employed as bioreceptors in a label-free electrochemical biosensor; immobilized on modified screen-printed carbon electrodes (SPCEs). The immuno-functionalized SPCEs were first characterized on a laboratory electrochemical workstation for proof-on-concept study using differential pulse voltammetry (DPV) electrochemical technique. An Android-based device is improvised as a portable electrochemical reader integrated with internet of thing (IoT) features which include cloud server and a dedicated website. Sensitivity achieved by the modified SPCEs on the portable device is superior compared to enzyme-linked immunosorbent assay (ELISA) method and lab-based electrochemical workstation. The miniaturized biosensor system has been successfully tested on cornfield for in-situ mycotoxins detection with simple sample extraction. Analysis performed on twenty samples were validated using chromatographic analysis. This biosensor-IoT system offers a potential application for real-time detection and the portable reader serves as an excellent tool for point-of-care in routine monitoring of harmful mycotoxins.

Keywords: aflatoxin B1, ochratoxin A, electrochemical biosensor, immunosensor, point-of-care, portable electrochemical device

1. Introduction

1.1 *Aspergillus* spp. and mycotoxins

Aspergillus is an oligotrophic fungus that can grow in an aerobic environment and is capable of producing a harmful secondary metabolite known as mycotoxin. In agriculture, mycotoxins contamination is one of the perturbing global food safety issues that need to be addressed. Its occurrence is widely spread infecting various important crops and commodities, [1] both during pre and postharvest conditions, and forages [2]; besides contaminating food products and animal feed. Among major crops that are susceptible to mycotoxin contaminations are peanuts, corn, rice, sorghum, chili, millets, and legumes [1]. Of recent, mycotoxins incidence in aquaculture also have been extensively reported, including fish feed [3, 4]. In food chain, storage environments such as poor aeration and high humidity are among factors that trigger and accelerate the growth of fungi, which consequently leads to mycotoxins production. Therefore, mycotoxins can exist in the field before harvest, postharvest, or during processing, storage, and feeding.

Among the most commonly observed mycotoxins that posed concerns to human health and livestock are aflatoxins, ochratoxin A, fumonisins, zearalenone, and deoxynivalenol. The harmful impacts of the mycotoxins have long been observed in both humans and animals [5]. Exposure to even low concentrations of mycotoxins in long term has been associated with liver diseases such as cancer, hepatitis, and jaundice, and to the extent of being carcinogenic. Aflatoxins and ochratoxins are principal toxins produced by *Aspergillu*s spp. Human exposure to mycotoxins can happen either directly or indirectly. Directly, we are susceptible to mycotoxins when obliviously consuming grains, cereal-based products, or food contaminated with the toxigenic fungus. Indirectly, on the other hand, mycotoxins can enter human food chain *via* compromised animal feed. In terms of economic impact, farmers with contaminated crops or sick animals will lose their income and agribusiness will be greatly affected [6]. The prevalent issue of mycotoxins still remains the biggest challenge for animal feed producers and therefore, regular monitoring of mycotoxins is imperative.

1.1.1 Aflatoxins

The term aflatoxin is derived from the name of its main fungal producer that is *Aflavus*. Aflatoxins, also commonly known as a postharvest mold, are listed as potent mycotoxins. They are mainly produced by *Aspergillus flavus* and *A. parasiticus*. Other aflatoxin-producing species, albeit less frequently encountered, include *A. nomius*, *A. bombycis*, *A. pseudotamari,* and *A. ochraceoroseus*. Grains or food products contaminated with aflatoxins are not safe to be consumed. These toxins are resilient and stable against any thermal, physical, and chemical treatments along the feed chain production. In farm animals, aflatoxicosis can affect the liver and interrupt the digestive system. This in turn has a negative impact on livestock production with a reduction in body weight and feed conversion rate. Aflatoxins can contaminate a variety of livestock feeds and cause enormous economic losses, estimated at between US$52.1 and US$1.68 billion annually for the U.S. corn industry alone [7]. Even more unfortunate, aflatoxin in the animal's body system can be carried to eggs, meat, milk, and organ tissues. Indirect consumption of aflatoxins through food by humans can also cause toxin poisoning such as imperfect growth, weakened immune system, and liver damage, and have the potential to cause cancer and death.

There are more than 20 types of aflatoxins, but the four main types are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). The terms B1, B2, G1, and G2 are addressed based on their fluorescence under ultraviolet light where B denotes blue and G denotes green color. Both AFB1 and AFB2 are produced by *A. flavus* while AFB1, AFB2, AFG1, and AFG2 are produced by *A. parasiticus* isolates. Meanwhile, aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) are hydroxylated metabolites of AFB1 and AFB2, respectively (**Figure 1**). Among all these types of aflatoxin, AFB1 has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer [8]. The order of toxicity of aflatoxin is as follows: AFB1 > AFG1 > AFB2 > AFG2 [9]. Aflatoxins have been reportedly found in peanuts, cattle feed, liquid milk, cashew nuts [5], and also grain corn [10].

1.1.2 Ochratoxin

There are three classes of ochratoxins, namely ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC) (**Figure 2**). They are produced by *Aspergillus* and

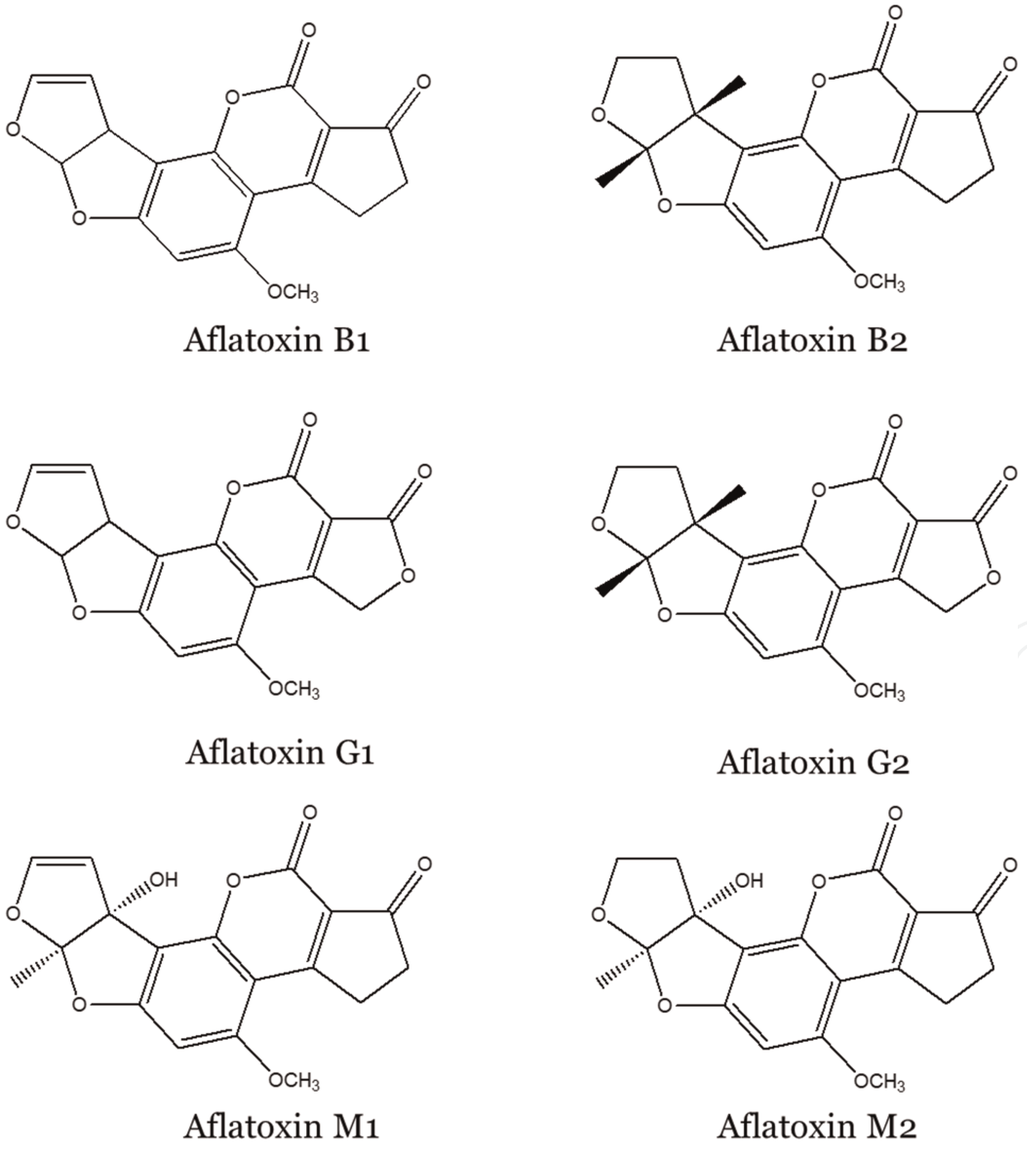

Figure 1.
Chemical structures of the main aflatoxins.

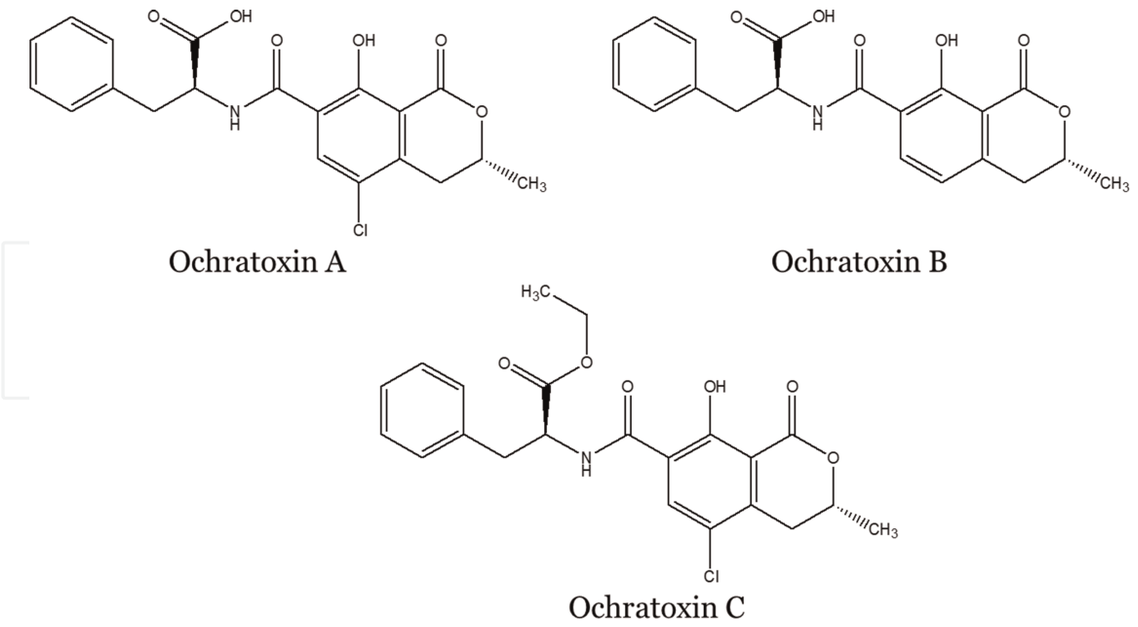

Figure 2.
Chemical structures of the ochratoxins.

Penicillium species, mainly *A. ochraceus*, *A. carbonarius*, *A. niger,* and *P. verrucosum*. Among these, OTA is considered the most abundant and harmful mycotoxin. OTA has been shown to be a potent nephrotoxic, hepatotoxic, and teratogenic compound. OTA has also been categorized as group 2B carcinogen for humans by the International Agency for Research on Cancer [8]. The intake of feed contaminated with OTA affects animal health and productivity and may result in the presence of OTA in the animal products. In acute cases, death may occur due to acute renal failure hence this toxin is regarded as nephrotoxic [11].

Ochratoxin commonly occurs in agricultural commodities, especially cereals this is corn and various foods such as cereal-based, wine, tea, coffee, cocoa, herbs, milk and milk products, poultry, fish, pork and eggs, fruits and vegetables, beans, dried products, infant foods as well as for poultry and other animal feeds. OTA is mostly found in the cereal grains such as maize, barley, oats, wheat, rye, etc. In a recent study, the abundance of ochratoxin A in grain corn is increasing in subregion of Asia in the second quarter of 2021 [10].

2. Mycotoxins and their detections

2.1 Current state detection for mycotoxins

Hitherto, instrumentation methods such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and gas spectrometry (GC) are regarded as gold standard for food contaminants analysis, including mycotoxins. The chromatography system is usually coupled with detectors such as fluorescence, ultraviolet–visible, or mass spectrometry. Even though these techniques are sensitive, they require extensive sample extraction, trained personnel, and laborious. Antibody-based assay or immunoassays method for aflatoxins and ochratoxins detection include enzyme-linked immunosorbent assay (ELISA), chemiluminescent

immunoassay (CLIA), fluorescence immunoassay (FIA), and lateral flow immunochromatographic assays (LFIA) [12]. Commercial ELISA kits also are widely available in the market for mycotoxins detection. However similar to chromatography analysis, ELISA method is still laboratory-bounded and requires a spectrophotometer to give accurate quantitative readings. Moreover, as ELISA is an optical-based technique, it is susceptible to light interference and hence is not suitable for on-site detection. LFIA strips also are being sought in the market as their analysis is straightforward, economical, and does not require skilled personnel. Nevertheless, the analysis is qualitative and only semiquantitative [13].

Besides immuno-based methods, spectrometric and spectroscopy techniques are also deemed sensitive for the detection of mycotoxin in food products. Near-infrared hyperspectral imaging has been successfully applied for the detection of fungal infection and OTA contamination in stored wheat and barley [14, 15]. Image processing techniques developed from machine learning is also gaining attention due to their nondestructive application for mycotoxins monitoring. Such has been applied for AFB1 in grains and feed commodities [16–19]. Machine learning methods, however, have limitations in terms of data volume, noises from the sensor, and calibration errors, besides being unable to ascertain types of mycotoxins produced.

Although tolerable limits for mycotoxins have been established, most contamination still exceeds maximum thresholds and hence continues to pose considerable risk to public health. As highlighted above, most of the reliable detections are lab-based and time-consuming. With this regard, when one requires immediate screening and on-site detection of mycotoxins, there is no readily available method. To address this, we have developed an immuno-based electrochemical biosensor for quantitative detection of *Aspergillus* spp. mycotoxins, namely AFB1 and OTA. Although the finding reported here is applied to grain corn (*Zea mays* L.), this developed biosensor platform can ultimately be applied to other food commodities.

2.2 Biosensor approach for mycotoxins detection

The first biosensor concept was introduced in 1962 based on an enzyme electrode for glucose sensing [20]. Ever since that, biosensor field has been receiving plethora of publications and vast applications in medicine [21–23], food safety [24, 25], agriculture [26–28], and environment [29]. By consensus, biosensor is defined as "*an analytical device, which exploits a biological detection or recognition system for a target molecule or macromolecule, in conjunction with a physicochemical transducer, which converts the biological recognition event into a useable output signal*" [30]. A biosensor system comprised of three essential components (**Figure 3**):

i. Detector—recognizes the biological element of interest (DNA, antibody, enzymes, cells, bacteria, etc.).

ii. Transducer—converts the biological element recognition to a readable signal.

iii. Output system—involves amplification and display of the signal.

Among the transducer mechanisms, electrochemical method is favorable in comparison to optical and mechanical. While optical biosensor is susceptible to light interference, electrochemical biosensor offers feasible application with simple sample extraction, rapid detection (within a few minutes), and point-of-care measurement.

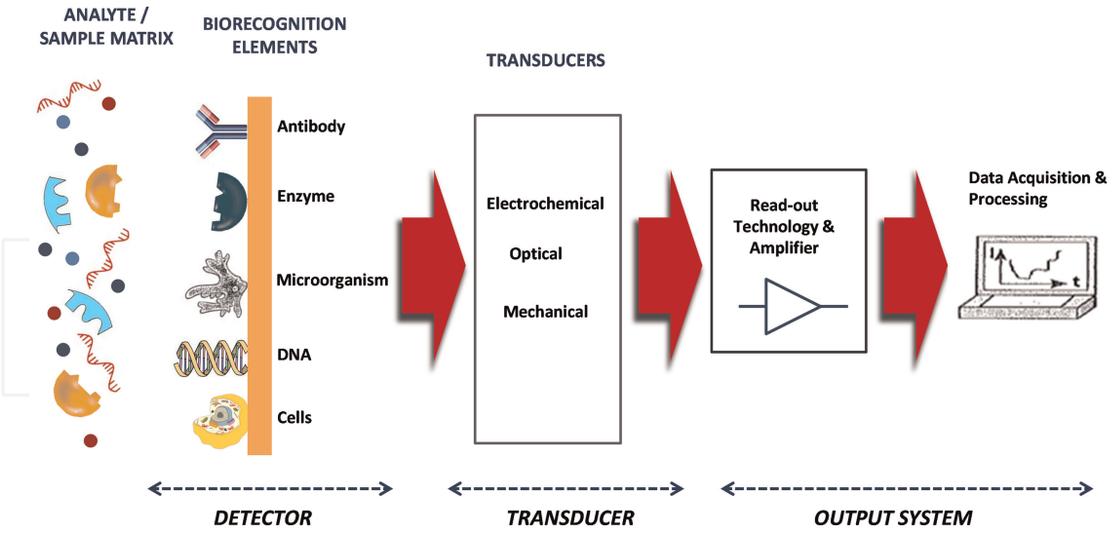

Figure 3.
Schematic diagram of biosensor comprising three components: Detector, transducer, and output system. Taken from ref. [31].

Main electrochemical sensing methodologies are voltammetry, potentiometry, impedimetric, conductometry, and amperometry. The recognition of biological elements (aptamer, enzyme, cell, antibody) with their targeted analytes by means of electrochemical measurement has been proven to provide reliability in numerous food contaminants detection, including mycotoxins in food and feed commodities [32–34]. Biosensors-based detection techniques have immense potential for mycotoxins detection in both research and industry as they are portable, simple, robust, sensitive, and cost-effective [35]. Of recent, aptamer, regarded as synthetic version of the antibody, also has been utilized in mycotoxin detection [36].

3. Strategies for advancing mycotoxins immunosensor

A miniaturized biosensor system with electrochemical tools and an integrated portable device for the detection of AFB1 and OTA mycotoxins is described in this study. The biosensor development employs in-house polyclonal antibody hence the term immunosensor. Research activities involved in mycotoxin immunosensor development are summarized in **Figure 4** as follows. In the first step, the antibody production is raised in rabbits as the animal host. After antibody purification and characterization, the antibody will be immobilized on screen-printed carbon electrodes (SPCEs), also addressed as sensor strips, for biosensing application. The immuno-functionalized SPCEs will be first optimized and tested on an electrochemical workstation for its proof-of-concept study. The strips are then attached to a handheld portable device integrated with I-of-Things (IoT) as point-of-care for rapid and real-time *in situ* analysis.

3.1 Components in biosensor integrated internet-of-things (IoT)

The integrated portable biosensor system for AFB1 and OTA detection with IoT system consisted of three main components (**Figure 5**): (i) an immuno-functionalized screen-printed carbon electrode (SPCE); (ii) a handheld electrochemical device with

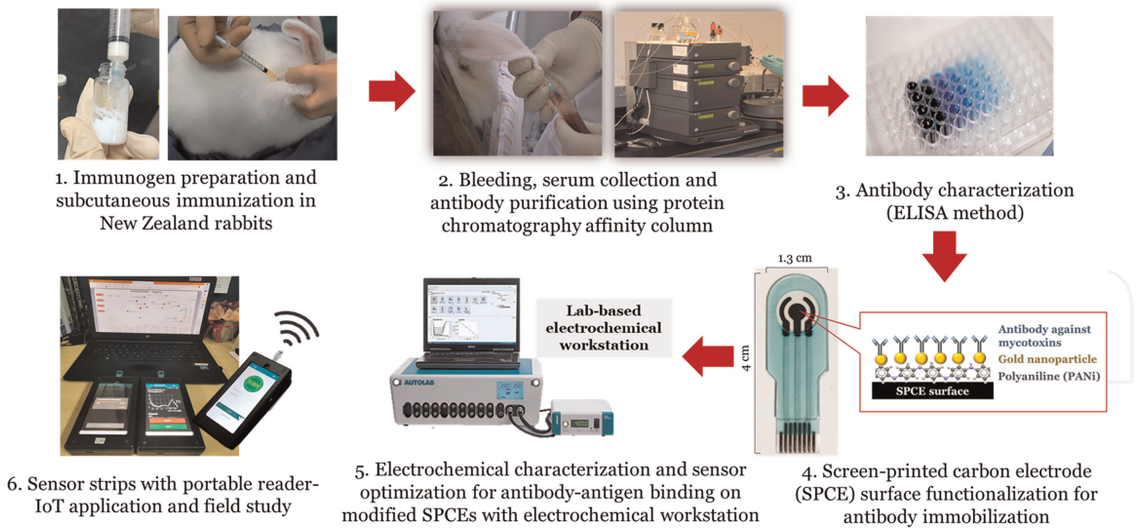

Figure 4.
Workflow for research activities involved in the development of immunosensor for mycotoxins detection.

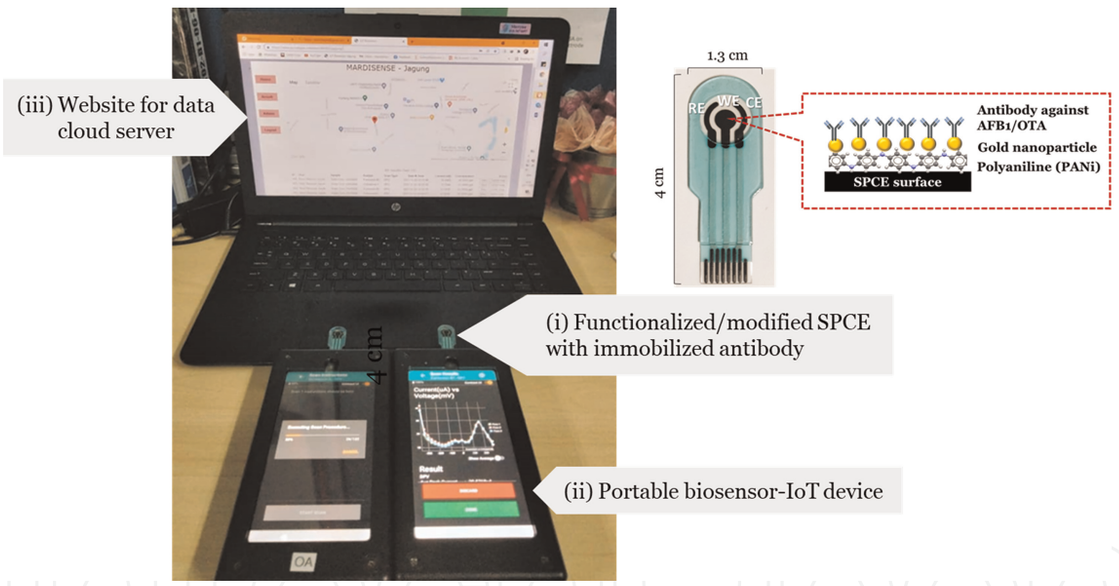

Figure 5.
Three main components of biosensor-IoT system. Inset*: A modified SPCE with polyaniline (PANi) and gold nanoparticle (AuNP) for antibody immobilization.*

electrochemical software; and (iii) a dedicated web server. The main part of the biosensor lies in the modified SPCEs. A plastic-based SPCE with single strip dimension of 1.3 cm (width) x 4 cm (length) is used in this study. The SPCEs have three carbon-based electrode channels: reference electrode (RE), working electrode (WE), and counter electrode (CE). WE is placed in the middle with a 4-mm circular well. The SPCEs are first modified with a conducting electroactive polymer (i.e. polyaniline, PANi) and nanogold (AuNP) network for antibody immobilization. The binding between the immobilized antibody and the targeted mycotoxin in samples will take place on the SPCE's WE surface.

Second component of the system is an improvised Android-based device with an electrochemical software and an electrode's scanner port as portable electrochemical

reader. To perform the analysis, the SPCE is first inserted into the reader's port. The signal generated from antibody immobilized on the SPCE and its targeted mycotoxins is measured by the scanner on the electrochemical device *via* differential pulse voltammetry (DPV) technique. The quantified results in terms of toxin concentration (i.e. parts per billion, ppb) are displayed on the device's screen. This handheld device has built-in mobile app equipped with global positioning system (GPS) function, which allows sampling locations to be recorded. The third component, the web server, allows authorization and electrochemical settings of the portable device by registered personnel. As the portable reader is integrated with IoT, the server will collect all the field test data to allow users to perform further analysis such as location-mapping as well as trend-mapping. The system can also be set to prompt alerts to mobile phones if the test data exceeds certain preset limits to indicate immediate actions are required.

4. Development of electrochemical immunosensor for mycotoxins detection

4.1 Antibody as bioreceptor

Despite choices of aptamers and antibody as bioreceptors in mycotoxin biosensor, antibody still remains the most useful tool for identifying contaminants as the analysis can be performed on-site [6]. The production of in-house polyclonal antibody against AFB1 and OTA for mycotoxins immunosensor is described here. In biosensor development, polyclonal antibody is preferred over monoclonal antibody due to its multiple epitopes availability that can recognize its target analytes, hence signals generated are higher compared to those of monoclonal.

4.1.1 Production of polyclonal antibody against mycotoxins

Polyclonal antibodies against AFB1 and OTA were produced in-house at Biotechnology & Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI). Antibody immunization against AFB1 and OTA was performed in four New Zealand rabbits (*Oryctolagus cuniculus*) with two replicates of rabbits for each toxin, denoted as A1 and A2 for AFB1, O1 and O2 for OTA. Animals protocol was reviewed and has been approved by the Animal Ethics Committee of MARDI (reference number 20190215/R/MAEC00045). For optimum antibody production and performance, immunization procedures were carried out adhering to the recommended guidelines [37].

Prior to immunization, blood was first taken and this preimmune batch is accounted as control study. Mycotoxins which have total molecular weight of >500 Da do not have immunogenicity property, hence as immunogen, they need to be coupled with a hapten in order to elicit immunity response in rabbits. Primary injection solution was prepared by homogenously mixing mycotoxins conjugated protein carrier with complete Freund's adjuvant (CFA) at 1:1 mixture. For subsequent immunogen, secondary injection solution was prepared by substituting CFA with incomplete Freund's Adjuvant (IFA). In total, eight immunization shots were performed and blood was collected until the fifth bleed within a duration of 5 months. Blood samples collected were purified with ammonium sulfate precipitation followed by nProtein A affinity column in order to obtain pure anti-AFB1 and anti-OTA IgG antibodies.

4.1.2 Characterizations of polyclonal antibody

Following antibody production and purification, the IgG needs to be further characterized to determine its performance, sensitivity, and selectivity. First, antibody titer is performed using indirect ELISA. The microtiters plates were coated with respective mycotoxins conjugated protein as the antigens. The performance of antibodies is evaluated by reducing the antibodies into several dilutions followed by optical measurement that is absorbance value. The purpose of titer is to determine which bleed of antibodies produced the best and most reliable result with the lowest dilution. These antibodies will then be used for further sensor development, thus, this step is exceptionally important. In general, a higher antibody titer indicates a better quality of fractionated antibody.

In both antibodies against AFB1 and OTA, preimmune antibodies that were used as control study showed no significant binding with the coated antigens (**Figures 6** and 7), indicating the successful production of the intended antibodies. From **Figure 6**, antibodies against AFB1 from third bleed onwards showed higher absorbance compared to the preimmune and the first two bleeds. The absorbance values recorded for both A1 and A2 rabbits exhibited similar range, indicating the reliability of the polyclonal antibody production in different hosts.

For antibodies against OTA, the absorbance of all bleeds (except preimmune) showed similar pattern until 10^{-3} dilution before absorbance reading gradually decreases upon further dilution (**Figure** 7). Antibodies from second bleed onwards

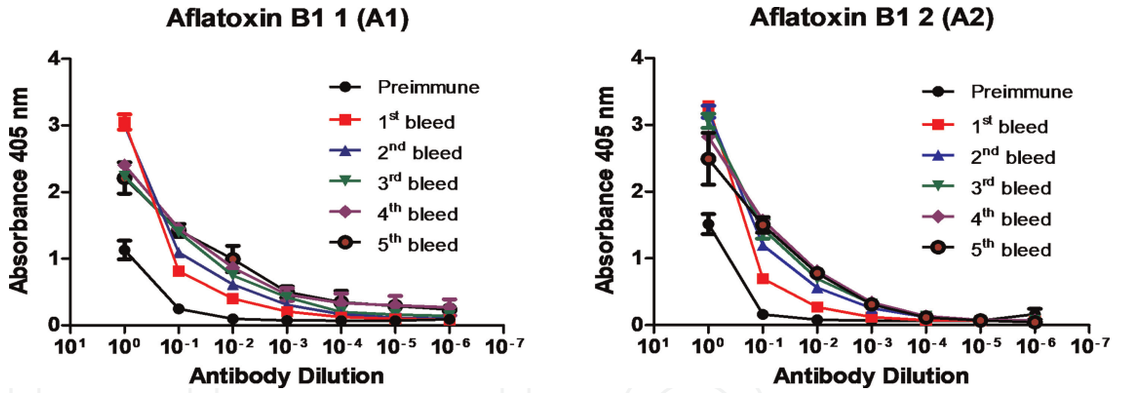

Figure 6.
Antibody titer for anti-AFB1 antibody produced from two different rabbits, A1 and A2.

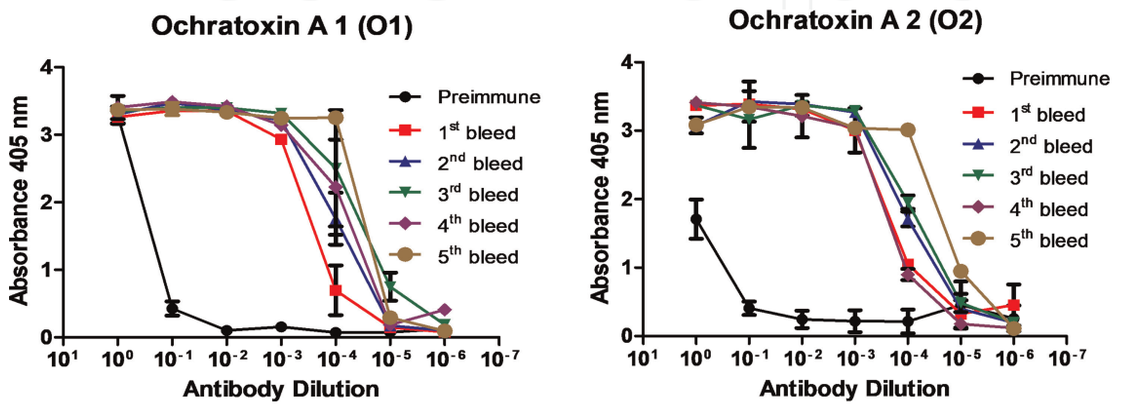

Figure 7.
Antibody titer for anti-OTA antibody produced from two different rabbits, O1 and O2.

showed the most prominent response compared to the first and preimmune bleed. Antibodies from third bleed exhibited the lowest concentration for antigen detection at 10^{-5} antibody dilution.

The amount of total protein in each antibody batch is then quantified by protein assay. Bicinchoninic acid or BCA protein assay is usually employed to determine the concentration of protein in the developed antibody. Bovine serum albumin (BSA) is used as the reference standard for antibody against mycotoxins assay. **Figure 8** displayed the concentration of antibody protein collected from different rabbits for anti-AFB1 and anti-OTA antibodies. The calculated concentration is based on BSA standard curve. Overall, the concentration was relatively low before introduction of mycotoxin into the rabbits' immune system. The protein concentration gradually increased as the rabbits built protective immune barrier against the mycotoxins after several injections. Some rabbits maintained the amount of antibodies in their bodies and some rabbits showed low tolerance of mycotoxins after four injections.

4.1.3 Cross-reaction studies

The purified IgGs are then subjected to cross-reaction study in assessing their specificity and sensitivity. The percentage of cross-reaction (CR) is obtained based on Eq. (1) while half-maximal inhibitory concentration (IC_{50}) values for linear response are calculated from Eq. (2). IC_{50} is one of the simplest and most accurate techniques to determine the performance of antibody toward specific target analyte in an assay. In this study, IC_{50} represents the 50% response of a series of mycotoxin concentrations (0–10 ppb) and corresponding electrochemical currents in a linear correlation, thus expressed in Eq. (2).

$$CR\ (\%) = \frac{IC50\ AFB1\ or\ OTA}{IC50\ toxins} * 100 \tag{1}$$

$$IC_{50} = \frac{(0.5 - c)}{m} \tag{2}$$

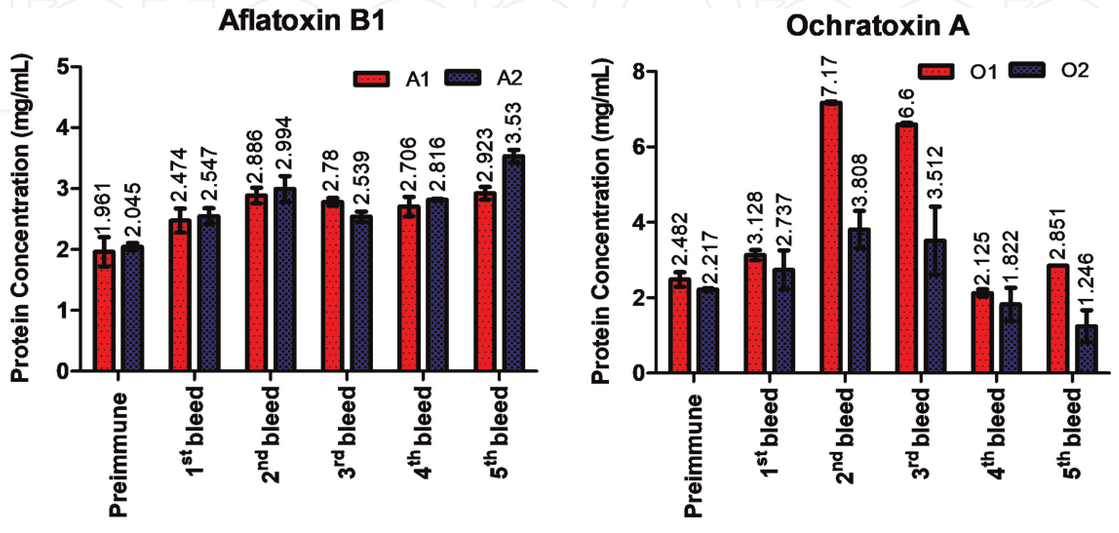

Figure 8.
Protein concentration found in antibodies against AFB1 (left) and OTA (right) for two rabbits.

The specificity of the anti-AFB1 polyclonal antibody produced was tested against other types of aflatoxins (i.e. AFB2, AFG1, and AFG2) and also other mycotoxins (ochratoxin A, ochratoxin B, fumonisin B1, fumonisin B2, and fumonisin B3). The anti-AFB1 antibody was found specific to AFB1, as indicated by the cross-reactivity studies shown in **Figure 9**. AFG1 showed cross-reaction with the antibody at 71% relative to AFB1 while AFB2 was at 65%. The cross-reaction study for AFG2 showed low cross-reactivity of less than 30%. The cross-reactivity order found was in the order of AFB1 > AFG1 > AFB2 > AFG2, corresponding with the order of toxicity reported [9, 38]. Meanwhile, when tested against other mycotoxins, anti-AFB1 antibody exhibited no cross-reaction with ochratoxins A and B, and fumonisins B1, B2, and B3. Overall, the in-house polyclonal antibody produced is highly sensitive toward AFB1, displaying high reactivity toward same group compounds and minimal cross-reactivity toward different group compounds. Similarly, anti-OTA antibody produced displayed nonsignificant cross-reactivity toward all groups of mycotoxins (**Figure 9**). The performance of OTA antibody in competitive assay was highly selective toward its respective target analyte.

4.2 Modified SPCEs for mycotoxins detection

Screen-printed electrodes (SPEs) fabricated by means of thick film deposition lend themselves well in biosensor construction. The miniaturization of three-cell electrodes integrated in one chip offers an economical, straightforward analysis and practicality that suit on-site analysis application [39]. The incorporation of nanomaterials and conducting polymers on the SPE's surface has successfully enhanced the sensitivity of the detection system [40, 41]. Here, the working electrode (WE) area on the SPCE was first modified by drop-casting a mixture of PANi and AuNP for antibody immobilization.

The combination of PANi and AuNP network was found to provide numerous binding sites for biomolecules and enhance current signals due to the Au nano-size and the properties of the conducting polymer, respectively. Antibody concentration of 0.1 mg/mL, which showed the most optimum electrochemical response toward the target analyte, was immobilized on the carbon WE surface (**Figure 10a**). The

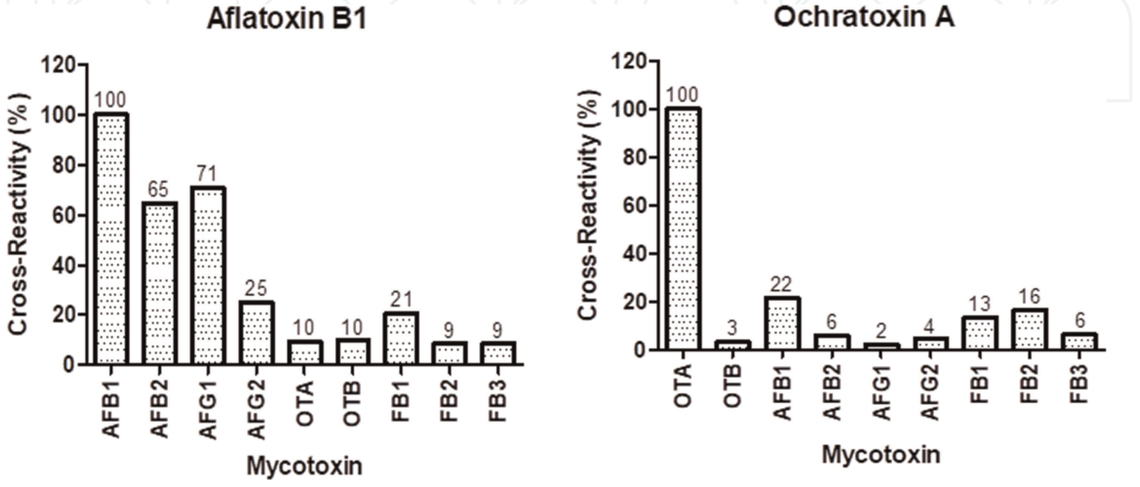

Figure 9.
Specificity study of anti-AFB1 antibody (left) and anti-OTA antibody (right) with their targeted mycotoxins and other mycotoxins groups. The percentage of cross-reaction was calculated based on IC_{50} readings.

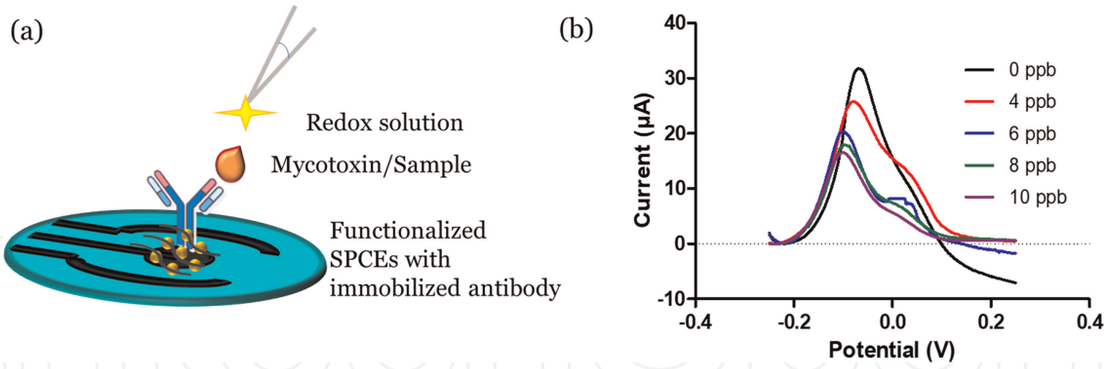

Figure 10.
(a) Working electrode (WE) of SPCE surface modification for antibody immobilization; and (b) differential pulse voltammetry (DPV measurement) for label-free approach for mycotoxin immunosensor.

electrochemical characterization from the SPCE modification has been detailed elsewhere [42]. The label-free electrochemical biosensor approach by means of DPV measurement (**Figure 10b**) was carried out by applying a redox solution of 5 mM ferricyanide/ferrocyanide in 0.1 M KCl on the SPCEs.

4.3 Sensor optimizations and standard curves development

Processed and nonprocessed grain corns can produce harmful mycotoxins along the production chain which can lead to serious health issues for livestock and human. In grain corn, *Aspergillus* species are the main producers of mycotoxins in maize, infecting both preharvest and during storage. Standard curves for AFB1 and OTA have been developed in grain corn as sample matrix model (**Figure 11**). The electrochemical measurement was carried out in undiluted and interference-free sample matrix on an electrochemical workstation. Peak current from voltammogram for each concentration was selected as the corresponding antibody-antigen response from electrochemical process and plotted on the graph. Overall, both AFB1 and OTA displayed a good linear correlation (R2) of 0.971 and 0.9685, respectively, in a broad working range of 0–10 ppb.

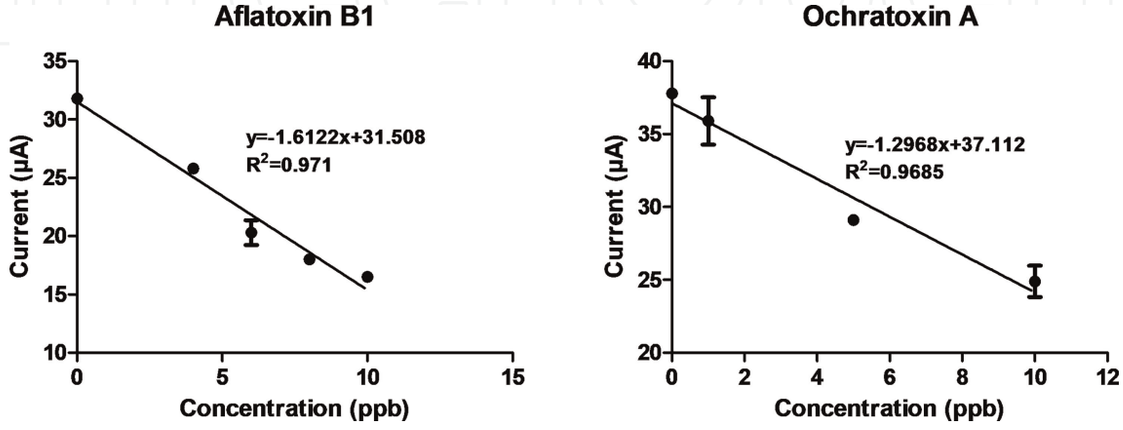

Figure 11.
Linear response from electrochemical workstation for a series of AFB1 (left) and OTA (right) concentrations with current reading in grain corn matrix.

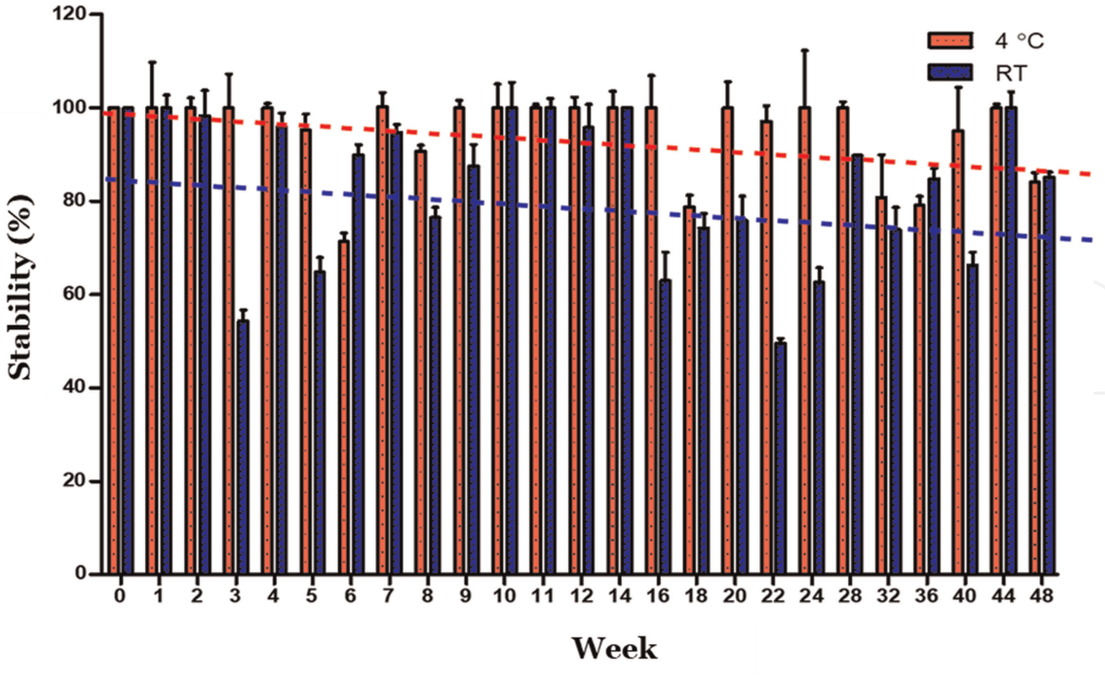

Figure 12.
Stability study of sensor strips with immobilized antibody against mycotoxin for a duration of 48 weeks stored in different temperatures (RT and 4°C).

4.4 Storage stability of the modified SPCEs

The stability of modified SPCEs with immobilized anti-AFB1 and anti-OTA antibodies was evaluated for the duration of 48 consecutive weeks. The sensor strips were stored without the presence of buffer at two storage temperatures: 4°C and room temperature (RT). This study is important to study the shelf-life of the functionalized strip in producing optimum results for long-term application. From **Figure 12**, it was found that storage in chilled environment showed higher antibody stability (90%) compared to ambient surroundings. The performance of the SPCEs started to decline below 90% stability after 14 weeks at 4°C and at the end of 48 weeks, the performance is still acceptable at 85% stability.

5. Electrochemical portable device as point-of-care

Portable electrochemical devices have a great potential as excellent tools for point-of-care testing technology. Detection of hazardous contaminants by means of portable electrochemical devices is a strategic approach for an efficient and rapid detection. With the complement of miniatured electrochemical cells, analysis can be conveniently performed on-site by nontechnical personnel. To facilitate on-site application and real-time detection of mycotoxins, a series of portable biosensor readers have been fabricated [39, 43, 44]. However, these readers, and even some commercially available handheld potentiostats, do not have latest features, particularly Internet-of-Things (IoT) integration. Recent trend in linking the portable device with IoT and cloud data storage has been made possible with the advancement of Fourth Industrial Revolution (IR 4.0).

In this study, an Android device is improvised as a universal portable electrochemical reader. A single unit of the portable reader is encased in a plastic box with dimensions of 16.8 cm (long) × 8.5 cm (wide) × 3.4 cm (height), powered by rechargeable lithium battery using USB cable through charger port. The reader which has a slot for SPCE attachment port consists of two main parts; an electrochemical transducer represented by a printed circuit board that converts signal from biology interactions reactions into readable and analyzable data; and a touch-screen Android-based device with developed electrochemical software application (**Figure 13**). In conventional electrochemical instrument, this unit is represented by a potentiostat. A Global Positioning System (GPS) tracking app is incorporated into the portable device, which allows users to pinpoint the real-time location of the field analysis performed. The handheld device has sufficient memory size of up to 32GB that can store over 100,000 measurements.

5.1 Interface for electrochemical portable reader and web server

The device interface is designed to be user-friendly, with the aim to allow users with minimal training to perform the testing themselves in the field. Authorized users will first need to log into the system before starting the measurement. This touch-screen device features drop-down menu, which is deemed convenient and practical for users' applications. To perform the testing, the user will need to insert the biosensor strip into the SPCE port on the reader, select the type of analyte (mycotoxins) to be tested, and press 'SCAN' to start the testing process. The interface for the Android-based device is shown in **Figure 14**. Once the scan is completed, the result is displayed on the device as concentration in ppb unit. The data then will be saved into the device, followed by the real-time transmission of the data together with GPS location and date/time to a central cloud server. The brightness of the screen can be adjusted; this feature is particularly important if the device is being used outdoor on the field. After

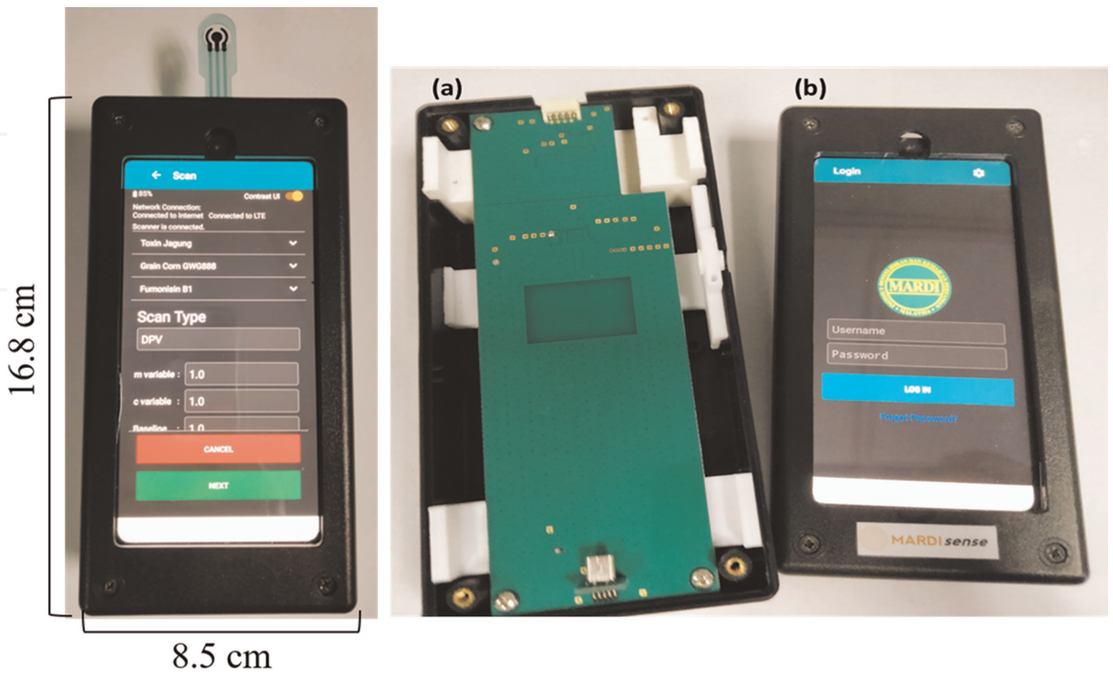

Figure 13.
An android-based portable electrochemical reader unit (16.8 x 8.5 x 3.4 cm) comprised of (a) electrochemical transducer and (b) touch-screen android-based device with developed electrochemical software.

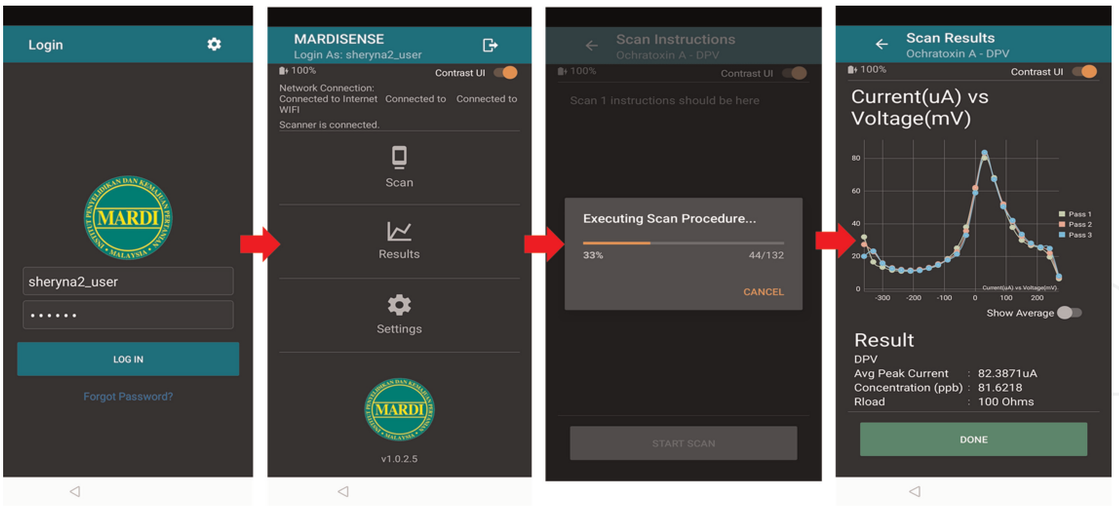

Figure 14.
Screen interface for portable reader application. User can select 'scan' *to begin the analysis or* 'results' *to view the analyzed results. Results are displayed in form of graphs and readily calculated in concentrations (ppb).*

a successful log-in, personnel can start a measurement, review results, or change the device setting.

All analyzed results on the portable readers will be stored on the cloud server with their data log details and pinned sample location. **Figure 15** displayed the web page for IoT server that acts as authorization, electrochemical setting, and data storage platform. The website can be accessed at http://mardisense.mardi.gov.my. Complete functions of each component were described in other publications [45].

5.2 Performance of portable reader as biosensing device

5.2.1 Sensor optimization on portable reader

Antibody concentration for both AFB1 and OTA was optimized at 0.1 mg/mL and was applied for cross-reaction study using a DPV measurement. The selectivity of immobilized in-house polyclonal antibody on the SPCEs was examined through cross-reactivity within the same toxins groups and other mycotoxins groups. As shown in

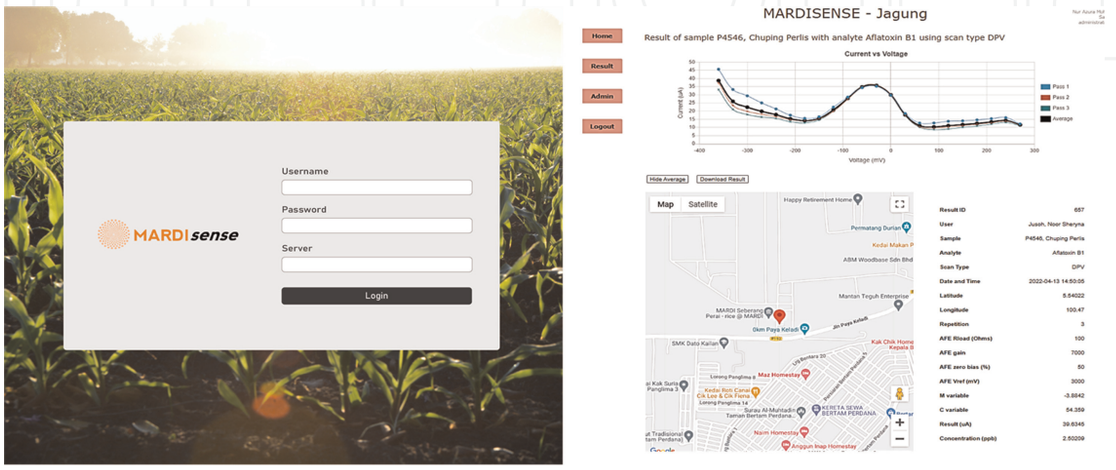

Figure 15.
Login interface of MARDIsense *website (left); and page displaying details of the analyzed data logs (right).*

Figure 16, the immuno-modified SPCEs were highly selective toward AFB1 and OTA, and demonstrated no cross-reaction with other mycotoxins. The cross-reactivity shown is less than 40%, hence the developed biosensor is still significantly sensitive and selective toward targeted toxins.

5.2.2 Sensitivity in sample matrix

The performance of developed sensor on the portable device was evaluated in undiluted and interference-free grain corn matrix. Standard curves for AFB1 and OTA are plotted as shown in **Figure 17**. Both AFB1 and OTA displayed an excellent linear correlation (R^2) of 0.9987 and 0.9836, respectively in a broad working range of 0–10 ppb. These indicate that the strategies of using the modified SPCEs on the portable reader are a success without compromising the sensitivity and accuracy of the biosensor system.

A recovery study was conducted for both AFB1 and OTA using spiked grain corn sample. This experiment is vital to evaluate the capability of the developed sensor to determine the presence of target analyte in sample. Prior to recovery study, the grain corns were first sterilized in 30% sodium hypochlorite (v/v) and autoclaved in ensuring a clean reference material for the spiking study. At concentrations of 10 ppb

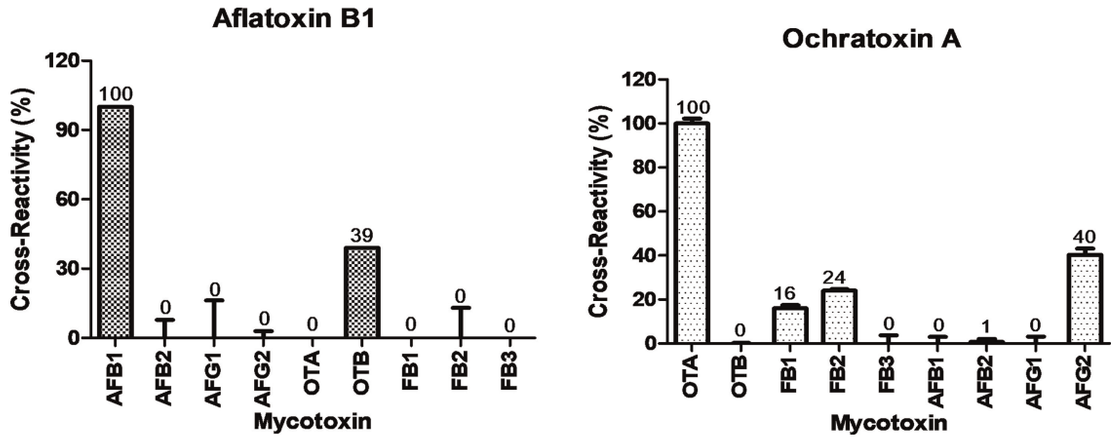

Figure 16.
Specificity study of the SPCEs with immobilized antibody with AFB1, OTA, and other mycotoxins at 6 ppb concentration.

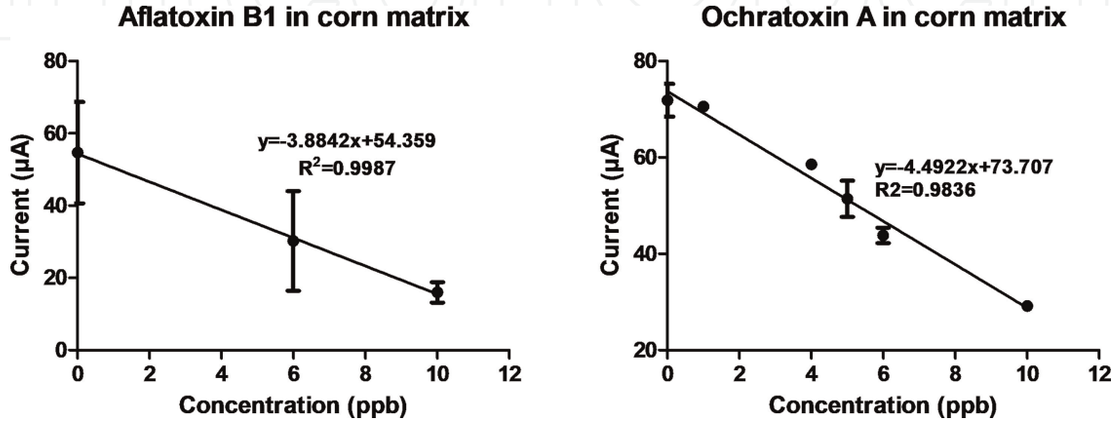

Figure 17.
Linear response from the portable device for a series of AFB1 (left) and OTA (right) concentrations with current reading in grain corn matrix.

spiked mycotoxins, the recovery was found to be in the acceptable range of 89–96% (**Table 1**).

5.2.3 Development of immunosensing methods for mycotoxin determination in sample matrix

The sensitivity of AFB1 and OTA detections in grain corn samples on the portable device is studied and compared with electrochemical measurements on a laboratory potentiostat workstation and an immunoassay technique that is enzyme-linked immunosorbent assay (ELISA). For the latter, a direct competitive ELISA was applied and the antibody-antigen response was recorded from the absorbance reading. All of the three detection means described here employed the in-house polyclonal antibodies against AFB1 and OTA as their bioreceptors. The laboratory potentiostat was utilized for laboratory testing while the portable device was intended for on-field testing. Standard curves for different AFB1 and OTA concentrations were developed, and parameters from the standard curves were compared, namely correlation coefficient (R^2), limit of detection (LOD), and limit of quantitation (LOQ) values.

From **Table 2**, all immune-based methods exhibited high R^2 values in broad working range of 0–10 ppb except for ELISA for OTA, which has a working range between 0 and 50 ppb. Electrochemical sensing method, both on the electrochemical workstation and the IoT-portable reader, displayed good correlation values, which are comparable with those of ELISA method for AFB1 and OTA. The other important parameter of determining the sensitivity of a developed method is the limit of LOD and LOQ values. LOD value represents the lowest concentration of an analyte that can be detected using an instrument. Lower LOD value implied higher sensitivity of an instrument, thus proving the better performance in detecting the target analyte. The

	Spiked (ppb)	Found (ppb)	Recovery (%)
AFB1	10	8.86	89
OTA	10	9.60	96

Table 1.
Recovery study of 10 ppb AFB1 and OTA in spiked grain corn on portable-IoT device.

	Mycotoxins/Parameters	AFB1	OTA
ELISA	R^2	0.9756 (0–10 ppb)	0.9903 (0–50 ppb)
	LOD	1.90 ppb	2.70 ppb
	LOQ	5.77 ppb	8.10 ppb
Electrochemical workstation	R^2	0.971 (0–10 ppb)	0.9685 (0–10 ppb)
	LOD	2.54 ppb	3.31 ppb
	LOQ	7.68 ppb	10.03 ppb
IoT-Portable reader	R^2	0.9987 (0–10 ppb)	0.9836 (0–10 ppb)
	LOD	0.84 ppb	1.72 ppb
	LOQ	2.55 ppb	5.22 ppb

Table 2.
Comparison of the performance for immuno-based detection methods for AFB1 and OTA in grain corn matrix.

MRL for both AFB1 and OTA in feed is 5 ppb and the calculated LOD values for both mycotoxins were much lower than the MRL for all immuno-based methods. In particular, the developed portable device recorded the lowest LOD and LOQ values of all for both AFB1 and OTA, signifying that the device was applicable for mycotoxins on-site testing.

5.3 On-field application of portable reader

In-situ determination of AFB1 was conducted at a cornfield in Perlis, northern region state of Malaysia (GPS coordinate 6.59835, 100.283) in March (pre-harvest season) and April 2022 (harvest season). Preharvest testing was carried out on 90-day-old grain corn for primary detection of AFB1 while harvest testing was carried out on 117-day-old grain corn. Samples (grain corn cob) were taken from five sampling plots, extracted using a simple sample extraction, and measured on-field (**Figure 18**). All samples showed detected AB1 concentration below the permitted level, which is 5 ppb [45].

5.4 Validation study with instrumentation method

Besides samples in Perlis, the developed biosensor system with the portable device was also tested on several other grain corn samples from Bachok and Seberang Perai, and used for AFB1 monitoring in stored grain corn with different packaging materials. A total of twenty-grain corn samples were analyzed with the portable reader and validated with HPLC-FL post-column method and UPLC-FLR. Correlation between the analyzed data and the instrumentation is tabulated in **Table 3**. LOD for the electrochemical portable reader sensor is 0.84 ppb while LOD for HPLC is 2.5 ppb. Data for HPLC method is presented by LOD reading, and it is indicative of either the AFB1 is not detected by the system or AFB1 concentration is below 2.5 ppb. Of the 20

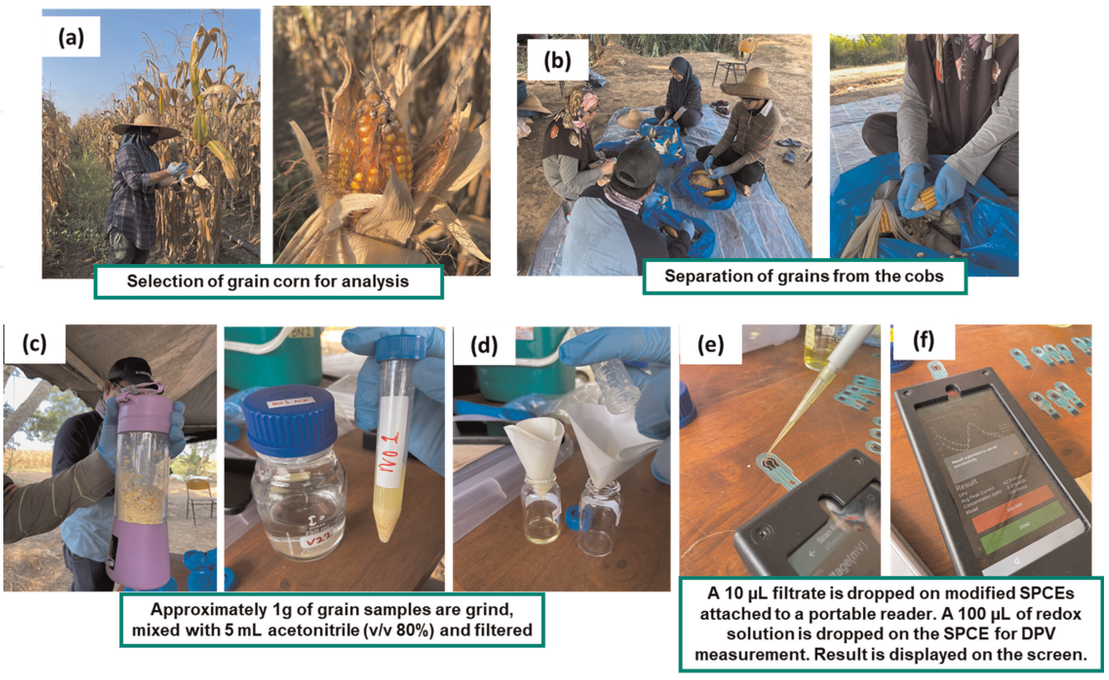

Figure 18.
On-site application for AFB1 detection in grain corn using the modified SPCEs and portable reader.

	[a]Portable device	[b]HPLC
Sample 1	0.96	<LOD
Sample 2	[c]n.d.	<LOD
Sample 3	n.d.	<LOD
Sample 4	n.d.	<LOD
Sample 5	n.d.	<LOD
Sample 6	n.d.	<LOD
Sample 7	n.d.	<LOD
Sample 8	1.25	<LOD
Sample 9	n.d.	<LOD
Sample 10	1.51	<LOD
Sample 11	1.25	<LOD
Sample 12	2.34	<LOD
Sample 13	3.42	<LOD
Sample 14	n.d.	<LOD
Sample 15	4.32	2.85
Sample 16	3.44	<LOD
	Portable device	[d]UPLC-FLR
Sample 17	n.d.	n.d.
Sample 18	n.d.	n.d.
Sample 19	<LOD	n.d.
Sample 20	2.13	n.d.

[a]LOD electrochemical portable device = 0.84 ppb.[b]LOD HPLC = 2.5 ppb.[c]n.d. = not detected.[d]LOQ UPLC-FLR = 1 ppb.

Table 3.
Detection of AFBI in grain corn sample on the portable reader and its validation study using instrumentation methods (HPLC and UPLC-FLR).

samples, ten samples were not contaminated by AFB1 as determined by the portable reader. Nine samples were detected with low AFB1 concentrations lower than the MRL permitted (5 ppb). Sample 15 recorded 4.42 ppb of AFB1 and was confirmed by the HPLC with 2.85 ppb. The discrepancies detection for validation with the UPLC-FLR method could be attributed to different sampling batches and duration of time for both experiments conducted, which affect the moisture content in the samples.

In general, the strategies of biosensor miniaturization for point-of-care using the modified SPCEs on the portable reader are successful without compromising the sensitivity and accuracy of the biosensor system. The portable reader offers a straightforward and direct application of AFB1 and OTA detection in grain corn with simple sample extraction as opposed to conventional methods. Furthermore, biosensor with portable reader allows the test to be performed rapidly on-site without the need to bring back the samples to the laboratory where the transportation and logistics factors may also contribute to the discrepancies in the analyzed results. The portable reader and miniaturized biosensor system described here can be used for mobile lab applications. Although the studies presented here highlighted mainly grain corn,

nevertheless the application of this developed system can be widened to other food commodities, particularly peanuts and rice for AFB1; and grains and coffee for OTA, to name a few. Moving forward, another point to be considered is the development of multi-mycotoxins detection on a dual or multiple working electrode [46].

6. Conclusions

A portable electrochemical device integrated with IoT system has been successfully designed for rapid and *in-situ* detection of *Aspergillus* spp. mycotoxins. Polyclonal antibody against AFB1 and OTA that were produced and purified in-house showed excellent sensitivity and selectivity toward the targeted mycotoxins. The antibodies are then further utilized and immobilized on modified SPCEs for the development of immunosensor system for mycotoxins detection. Using grain corn as matrix sample model, electrochemical measurements achieved by DPV for AFB1 and OTA standard curves development on the portable IoT device are superior to the ELISA method and laboratory electrochemical workstation. Good linearity was obtained in working range of 0–10 ppb (MRL 5 ppb) with excellent LOD and LOQ values. *In-situ* analysis of AB1 has been successfully conducted at a local cornfield in Perlis, northern Malaysia, using the portable device integrated with IoT as point-of-care tool. Analysis of 20 samples with the portable reader correlated well with HPLC and UPLC-FLR methods. In general, biosensors posed major advantages over the conventional method for mycotoxins detection in terms of assay duration, detection limit, and portability. With IoT integration and web server that can store data logs and pinpoint sampling locations, the portable device posed an excellent tool for mycotoxins routine monitoring in near future.

Acknowledgements

The authors would like to thank the Ministry of Agriculture and Food Industry of Malaysia for the financial support under research fund KRB 167. Gratitude also goes to other team members involved in this study and Mr. Hashim Ab Rahim from Persada Bumi Enterprise for the sampling plot at the cornfield.

Conflict of interest

The authors declare no conflict of interest.

Fabrication of portable device

Electrochemical portable devices (Version 1) with cloud server integration were fabricated and developed by Biogenes Technologies Sdn. Bhd., Malaysia.

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