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# Development of a cellular biosensor for the detection of aflatoxin $B_1$, based on the interaction of membrane engineered Vero cells with anti-$AFB_1$ antibodies on the surface of gold nanoparticle screen printed electrodes

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ABSTRACT

The development of methods for the detection of aflatoxin $B_1$ ($AFB_1$) in foods is a very important practice for ensuring food quality and safety. Most tests of $AFB_1$ are still conducted with conventional methods (i.e. antibody-based ELISA tests, high performance liquid chromatography – HPLC); however biosensor methods are being developed to date as screening tools for field analysis. Compared to immunology/ELISA-like tests or chromatography methods, biosensors are able to provide rapid, sensitive, robust and cost effective quantitative methods for on-site testing. In this work we propose a cellular biosensor based on Vero cells, membrane engineered with anti-$AFB_1$ antibody as the biological recognition element reacting with $AFB_1$ molecules on gold nanoparticle/screen printed electrodes (SPEs) (three electrode system). In order to culture the cells on the SPEs surfaces the working electrodes were coated with poly-L-lysine to facilitate cell adhesion. The SPEs were connected to a potentiostat device through a transducer and chronoamperometric (CA) and cyclic voltammetric (CV) measurements were performed. Quantitative results obtained using the cellular biosensor method for $AFB_1$ were compared to those obtained using the HPLC method in *pistachio* samples spiked with $AFB_1$. The method displayed good sensitivity ($r^2 = 0.87$) and detection limit (0.5 ng/mL).



## 1. Introduction

Mycotoxins are toxic secondary metabolites that can occur in food and feed in conditions that promote mould growth (Boonen et al., 2012). For the purpose of the present study aflatoxin B1 ($AFB_1$) was chosen as it belongs to a group of highly toxic difuranocoumarin derivatives that are produced by many strains of *Aspergillus flavus* and *Aspergillus parasiticus* and can contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favourable to mould growth (Cole & Cox, 1981; Creppy, 2002;; Waliyas & Reddy, 2009). Specifically peanuts, corn and cereal crops, before or after harvest are susceptible to AFB1 contamination (Ellis, Smith, & Simpson, 1991; Radoi, Targa, Prieto-Simon, & Marty, 2008). $AFB_1$ is particularly interesting for analysis because of the current regulatory decisions (European Commission Regulation (EC) 1881/2006; FAO, 2001; 2004 and FAO/WHO, 2007) according to which the allowable level for aflatoxins in Europe is just 4 mg/kg (ppm) of total aflatoxin or 2 ppm of aflatoxin $B_1$. The International Agency for Research on Cancer (IARC) has classified aflatoxin $B_1$ as a group 1 human carcinogen (IARC, 1993). This toxin exhibits carcinogenic, teratogenic and mutagenic properties and has been isolated from a wide variety of agricultural products (Moss, 2002). $AFB_1$ can enter the food chain mainly by ingestion via the dietary route in humans and animals; it has been shown that the intake of $AFB_1$ over a long period of time, even at very low concentrations may be highly dangerous (Miraglia, Brera, & Colatosti, 1996).

Many analytical methods have been developed for the determination of aflatoxins including thin-layer chromatography (TLC) (Fernandez, Belio, Ramos, Sanz, & Saez, 1997;; Ramesh, Sarathchandra, & Sureshkumar, 2013), antibody-based, indirect

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Enzyme Linked Immunosorbent Assays (ELISA) (Rossi et al., 2012;; Sun, Gu, Li, & Dong, 2015), often coupled with lateral flow devices (Maragos & Busman, 2010) and high-performance liquid chromatography (HPLC) (Jaimez et al., 2000;; Yazdanpanah et al., 2013). According to Kovacs, cell-based sensors utilize the ability of cells to selectively respond to complex mixtures of signals in a way that makes them highly attractive for detection of chemical and biological analytes, for detection of environmental toxins and for drug screening (Kovacs, 2003). A previous study from Rasooly et al. has shown that low levels of $AFB_1$ stimulate monkey Vero kidney cells, whereas high levels kill the cells (Rasooly, Hernlem, & Friedman, 2013).

The purpose of the study was the development of a cell based biosensor with Vero cells (plain or membrane engineered with anti-$AFB_1$ antibodies) as the biosensor's biological part for the detection of $AFB_1$ in pistachio matrices. In the present work, anti-$AFB_1$ antibodies were inserted to Vero cell membranes through electroinsertion and osmotic insertion, in order to be used as the biosensor's biological part.

The results were compared afterwards with the cellular biosensors' results. The biosensor was based on a potentiometric method (cyclic voltammetry, chronoamperometry) employing gold nanoparticle-modified carbon screen printed electrodes for the determination of $AFB_1$ with plain Vero and Vero-*anti*-$AFB_1$ based cellular biorecognition elements. In the near future they will play a key role in the field of cellular biosensors of high selectivity and specificity. In addition, Vero cell viability and total protein concentration have been assessed. In the second part, an analytical method was developed for the determination of $AFB_1$ in pistachio and nut matrices after extraction by the immunoaffinity column (IAC) method.

## 2. Materials & methods

### 2.1. Reagents

Vero cell culture was originally purchased from LGC Promochem (Teddington, UK). Dulbecco's Modified Eagle's Medium, fetal bovine serum, L-glutamine, penicillin/streptomycin, and trypsin/EDTA were purchased from Biochrom AG (Berlin, Germany). Dimethylthiazol-2-2,5 diphenyl tetrazolium bromide (MTT) was provided from MP Biomedicals (Solon, OH) and anti-$AFB_1$ mouse monoclonal IgG (200 μg/mL) from Santa Cruz Biotechnology Inc (Dallas, TX). The Immunoaffinity columns (IAC) AflaClean™ (3 mL widebore) were purchased by the company LC Tech (Germany). Finally, the $AFB_1$ standard from *Aspergillus flavus* (CAS Number 1162-65-8) and all other reagents were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture

Vero cells from African Green monkey kidney were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), l-Alanyl-*l*-Glutamine, Pyruvate and penicillin/streptomycin, in a standard incubator (95% humidity, 5% $CO_2$) and subcultured three times per week. For the subcultures, cells were collected with trypsin/EDTA, centrifuged 2 min at 1000 rpm and resuspended in fresh DMEM.

### 2.3. Membrane-engineered Vero cells with anti-$AFB_1$ antibodies (Vero-anti-$AFB_1$ cells)

Membrane-engineered cells were created either by electroinserting or by osmotically inserting mouse monoclonal anti-$AFB_1$ antibodies into the membrane of Vero cells following the modified protocols of Zeira et al (Zeira et al., 1991). and Moschopoulou and Kintzios (Moschopoulou & Kintzios, 2006), respectively. Briefly, for the electroinsertion method, the cells (2.5 × 106/mL) were centrifuged at 100*g* for 3 min and then resuspended in PBS (pH 7.4). Subsequently, cells were incubated together with different concentrations of anti-$AFB_1$ antibodies (0, 1, 1.4, 2, 6 and 10 μg/mL) for 20 min on ice. Then, the cells-*anti*-$AFB_1$ antibodies mixture was transferred to appropriate electroporator (Eppendorf Eporator, Eppendorf AG, Germany) cuvettes and electroinsertion was performed by applying an electric field (two pulses) at 1800 V/$cm^{-1}$. After electroinsertion, cells were incubated at 37 °C for 1 h. Finally, the cells were centrifuged at 100*g* for 3 min and resuspended in PBS (pH 7.4). This step was repeated twice.

Additionally, for the osmotic insertion a modified protocol of Okada and Rechsteiner (Okada & Rechsteiner, 1982) was followed. Vero cells (2.5 × 106/mL) were incubated with the same concentrations of anti-$AFB_1$ antibodies (0, 1, 1.4, 2, 6 and 10 μg/mL) in a hypertonic DMEM solution (0.5 M Sucrose, 10% PEG, DMEM) for 10 min at 37 °C. Then, the cells were centrifuged at 100*g* for 3 min and the supernatant was removed. The cells were resuspended in hypotonic DMEM solution (DMEM:$H_2O$ = 6:4) and were incubated for 2 min at 37 °C. Finally, the cells were centrifuged at 100*g* for 3 min and were incubated for 2 h in DMEM.

### 2.4. Viability test

MTT viability test was performed as described previously (Mosmann, 1983) with the following modifications. Cells were seeded on 96-well culture plates at 5 × 103 cells/well for 30 min, 24 h and 48 h after modification with the anti-$AFB_1$ antibodies. Then, MTT was added to the supernatant at a final concentration of 0.5 mg/mL and the plates were incubated for 3 h at 37 °C. After MTT formazan was formed, the medium was removed and replaced by 200 μL of dimethyl sulfoxide (DMSO) to solubilize the converted purple dye. The absorbance of this extract was measured at 560 nm in an Infinite M200 Pro plate reader. For the data analysis Magellan™ software was used. Cell viability was expressed as the relative formazan formation in treated samples as compared to control cells. The protein concentration of the different modifications was determined by the Bradford method (Bradford, 1976).

### 2.5. Sensor fabrication

All electrochemical experiments were performed with a PG581 potentiostat (Uniscan, UK) (Fig. 1A) with a boxed connector for screen printed electrodes with dimensions 8.0 × 4.0 × 3.5 cm (DropSens, Spain). Cyclic voltammetry and chronoamperometry experiments were performed with gold nanoparticle-modified (AuNPs) screen printed carbon electrodes with dimensions 3.4 × 1.0 × 0.05 cm (Fig. 1B). Working electrode (4 mm) is made of GNP-Carbon, Counter electrode is made of carbon; reference electrode and electric contacts are made of silver (DropSens, Spain). The membrane engineered cells were incorporated in the biosensor system according to the principles of the Bioelectric Recognition Assay - BERA (Moschopoulou, Valero, & Kintzios, 2012). Plain Vero cells or Vero-*anti*-$AFB_1$ cells were cultured on the electrode surface for 24 h (37 °C, 5% $CO_2$) after coating with poly-L-lysine (Fig. 1C). At the time of the measurement the SPEs were transferred to a boxed connector device that served as an interface between the electrodes and the potentiostat (Fig. 1D). Each measurement lasted 6 min, at a rate of 2 Hz.

### 2.6. Recording and data processing

Cyclic voltammetry was performed between −0.5 and 0.5 V