CRANFIELD UNIVERSITY

MOHAMAD KAMAL ABDUL KADIR

DEVELOPMENT OF IMMUNOSENSORS FOR MYCOTOXINS ANALYSIS

CRANFIELD HEALTH

PhD THESIS

Academic Year: 2006 – 2009

Supervisor: **Dr. Ibtisam E. Tothill**

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Aflatoxin B_1 (AFB_1) and fumonisins (Fms) are mycotoxin contaminants found in peanuts and corn, respectively, and are known to be immunosuppressive and carcinogenic compounds. Therefore, the development of rapid and sensitive method for detecting these toxins especially for field analysis is required for risk assessment and management. The work presented in this thesis reports on the construction of sensor platforms capable of fulfilling these requirements. The use of, screen-printed thick film electrodes, gold nano-particle application and microelectrodes on a silicone support were investigated as suitable sensor platforms. The development of indirect and direct competitive immunoassay formats for the electrochemical immunosensor construction was undertaken for AFB_1 and Fms determination.

A spectrophotometric assay was initially developed as a first step procedure using microtitre plates for immunoreagents concentration and conditions selection before being transferred to the surface of the screen-printed gold electrode (SPGE) and then the microelectrode array (MEA) sensor. Detection was performed by chronoamperometry monitoring the reaction of tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) catalysed by horseradish peroxidase (HRP). The performance of screen-printed gold electrode (SPGE) immunosensor was compared to 3,3-dithiodipropionic acid (DTDPA) modified SPGE, immuno gold nano-particle modified SPGE and amine silane modified microelectrode array (MEA). Surface modification of MEA was successfully undertaken using silane and phenylene di-isothiocyanate (PDITC) chemistries for the covalent binding of the recognition system to the silicon surface area of the microelectrode arrays. The immunosensor format was transferred to a gold microelectrode array based on a silicone support for the purpose of signal sensitivity enhancement and miniaturisation for multiplex detection in the prospect of field analysis.

The developed of the gold immunosensor achieved a detection limit of 5 ng L^{-1}, 1 ng L^{-1} and 1 ng L^{-1} for AFB_1 using indirect assay on DTDPA thiol modified SPGE, immuno gold nano-particle modified SPGE and modified microelectrode array (MEA), respectively. While, the direct competitive method for fumonisins detection

on the SPGE sensor and modified MEA achieved a limit of detection was 0.5 µg L^{-1}. The sensors were also used for AFB_1 and Fms determination in peanuts and corn samples, respectively, and also validated using a standard HPLC and a commercial ELISA kit. Samples analysis involved the rapid extraction (without clean-up) and pre-treatment using solid phase extraction (clean-up) before measuring using the developed immunosensor platforms. The results achieved were found to be in average 82.7% (without clean-up) and 103.1% (clean-up) using gold-particle SPGE and 85.7% (without clean-up) using MEA for AFB_1 detection in peanut, respectively. While for Fms detection in corn sample was achieved in average of 73.5% (without clean-up) and 98.6% (clean-up) using SPGE and 87.3% (without clean-up) using MEA, respectively. The developed immunosensors (gold particle SPGE, SPGE and MEA) had a satisfactory agreement with HPLC and ELISA kit. The performance of the proposed sensors are highly sensitive and provide analytical system capable of detecting very low level of toxin within the required legislative EU limit of analyses.

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NOTATION

Ab	Antibody
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_1.BSA	Aflatoxin conjugated with BSA
AFB_1-HRP	Aflatoxin B_1 conjugated with HRP
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
Ag	Antigen
Ag/AgCl	silver/silver chloride
Anti-IgG	Anti-IgG (H+L)
Anti-IgG-HRP	Anti-antibody IgG labeled with HRP
AOAC	Association of Analytical Communities
B	Background
BSA	Bovine Serum Albumin
CE	Carbon counter electrode
C_L	Constant region light chain
CMD	Carboxylmethyl dextran
COMM	Corn Open Market Malaysia
CPB	Citrate phosphate buffer
CR	Cross reactivity
CTUK	Corn Tesco UK
CV	Cyclic voltammetry
CV%	Coefficient of variation
DON	Deoxynivelanol
DMF	Dimethylformamide
DPV	Differential pulse voltammetry
DTDPA	3,3-dithiopropionic acid
EC	European community
ECL-ELISA	Enhanced chemiluminescent
EDC	*N*-ethyl-*N*'–(3-dimethylaminopropyl)-carbodiimide
ELEM	Equine leukoencephalomalacia
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European union
Fms	Fumonisins
Fms-HRP	Fumonisins conjugated with HRP
FmB_1	Fumonisin B_1
FmB_2	Fumonisin B_2
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GC	Gas Chromatography
H_2O_2	Hydrogen peroxidase
H_2SO_4	Sulphuric acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer

IAC	Immunoaffinity Column
IC_{50}	Adequate sensitivity
IC-ELISA	Indirect competitive ELISA
IDA	Interdigiated microelectrode array
IgG	Immunoglobulin G
IgG-HRP	Anti-IgG labeled enzyme HRP
IPA	Intermittent pulse amperometry
IUPAC	International Union of Pure and Applied Chemistry
KCl	Pottasium chloride
KLH	Keyholelimpet Hemacyanin
LC	Liquid chromatography
LOD	Limit of detection
MAb	Monoclonal antibody
MAbAFB1	Monoclonal antibody against aflatoxin B_1
MAbFms	Monoclonal antibody against fumonisins
MEA	Microelectrode array
MS	Modified surface
MW	Molecular weight
Na_2CO_3	Natrium Carbonate
$NaHCO_3$	Natrium hydrogen carbonate
NBD-F	4-fluoro-7-nitrobenzofurazan
nd	Non detected
NDA	maphthalene-2,3-dicarboxaldehyde
NH_4OH	Ammonium hydroxide
NHS	*N*-hydroxysuccinimide
NIV	Nivalenol
NTD	Neural tube defects
O_2	Oxygen
OPA	o- phthadialdehyde
OT	Ochratoxin
OWLS	Optical Waveguide Lightmode Spectroscopy
Ox	Oxidised
P	Patulin
PBS	Phosphate buffered saline
PBS-T	0.05% tween-20 (v/v) to the PBS
PDITC	1,4-phenylene diisothiyanate
POMUK	Peanut Open Market UK
POSM	Peanut Open Market Malaysia
PSA	Prostate specific antigen
PSS	Peanut Supermarket Malaysia
PTUK	Peanut Tesco UK
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
QCM	Quartz Crystal Microbalance
RE	Silver/silver chloride reference electrode
Red	Reduced
RSD	Relative standard deviation
SAM	Self assembled monolayer
S	Signal
S/B	Signal/Background

SPE	Solid phase extraction
SPGE	Screen-printed gold Electrode
SPGE-Ercon	Screen-printed gold Electrode – Ercon
SPGE-Dupont	Screen-printed gold Electrode – Dupont
SPR	Surface Plasmon Resornance
svFv	Single variable chain antibody
T-2 toxin	Trichothecenes 2
TCNQ	Tetracynoquinodimethane
TMB	3,3',5,5'-tetramethylbenzidine dihydrochloride
$TMB_{(ox)}$	TMB oxidised
$TMB_{(ox)}$	TMB reduced
TLC	Thin Layer Chromatography
TTF	Tetrathiafulvalene
UK	United Kingdom
USA	United State of America
UV	Ultraviolet
V_L	Variable region Light chain
WE	Working electrode
WHO-IARC	World Health Organization-International Agency for Research on Cancer
ZEN	Zearalenone

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Foods for human consumption are mainly produced from plant and animal sources. However, the food is risky if it is contaminated or exposed to fungal contamination. The most common fungi that may occur in food and food products particularly in grains (maize, wheat, barley), nuts (peanut, groundnut, Brazilian nuts, pistachio) and fruits (apple, grape) are *Acremonium, Alternaria, Aspergillus, Chaetomium, Cladosporium, Fusarium, Paecilomyces, Penicillium, Stachybotrys,* and *Trichoderma* (Hussein and Brasel, 2001; Davis, 2001). Some of these fungi have been known to produce a group of chemical toxic secondary metabolites, known as mycotoxins. Mycotoxins have been identified and characterised, and include: aflatoxins, fumonisins, deoxynivalenol (DON, or vomitoxin), ochratoxins, zearalenone and patulin. They show significant diversity in their chemical structures and biological activity (Bhatnagar *et al*., 2002).

Whenever mycotoxins are detected and are present in food and feed, peoples and animals are being exposed to the toxins. Exposure to mycotoxins can result in (probable and possible) acute toxic or chronic carcinogenic, mutagenic, teratogenic or estrogenic effects, causing health hazards to humans and animals (Raisuddin *et al*., 1993). The effects are mediated by damage to cells of all the major organs, such as the liver, kidney, lungs, endocrine and the immune system (Bhatnagar *et al*., 2002).

Mycotoxin contamination is not only a cause of health hazards to humans and animals but is also a global problem for the agricultural economy. A thousand million tonnes per year of foodstuffs losses and almost a billion dollars a year of crop losses are reported because more than 25% of the world's agriculture is contaminated with mycotoxins producing fungi (FAO, 2003).

There is an increasing awareness of the natural toxin hazards imposed on both human and animal health and the need for safe food. A major goal for consumers, food producers and agro-food companies is to prevent and control mycotoxin contamination in foods and feeds for human and animal health safety. There are legislative and other organisations in many countries worldwide that able to control and regulate the policy for mycotoxin limitation. For example, the Food and

Agriculture Organisation (FAO) presented a policy regarding the legislation on the level of mycotoxins in raw foods and feeds and also end products, in order to protect consumers' intake (FAO, 2003). Therefore, to control and monitor the mycotoxins level, a need exists for analytical tools which are reliable, rapid, robust, sensitive, economical and easy to use assays for screening and monitoring of food contents. The analytical devices must be suitable for use in raw material examination in food and feed production.

Chromatography methods have been applied for the determination of mycotoxin content including: Thin Layer Chromatography (TLC) (Betian, 1985), High Performance Liquid Chromatography (HPLC) (Shepard, 1998; Jaimez *et al*., 2000), and Gas Chromatography (GC) (Scott, 1992). Confirmatory methods generally use sophisticated instruments (HPLC and GC) which require technical skill and give very sensitive results but these methods are also expensive, with a slow output of results caused by the need for extraction and cleanup procedures, while screening methods generally require minimal technical expertise.

In recent years, immunoassays and immunosensors have been developed to replace traditional analytical techniques which rely on the antibody-antigen binding reactions (Tothill and Turner, 2003). The adopted technique for immunosensors is based on the combination of specific antigens and antibodies in a solution on the surface support coupled to a signal transducer. Currently, the development of this device is for the purpose of moving to more rapid, sensitive methodologies and cost effective screening in large scale analysis. Antibody-based methods such as the immunofiltration assay, dip-stick test, affinity column, enzyme-linked immunosorbent assay (ELISA) (Zheng *et al*., 2006) and biosensors (immunosensors) have also been widely used for screening purposes (Maragos, 2001). Nowadays, immunosensors are considered as a major development in screening methods for use in mycotoxin determination.

Advances in microfabrication technology have created new products and materials such as microelectrode devices. Based on adaptation of immunosensor techniques, these devices are being used in microsensor systems in an effort to improve upon the speed, accuracy, sensitivity and cost of analysis. The bio-interface between the

analyte/receptor and microelectrode is also a main factor in designing a successful microsensor. The interface of microelectrodes can also be made more selective, thus reducing interference compounds. In this work, immunosensors based on screen-printed electrodes and also microelectrode arrays will be developed for mycotoxin analysis.

1.2 Mycotoxins

The term mycotoxin is originally from the Greek language: *mykes* meaning fungus and *toxikon* for toxin (Rustom, 1997). The first significant case was in 1960, when 100,000 turkeys died in a turkey 'X' disease outbreak in Great Britain. The cause of death was identified as aflatoxin B_1 and B_2 produced by *Aspergillus flavus* contaminated feed (peanut meal) (Daly *et al*., 2000). From 1960 to 1970, it was confirmed that some of the mycotoxins producing fungi are also the cause of animal diseases and death. After that time, it became clear that the fungal metabolites (mycotoxins) have been and are responsible for causing illness and death not only in animals but also in humans.

Mycotoxins are natural chemical secondary metabolite products produced by saprophytic fungi growing on foodstuffs or animal feeds. There are three main fungi of concern, which are *Aspergillus, Fusarium* and *Penicillium*, and comprise the largest number of mycotoxin-producing species during food handling and storage. Fungi growth and mycotoxins production are affected by many factors, which can be classified as either physical, chemical, biological, or macro- and micro-environmental factors: moisture level, correct temperature, mechanical injury, aeration (carbon dioxide, oxygen), nature of substrate, mineral nutrition, plant stress and others. For example, *Aspergillus* grows under high temperatures from 25 to 37 °C and relative humidity > 85% and these conditions are found in tropical and subtropical climates (European Mycotoxin Awareness Network, EMAN). However, temperate conditions at a lower water activity are the optimum conditions for *Fusarium* and *Penicillium* species growth and mycotoxin production. The presence of these fungi in or on a food product does not unequivocally mean the presence of mycotoxins but does mean the possibility of mycotoxin contamination.

The chemical secondary metabolites of mycotoxins are synthesized by a greater variety of pathways in fungi. For example, an aflatoxin is a representative polyketide mycotoxin derived by the cyclization of polyoxymethylene route. Other mycotoxins are produced by the terpenoid biosynthesis route (e.g. trichothecenes), amino acid (e.g. ochratoxin) and tricarboxylic (e.g. rubratoxin) (Smith & Moss, 1985). Based on their pathway, mycotoxins present significant diversity in their chemical structures and biological activity (Bhatnagar *et al*., 2002).

The diseases caused by the ingestion of mycotoxins contaminated foods and feeds are called ‘’mycotoxicoses’’, which affect human and animals most commonly liver, and kidney. According to the World Health Organization-International Agency for Research on Cancer (WHO-IARC) mycotoxins such as aflatoxins (AFs), ochratoxins (OTs), trichothecenes, zearalenone (ZEN), and fumonisins (Fms) have been evaluated as having a carcinogenic potential (IARC, 2002). Naturally occurring aflatoxins were classified as carcinogenic to humans (liver) while ochratoxins and fumonisins were classified as possible carcinogens and affect the kidney and cytotoxin. Trichothecenes and ZEN, however, were not classified as human carcinogens (alimentary and circulatory). Furthermore, some of the mycotoxins show immunosuppressive activity by inhibiting protein biosynthesis, which can occur in different ways such as: “(1) inhibition of transcription (e.g. aflatoxin), (2) inhibition of the phenylalanine tRNA synthetase (e.g. ochratoxin) or (3) inhibition of the translation through binding to the eukaryote ribosome (e.g. Trichothecene-2 toxin) (Adams, 1995).

The toxins are chemically and structurally diverse and stable and hence pose a threat to human and animal health. Concerns regarding their high toxicity resulted in the U.S. Food and Drug Administration (FDA) and the European Union (EU) setting strict regulatory limits on the permissible levels of these toxins in food. Approximately 100 countries have their own regulatory limit exposure for at least 13 mycotoxins to protect from health and economic risks (Dohlman, 2006). The European Union (EU) has set limits for 40 mycotoxin–food combinations including AFs, deioxynivalenol (DON), zearalenone, fumonisins, patulin and ochratoxin A; according to the European Commission Regulation (EC) No 123/2005 of 26 January 2005 (amending Regulation (EC) No. 466/2001). Other regions such as Africa, Asia/Oceania, and Latin America have concentrated on total aflatoxin regulations.

Selected mycotoxins are described briefly in the following sections. Table 1.1 shows the major mycotoxins, the producing fungi in agricultural crops, and the food and feed they contaminate. In this study two types of mycotoxins – aflatoxins and fumonisins – will be investigated. This is to improve the analysis techniques and lower the level of detection of them.

1.2.1 Aflatoxins

Aflatoxins are a group of highly toxic fungal secondary metabolites produced by the fungi *A. flavus* and *A. parasiticus* (Deiner *et al*., 1987; Kurtzman *et al*., 1987). The major occurring aflatoxins are aflatoxin B_1 (AFB_1), B_2 (AFB_2) (produced by *A. flavus* and *A. parasiticus*) and G_1 (AFG_1) and G_2 (AFG_2) (produced by *A. parasiticus*) (Davis, 1983), plus two additional metabolic products, M_1 (AFM_1) and M_2 (AFM_2) (Patterson, 1978) (Figure 1.1). The AFBs and AFGs are named after the Blue fluorescence and Yellow Green fluorescence respectively, exhibited by the compound under UV light. Aflatoxin M_1 and M_2 are produced by metabolism (hydroxylated derivatives) of B_1 and B_2 in animals' bodies (Patterson, 1978). AFMs are usually excreted in the milk of dairy cattle and other mammalian species that have consumed aflatoxin-contaminated food or feed.

Common aflatoxin contamination is found in a wide variety of commodities including nuts, spices (Ali, 2000), maize (Vincelli and Parker, 1995) and cottonseeds (Sekul *et al*., 1977). The production of aflatoxin on these commodities depends on several factors including the type of mould present, pH, temperature, water activity, type of substrate, and the presence of various chemicals in the substrate as well as the extent of product damage.

In general, the level of aflatoxin produced in oily products is always higher than in starchy products and the amount produced is closely related to the type and quantity of fatty acids present. In normal cases, the maximum level of aflatoxin production was achieved within 6-10 days (Abidin and Smith, 1987a).

Table 1.1: Common mycotoxin concerns, commodities affected and fungus.

Toxin	Producing fungi	Commodities	Main effects on health
Aflatoxin (B_1, B_2, G_1, G_2, M_1 & M_2)	*A. flavus* and *A. parasiticus*	Nuts, corn, cottonseed, palm kernel, spices	Carcinogen, acutely and chronically toxic (liver, kidney, trachea) (Stoloff, 1983)
Fumonisin (B_1, B_2 & B_3)	*F. proliferatum* and *F. verticillioides*	Corn (Shephard, 1998)	Carcinogen (liver, kidney), pulmonary oedema, neurotoxicity (Pagliuca *et al.*, 2005)
Ochratoxin (A, B & C)	*A. ochraceus*, *Penicillium verrucosum*	Beans, cereals (mainly barley, corn, rye and wheat), cocoa, coffee, dried fruits, grapes, (Pittet & Royer, 2002).	Carcinogen (kidney, liver) Impairment of immune system
Zearalenone	*Fusarium* spp.	Grains	Strong oestrogenic activity (uterus-oestrogen) (Berthiller, 2006)
Trichothecenes (DON, NIV, T-2 toxin)	*Fusarium* spp.	Corn, wheat and barley (Jelinek *et al.*, 1989)	Acute gastro-intestinal illness, alimentary toxic aleukia, pulmonary hemosiderosis (Eskola *et al.*, 2001).
Patulin	*Aspergillus* spp. *and Penicillium* spp.	Apple, apple juices and apple juice products (Gokmen *et al*., 2005)	Haemorrhaging (brain and lungs) (Beretta *et al*., 2000)

DON; deoxynivalenol, NIV; nivalenol, T-2 toxin; trichothecenes 2

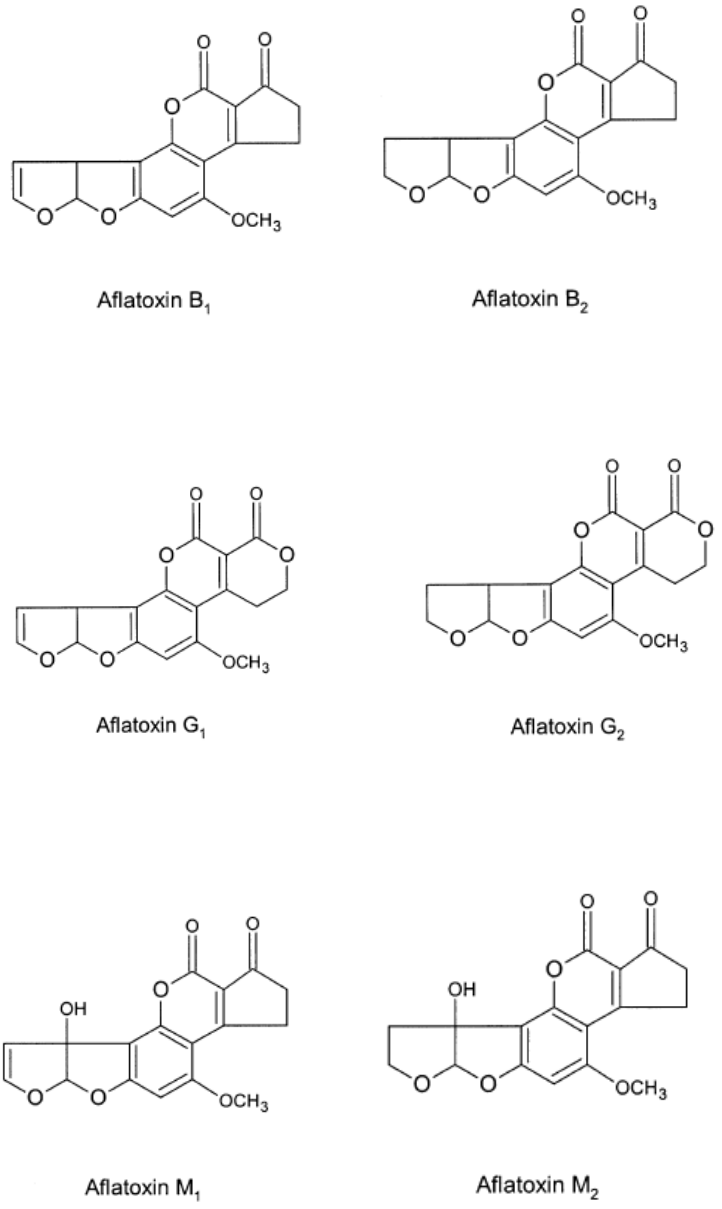


Figure 1.1: Chemical structures of aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 (Hussein and Brasel, 2001)

Among the group of AFs, AFB_1 is considered very toxic and the most prevalent compound (Khoury *et al*., 2008). According to the International Agency for Research on Cancer (IARC) the order of toxicity of aflatoxin is AFB_1> AFG_1> AFB_2> AFG_2 and are all classified as carcinogenic to humans and animals (Chiavaro *et al*., 2001; Ammida *et al*., 2004). They are acutely and chronically toxic, producing four distinct

effects: *acute liver damage; liver cirrhosis; induction of tumours; and teratogenic effects* (Stoloff, 1983). Meanwhile AFB_1 is positively associated with cell liver cancer and linked to human hepatocellular carcinoma.

To prevent the consumer from the risk of aflatoxin effects, in 2002 approximately 90 countries had regulations on the maximum aflatoxin limit in food and feeds (Table 1.2).

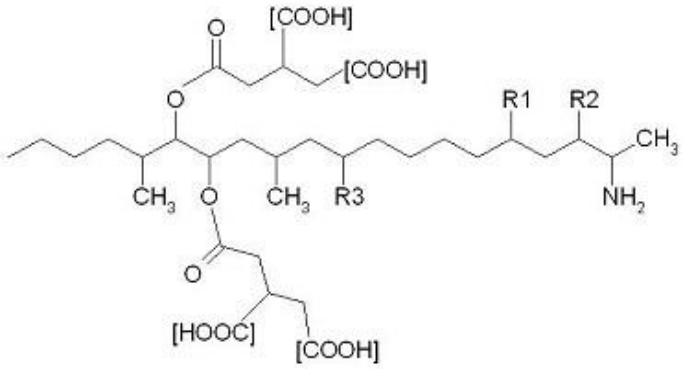
Table 1.2: The maximum levels for aflatoxins and the number of countries in 2002 which have regulations based on ranges (van Egmond and Jonker, 2004).

Aflatoxin	Range ($ng\ g^{-1}$)	No. of Countries
B_1 in foodstuff	1-20	57
$B_1+B_2+G_1+G_2$ in foodstuff	1-35	75
M_1 in milk	0.05-15	59
B_1 in feedstuff	5-50	45
$B_1+B_2+G_1+G_2$ in feedstuff	0-50	17

1.2.2 Fumonisins (Fms)

The history of fumonisins started in 1988, when a researcher from South Africa first described the fumonisins produced by *Fusarium* as dangerous (Marasas, 2001). Fumonisins were first isolated in that year from a culture of *F. verticillioides* by Gelderblom and others (Gelderblom *et al.*, 1988). The isolation and characterisation of fumonisin came after active research into the causal agent(s) for equine leukoencephalomalacia (ELEM), a syndrome characterised by liquefactive necrotic lesions in horse (Marasas, 2001), and human oesophageal cancer in some populations in the Transkei region of South Africa.

Fumonisins are categorised into A, B, C and P series of main groups (Wang *et al.*, 2006). The B-series of fumonisins (FmBs) are the most abundant toxic compounds produced mainly by *F. verticillioides* and *F. proliferatum* (Feng and Fun, 1999). The chemical structure of the B series of fumonisins is a long chain of 20 carbon atoms in the backbone, with hydroxyl, methyl and tricarballylic acid moieties shown in Figure 1.2 (Wang *et al.*, 2006). The major compounds of fumonisin are Fumonisin B_1 (FmB_1) and Fumonisin B_2 (FmB_2). The most abundant and toxic is FmB_1 followed by FmB_2.



	R1	R2	R3
FB_1	OH	OH	OH
FB_2	OH	OH	H
FB_3	H	OH	OH
FB_4	H	OH	H

Figure 1.2: General structure of fumonisins (Wang *et al*., 2006).

These toxins are natural contaminants of cereal grains worldwide and are mostly found in corn and its products. Fumonisin-contaminated grains have been linked to various diseases: liver and kidney toxicity and carcinogenicity, pulmonary oedema, immunosuppression and neurotoxicity (Pagliuca *et al.*, 2005). It is also linked to the risk effect of oesophageal cancer in humans and possibly connected with neural tube defects (NTD) in the Transkei region of South Africa, as well as in China and South

Texas, USA (Shephard, 1998; Lino *et al*., 2006). According to the International Agency for Research on Cancer (IARC), FmB_1 is classified as a potential carcinogen (probable human carcinogen) (IARC, 2002).

The FDA has advised a maximum level of fumonisins concentrations in corn and corn-based product for the safety of human consumption. The safety concern is based on animal studies (Table 1.3).

Table 1.3: Maximum level of fumonisins in corn and corn-based products (http://www.cfsan.fda.gov).

Corn and corn-based product	Total fumonisin (FmB_1 to FmB_3) (mg L^{-1})
De-germed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content of < 2.25 %, dry weight basis)	2
Whole or partially de-germed dry milled corn products (e.g. flaking grits, corn grits, Corn meal, corn flour with fat content of ≥2.25 %, dry weight basis)	4
Dry milled corn bran	4
Cleaned corn intended for mass production	4
Cleaned corn intended for popcorn	3

1.3 Analytical methods used for aflatoxin and fumonisin analysis

Several methods for the determination of mycotoxins have been developed and some of these have been established as standard techniques. Therefore, an accurate quantitative analysis result of mycotoxin level in samples is important for quality control, for mycotoxin control procedure, and for the mycotoxin exposure in humans.

Sampling procedures, extraction and clean-up steps, separation, and detection are very important aspects of the application of analytical methods.

Sampling

The first step in mycotoxins analysis is a sampling of the foods such as grains. Priority interest should be given to sampling procedures because usually the largest source of errors in the analytical method is in sampling. To reduce sampling errors, it is very important to increase the sample size or obtain a representative sample of the bulk material (Whitaker, 2004). An analytical method of mycotoxin inspection of agricultural commodities such as peanuts, shelled corn, nuts and grains should be composed of three steps. Step 1: single and plural samples are taken from a lot. Step 2: each sample is comminuted to reduce the sample size, and single or plural subsamples are removed as representative of the sample analysis. Step 3: the mycotoxin contamination level of the lot is estimated from the analyses (Whitaker, 2004). The ideal sampling procedure should assure the highest probability of detecting mycotoxins even when contamination is low.

Extraction and Clean-up

To analyse food samples, the mycotoxin must first be extracted from the food matrix. The standard analytical technique for the extraction of mycotoxin from food is by solvent extraction. Generally, organic solvents such as chloroform, dichloromethane, acetone, methanol and acetonitrile are commonly used. These solvents may be used in combination with water and electrolytes. The selection of solvent for extraction and redissolution can have a great influence on the recovery of mycotoxins. Thus, the solvent employed has to be taken into account when considering individual properties of toxin and solvents, especially those with polarities.

The issues of sample matrix are more complex for the analysis of mycotoxins in food because more than one interference materials is present. To solve the problem, the extraction process then performed on the toxin sample has to include an extensive clean-up process to remove the interference substances before the analysis is run (Chu, 1992; Scott, 1993). The major clean-up techniques used include: dialysis, aqueous anionic precipitation, column chromatography and solvent partitioning. The column chromatographic method is widely employed for the clean-up of mycotoxins.

Columns packed with silica gel, florisil, alumina, charcoal, ion exchange resin and antibody-based affinity columns have been used. Recently, smaller disposable pre-pack columns such as a Solid Phase Extraction column (SPE) have been used, which can be commercially obtained from Waters, Varian and others.

Different samples with different clean-up techniques used in HPLC systems influence the recovery of the toxin being detected. Using gel permeation in the clean-up process has allowed a level of detection down to 1.0 μg kg^{-1} of AFs in samples of wheat and maize, and average recoveries are 80% and 70%, respectively (Hetmanski and Scudamore, 1989). Also it has been shown that using a five times re-used SPE column in the clean-up process to analyse AFs in groundnut samples from Malaysia still obtained an 85% to 94% recovery with 92% of the samples being found to be contaminated with aflatoxin in the range of 0.3–62.1 μg L^{-1} (Abidin *et al*., 2003).

Separation, detection and determination

The generally accepted method for the separation from matrix interference and the detection of mycotoxins is by chromatography methods which include Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC). For mycotoxin analysis, the separation using the TLC and HPLC procedures is widely employed, because most mycotoxins are non-volatile.

By using the TLC procedure, aflatoxins and fumonisins can be detected as fluorescent spots under ultraviolet (UV) light. For HPLC, detectors such as diode array, fluorescence, refractive index and mass spectrometry can be employed for toxin analysis. The choice of any apparatus and detector depends on the physico-chemical properties of the mycotoxin to be analysed and the sensitivity required. For the confirmation of the identity of mycotoxins using HPLC analysis, different mobile phases and columns can be used, and also reacting pre-or post column derivatization and measuring several wave lengths of fluorescence can be applied. More details on these techniques can be found in section 1.3.2.

Recently, immunological techniques based on specific antigens and antibodies have also been developed for mycotoxin detection, and these include Lateral Flow, ELISA and immunosensors. ELISA, with a simple extraction and clean-up procedure, has

been reported to give very sensitive and selective analysis. The analysis method using the ELISA technique can be a semi quantitative or quantitative test (Asis *et al*., 2002).

Analytical methods which use TLC and ELISA techniques are suitable for screening purposes, while HPLC methods are suitable for confirmation and used for a large scale analysis in laboratory settings. All the analytical methodology must allow determination of mycotoxins at least down to the specified regulatory level.

1.3.1 Thin Layer Chromatography (TLC) method

TLC is the conventional method used for separation, purity assessment and identification of organic compounds to determine a large number of mycotoxins. It consists of three main components including a stationary phase (silica immobilised on plastic or glass plate), a mobile phase (solvent and acids) and samples or standards (liquid or dissolved in volatile solvents).

The determination technique involves both extraction and purification before measurement, including extraction from the samples using a combination of acetonitrile, methanol and water; column filtration for sample clean up; samples spot at chromatography plate, using an aqueous organic solvent as a mobile phase, and observation by fluorescent / UV light or by a suitable reaction procedure (Betian, 1985; Mermet *et al*., 1998).

The TLC method was considered by Association of Analytical Communities (AOAC) the official method to identify and quantify aflatoxins. The detection limits using the TLC method for total aflatoxin are 2 µg L^{-1}. (www.eurofinsus.com). The method had been used to determine aflatoxin B_1 in peanut and corn where levels of contamination ranging from 5-25 µg kg^{-1} in peanut and 5-50 µg kg^{-1} in corn were reported (Park *et al*., 1994).

The TLC method is still used for screening purposes especially in food manufacture since TLC does not require expensive instruments such as GC and HPLC. However,

this method will produce a large amount of solvent waste from expensive (HPLC grade) solvents that may create a hazard to workers and pollution to the environment.

1.3.2 High Performance Liquid Chromatography (HPLC) Method

Several mycotoxins are polar chemical compounds, which are soluble in polar solvents (methanol, acetonitrile) and water and therefore reversed phase HPLC is ideally suitable for the determination of mycotoxins (Shephard, 1998). Coupled with fluorescence detectors, fumonisins (Sydenham and Shephard, 1996) and aflatoxins (Sharman and Gilbert, 1991) have been separated and detected by HPLC.

Generally, the analytical methods involved are: *extraction* procedures, by solvent extraction with single or combination polar organic solvent such as methanol, acetonitrile and water (Nielsen, 2002); *clean-up* process, commonly employing a strong anion exchange (sax column), solid phased extraction (SPE) (Abidin and Husni, 2002) and immunoaffinity column (IAC) (Pagliuca *et al*., 2005); *separation*, whereby reversed phase column and C_{18} column is the most commonly used method. The analysis also involves: pre or post column derivatization for low level of detection with different reagents such as *o*-phthadialdehyde (OPA) (Pagliuca *et al*., 2005); naphthalene-2,3-dicarboxaldehyde (NDA) and 4-fluoro-7-nitrobenzofurazan (NBD-F) (Lino *et al*., 2006) for fumonisins; and with bromination and iodination for aflatoxins (Henry *et al*., 2000); lastly, *detection and quantification* by HPLC coupled with fluorescence or a mass spectrometry detector.

For the detection of low levels of aflatoxin and fumonisin in the range of µg kg^{-1}, pre or post column derivatization is needed because these toxins are quite weak emitters of fluorescent light. HPLC method coupled with mass spectrometry is one such technique without a derivatization procedure (Shephard, 1998). However, this method is expensive and more complex.

To avoid hazards from chemical reagents, different techniques of derivatization using non-chemical reagents have also been studied (Trucksess and Maragos, 2001). The system is photochemically sensitive post column to determine AFB_1, AFB_2, AFG_1

and AFG_2. The use of a photochemical reactor system is equivalent to the iodine method for AFs. This photochemical system consistently gave slightly higher values for AFB_1 and AFB_2 in peanut where the Pearson Correlation Coefficients for aflatoxin B_1 and aflatoxin B_2 were 0.9994 and 0.9874, respectively (Walking and Wilson, 2006).

In the case of fumonisin determination, more than 90% of laboratories reporting fumonisin levels in corn and corn-based product use pre-column derivatization followed by HPLC. Fumonisin derivatives were analysed by pre column derivatization using o-phthaldialdehyde (OPA), where average recoveries in the ranges of 102.6% and 95.1% for FmB_1 and FmB_2 spiked corn based product, were found respectively (Solfrizzo *et al*., 2001). The detection limit for both FmB_1 and FmB_2 was 5 μg kg^{-1}. However, with a different derivatization of naphthalene-2,3-dicarboxaldehyde (NDA) the average recovery was 79% FmB_1 and 99.6% FmB_2. The detection limit of corn fortified was 20 μg kg^{-1} and 15 μg kg^{-1} for FmB_1 and FmB_2, respectively (Lino *et al*., 2006).

The advantages of using the HPLC method include: automated system with PCs, automatic injection with larger samples, low detection limit and highly accurate qualitative and quantitative determination (Nielsen, 2002). This method is also suitable for confirmatory mycotoxin analysis. However, as mentioned above, HPLC methods need experienced personnel to run, expertise to handle, and costly equipment and maintenance.

1.4 Immunochemical Analysis

To minimize the interferences by co-extracted compounds from food and feed samples in which mycotoxins occur, the conventional methods for chemical analysis of mycotoxins consume a large amount of time and solvent, need several clean-up steps, and sometime require practical experiences. The specific antibodies have been produced against mycotoxins such as aflatoxins and fumonisins (Azcona-Olivera *et al*., 1992b; Fukuda *et al.*, 1994). Using the antibodies, simple and rapid methods of immunochemical analysis such as Enzyme Linked Immunosorbent Assay (ELISA)

(Asis *et al*., 2002), Immunoaffinity Column (IAC) chromatography (Stroka *et al*., 2000) and Immunosensor (Tothill, 2003) have been developed. The major advantages of the immunological methods are that they are highly specific, simple, rapid and do not use toxic solvents. In this section, the antibody molecule and the principle and application of immunoassay (ELISA and Immunosensor) and IACs are described.

1.4.1 The Antibody (Ig) molecule

An antibody (Ab) is a glycoprotein called an immunological protein (immunoglobin) produced by immune cells of the body in response to a foreign molecule (antigen (Ag). They are produced in large amounts by plasma cells developed from precursor B lymphocytes (B-cell). There are five classes of immunoglobulins such as IgA, IgD, IgE, IgM and IgG. Each is differ from the others in function, number of binding sites, molecular mass, amino acid composition and carbohydrate content.

The smallest antibody and most abundant of all immunoglobulins (75-80%) in serum is IgG (Junquiera & Jose, 2003). IgG consists of four tightly folded polypeptide chains in a 'Y' shape and has an average molecular mass about 146-160 kD (Figure 1.3). The IgG molecule has two different side chains: heavy chains (H) of 55-77 kD and light chains (L) of about 25 kD (Stadlmann *et al*., 2008).

The half of the L chain toward the carboxyl terminus is referred to as the constant region (C_L), while the amino-terminal half is the variable region of the light chain (V_L). The portion of the Ig molecule that binds the specific antigen is formed by the amino-terminal (-NH_2-) portion of both the H and L chains. The flexibility of the hinge region permits the two antigen binding sites to operate independently. They bind specifically to the antigen through its antigen–binding sites called epitopes.

The stem portion of the Y shaped Ig is called the Fc fraction and is a dimer of the last heavy chain domain. The F(ab')$_2$ fraction is the upper part. A monomer of the F(ab')$_2$ fraction, including only one light chain and one upper heavy chain, is called a Fab fraction. The whole Ig molecule can be used in immunoassay. IgG makes up

approximately 80% of the total immunoglobulin in human serum and is the most common class of antibody used in immunoassay ((Junquiera & Jose, 2003).

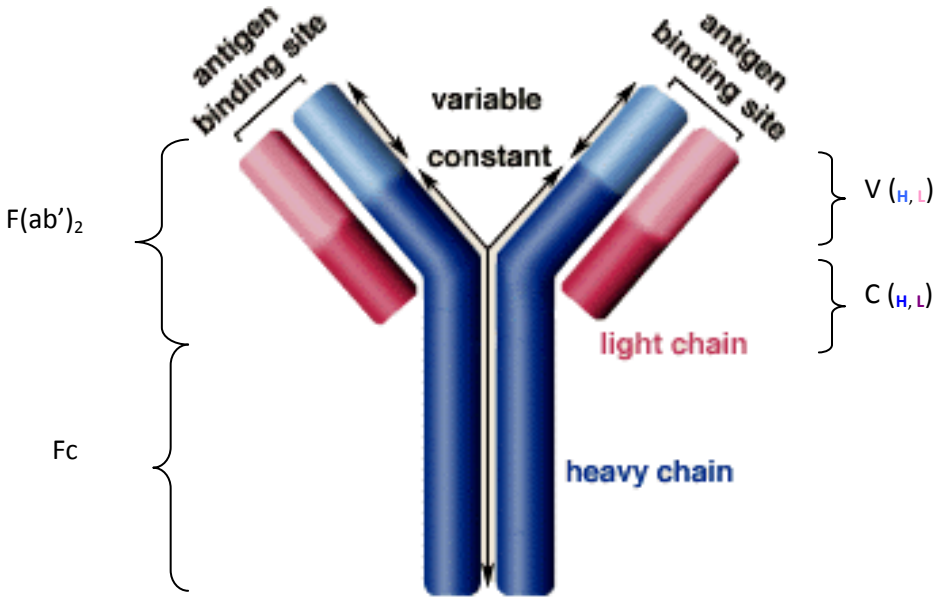


Figure 1.3: A simplified model of an antibody. V is a variable region and C is a constant region. H = heavy chain and L = light chain (Stadlmann *et al*., 2008).

There are two types of specific antibody: polyclonal and monoclonal antibodies. Nowadays, the production of both antibodies specific to different antigens have been commercialised and are widely used in the laboratory for developing a range of immunochemical techniques.

1.4.1.1 Polyclonal antibody (PAb)

Polyclonal antibodies are specific antibodies and can be produced through the injection of a specific foreign antigen into animals such as a mouse, rabbit or goat. The animals must first be immunised with a suitable antigen-adjuvant before the re-injection of the antigen without an adjuvant. Serum is harvested from the animals, and the antibody is purified from it. A polyclonal antibody preparation may contain up to

10,000 different types of IgG, some of which are specific to the antigen of interest. The binding of the antibody to the antigen is accomplished by many noncovalent forces: hydrogen bonds, Van der Waals, and electrostatic and hydrophobic effects**.** The most frequently used method for the production of the mycotoxin polyclonal antibody is multiple-site immunisation of rabbit with the mycotoxin-protein conjugate followed by boosting (Abouzied *et al*., 1993; Thirumala-Devi *et al*., 2000).

The advantage of polyclonal antibodies is their ability to form large insoluble immune complexes with antigen, and to agglutinate cells easily, so that the reaction can be measured and determined photometrically. This indicates that a polyclonal antibody is broad binding in nature, as a useful property and can be utilised in many immunoassays especially as an antigen captures antibody preparation. It is also cost effective and cheap to produce and can be used to capture a class of analytes.

1.4.1.2 Monoclonal antibody (MAb)

Monoclonal antibodies (MAbs) are most often produced in animals such as mice. The following steps describe the MAb production. After immunizing an animal with antigen and obtaining immune cells from its spleen, the spleen cells are fused with myeloma cells (tumor of B lymphocytes) by adding polyethylene glycol. Then the fusion mixture (spleen cells, myeloma cell and fused cell) is set up in a culture with a medium containing hypoxanthine, aminopterin and thymidine (HAT medium), and the fused cells will survive because they have the immortality of the myeloma and the metabolic bypass of the spleen cells. Some of them will also have the anti-X antibody-producing capacity of the spleen cells. The supernatant of growing cells are tested for the production of the desired antibody with ELISA and positive cells are diluted so that there is only one cell in each well. As the hybrid (fused) cells grow, antibodies are secreted into the cell culture media, or produced in animals' ascites fluid or a hollow-fiber production system (Campbell, 1996; National Academy Press, 1999).

Since a hybrid cell only has one spleen cell (secreting one type of antibody) as its basis, monoclonal antibodies are chemically identical. On the basis of their well-defined epitope specificity, purity, and relative ease of production, monoclonal

antibodies are often preferred for use in immunoassay. In term of requiring constant availability of standardised reagent, MAb specificity can be prepared and maintained *in vitro* in unlimited quantities from frozen hybridoma cell lines (Savard *et al*., 2003).

Different from polyclonal antisera, MAbs are more specific as a reagent in assays for soluble antigens. However, in certain immunological tests, some MAbs may not be as sensitive as polyclonal antibodies. For example, when MAbs are used in an ELISA system they may be too specific but less sensitive, reacting only to certain antigens and not to others (Varma *et al*., 2002). Not only will an assay using MAbs replace many of the methods currently available, but use of these antibodies will also permit unique approaches to rapid and accurate diagnosis test.

Table 1.4 below lists the comparative criteria for the production of both antibodies.

Table 1.4: Comparison of monoclonal and polyclonal antibody production

Criterion	Monoclonal	Polyclonal
Purity of antigen	Not significant	Significant
Price	Initially high	Low
Time required	2-6 months	1-2 months
Concentration	1-25 µg mL^{-1} (culture sup.) 0.5-5 mg mL^{-1} (ascites fluid)	$\geq$ 1 mg mL^{-1}
Cross-reactivity	Some	Some
Affinity	Homogeneous	Heterogeneous

In the case of mycotoxin analysis, these antibodies have been used especially in immunochemical analysis such as the production of the immunoaffinity column (IAC) and the ELISA kit. The major advantages of these immunological methods are that they are highly specific, simple, rapid and avoid the use of toxic solvents.

1.4.2 Immunoaffinity column (IAC)

The immunoaffinity column is a type of separation technique of chromatography based on molecular binding interaction. Generally, IACs contain an anti-mycotoxin antibody covalently attached to an appropriate support (stationary phase) and placed in a mini column. The mycotoxin in the sample binds specifically to the anti-mycotoxin antibody, while unbound impurities are removed by washing. The bound mycotoxin is then separated by eluting with an appropriate solution (such as methanol and acetonitrile). The IACs prepared using anti-mycotoxin antibodies are highly specific, simple, rapid and does not use toxic solvent. Recently, with the availability of commercial IACs for AFs and fumonisins, these IACs have become a powerful technique in the clean up stage of the analysis. Many companies are producing commercial IACs for AFs such as VICAM, Rhone-Diagnostic Technologies (RDT), R-Biopharm and NEOGEN, while there are Fms such as VICAM and RDT. For example, in these commercial IACs, a method for determining AFs in corn, raw peanut and peanut butter using the AflaTest column (VICAM) coupled with HPLC has been developed.

1.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a common method based on affinity interactions (Janeway & Travers, 1999). Immunoassays are analytical tests based on the selective and sensitive antibody-antigen (Ab-Ag) interaction immobilised on a solid phase support for samples components detection (Hage, 1999). Immunoassays are classified into two main categories: homogeneous and heterogeneous, which in turn can be subdivided into competitive and non-competitive formats (Tothill, 2003). The difference between homogeneous and heterogeneous is in the separation steps of the assay. A heterogeneous assay requires the separation of the bound antibody-antigen and free phases before the end-point is determined. These assays also require washing of the solid phase to remove unreacted reagents after incubation. The heterogeneous immunoassay format is the most commonly used format for diagnostic testing and often referred to as Enzyme Linked Immunosorbent Assay (ELISA) when an enzyme is used as the signal molecule.

ELISA involves a coating process with antibodies (e.g. a microwell coated with an anti-antigen antibody), blocking the un-reacted sites with a reagent, and then the sample or standard toxin mixed with antigen-conjugated with enzyme for competition step (Lee *et al*., 2004). Between these steps (coating, blocking, competition and detection), a washing procedure is needed. Finally, the activity is measured with an ELISA reader. ELISAs have been developed for AFs and Fms and are reported to be very sensitive, selective, rapid and simple without clean-up processes for semi quantitative and quantitative tests. Recently, these ELISAs have been commercially available, and some ELISA kits have been adopted as the AOAC's International Official Methods.

1.4.3.1 Competitive immunoassay tests

In the case of mycotoxin detection, a competitive heterogeneous assay is a suitable format. Mycotoxins are low molecule substances (haptens), and a competitive method is the preferred option. There are two types of competitive assay: direct detection and indirect detection.

Direct detection assay

This assay is based on the competition between a labelled and an unlabelled analyte (antigen) in the reaction with an antibody on the solid phase (immobilised antibody) (Figure 1.4 (a) and (b)) (Tothill, 2003). The amount of labelled analyte or hapten bound to the antibody site is then measured. The direct competition assay based on pre-coated 2º antibody (anti-IgG) immobilised on the well is to improve the orientation of the specific binding (Figure 1.4 (b)), and enhance the sensitivity of the assay (Lee *et al*., 2004; Lee and Rachmawati, 2006).

Indirect detection assay

The assay is based on the analyte-specific antibody binding to the analyte and then being detected by a labelled secondary reagent such as anti-immunoglobulin antibodies (secondary antibodies) (Figure 1.5) (Elisabete *et al*., 2000; Tothill, 2003; Lee *et al*., 2004; Ammida *et al*., 2004). The amount of labelled anti-antibody bound to the anti-antigen antibody site is then measured.

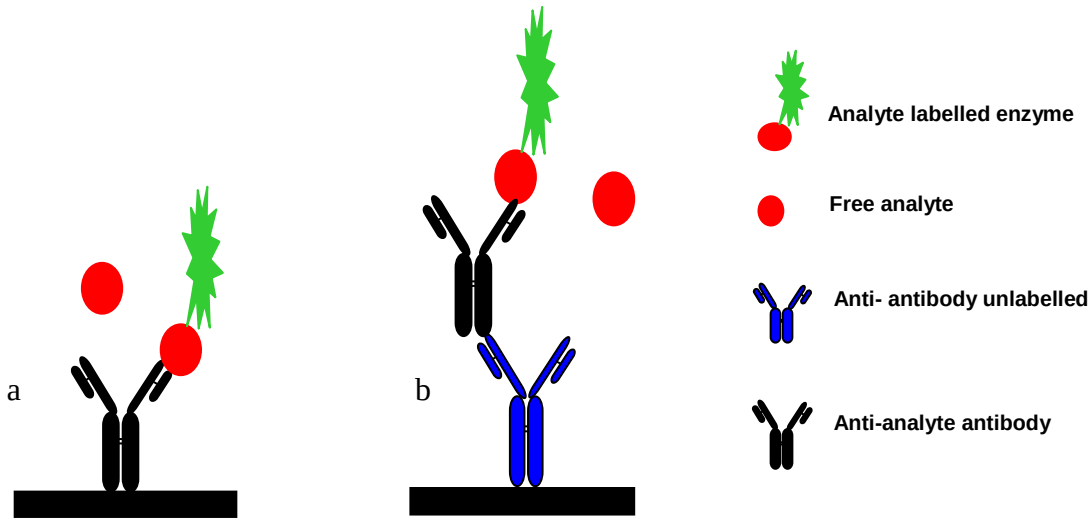


Figure 1.4: Example of direct competitive assay based on two different formats: (a) Antibody coated well before competition of analyte/analyte-labelled. (b) Anti-antibody pre coated well before being coated with an analyte specific antibody and followed by competition analyte/analyte-labelled.

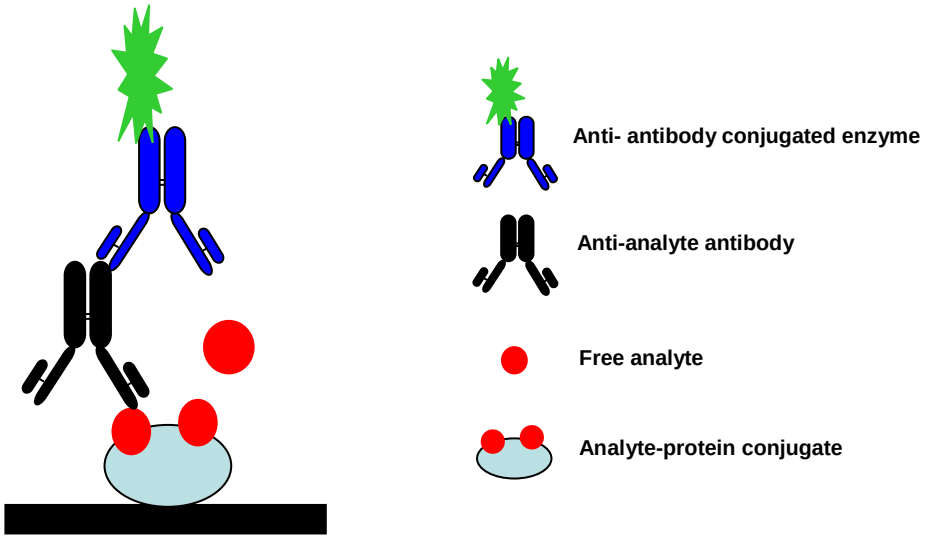


Figure 1.5: Example of indirect competitive assay (immobilised hapten).

The determined enzyme activity after competition for both assays is inversely proportional to the analyte concentration. This is because the greater the response, the fewer analytes in the unknown sample which are available to compete with the labelled analytes.

1.4.3.2 Detector labels and substrates used in immunoassays

Colorimetric methods are the most commonly used detection methods for ELISA. The advantages of a colorimetric endpoint are that visual evaluation can be used in large scale screenings and chromogenic substrates provide long lasting stability of a coloured product after the reaction is complete. Substrate reaction times are 10-30 minutes. The colour intensity in the well is formed by the substrate reacting with the enzyme and is inversely proportional to the target species concentration. For example, in the RIDASCREEN commercial test, urea peroxide acting as an enzyme substrate is added with a chromogen. The enzyme will break down the urea peroxide and the by-product will cause a colour reaction with the chromogen, which is then measured using a spectrophotometer. The selection of enzyme is reasonably broad and horseradish peroxidase (HRP) is reported to be more favoured than alkaline phosphates, followed by β-galactosidase.

1.4.3.3 Data analysis

The data collected from using standard reagents in an ELISA experiment is interrogated to plot a standard curve before the quantity of analyte in the unknown samples are measured and quantified. The competitive standard curve is inversely proportional between the correlation of signals found and the analyte concentrations. The response of the signal is relative to the amount of enzyme conjugated bound to the support. This is based on the competition of analyte and enzyme conjugated that are bound to the antibody immobilised on the support, i.e. the more enzymes that are conjugated bound (fewer analytes) the higher the signal (Figure 1.6).

The curve is fitted using a non linear four-parameter as shown by the following equation (Neagu *et al.,* 2006):

$$y = [(a - d)/(1 + (x/c)^b)] + d \quad \text{.....(1.1)}$$

Where parameters ***a*** and ***d*** are the maximum and minimum signal, respectively, ***b*** is a slope-related term at the inflection point (IC_{50}), and ***c*** is the concentration at the inflection point (50% ***B/B₀***).

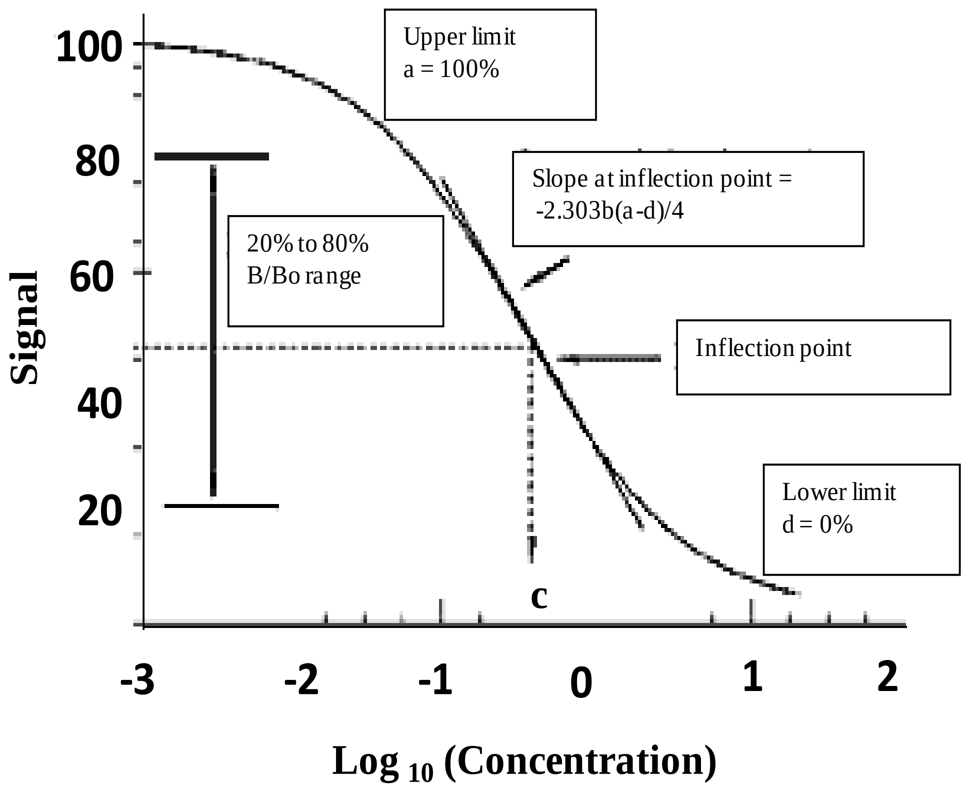


Figure 1.6: A typical standard curve of ELISA competitive assay (Biomax Co. Ltd).

If the absorbance values need to be converted to percent values, the calculation must be done as in the following equation:

% relative binding = (B/B₀)* x *100 ……………………………………………(1.2)

This is where ***B₀*** and ***B*** are the absorbance values of a non competition analyte (***a – d***) and competition (***y – d***), respectively. From this equation the value for 20%, 50% and 80% of ***B/B₀*** are determined.

Many reports by researchers show they have self developed an ELISA protocol based on direct and indirect competitive assays that produced different limits of detection (LOD). The calculation is based on the following equation (Warwick, 1996):

$$\mathbf{LOD} = \boldsymbol{x}\,[(\boldsymbol{a} - \boldsymbol{d}/\,(\boldsymbol{a} - \boldsymbol{d}) - 3\sigma) - 1]^{-1/b} \quad \ldots\ldots(1.3)$$

where σ is the standard deviation of the zero value.

Table 1.5 lists the different ELISA assays developed for aflatoxin B_1 and fumonisin detection by different researchers. Different formats of competitive assays produced different limits of detection (LOD). The sensitivity of the detection also strongly depends on the ability of the specific antibodies used in the test. For example, the screening of FmB_1 in corn using a monoclonal antibody based on an indirect competitive ELISA (IC-ELISA) has achieved detection limits of 76 µg L^{-1} (Barna-Vetro *et al*., 2000). With the same format, using a polyclonal antibody, the detection limit was 112 µg L^{-1} (Christensen *et al*., 2000). Therefore, the different assay formats and reagents need to be examined in order to optimize and characterise the method for the target toxin.

1.5 Immunosensors

1.5.1 Background

Immunosensors are based on biosensor technology, which is the technique for new rapid, sensitive and reproducible methodologies for routine analysis. A biosensor is an alternative method to traditional analytical techniques, for the future, using miniature and inexpensive devices for testing (Velasco-Garcia & Mottram, 2003**)**. This is because "*biosensors are analytical devices incorporating a biological material, a biologically derived material or a biomimic as the recognition molecules, which is either intimately associated with or integrated within a physicochemical transducer or transducing microsystem*" (Figure 1.7) (Tothill and Turner, 2003).

Table 1.5: The sensitivity of different ELISA method developed for AFB_1 and Fumonisin (Fms) detection.

Mycotoxins	Competitive formats	LOD µg L^{-1}	Authors
AFB_1	Direct	10	Lee *et al.*, 2004
AFB_1	Direct	0.2	Lee & Rachmawati, 2006
AFB_1	Indirect	0.9	Lee & Rachmawati, 2006
AFB1	Indirect	0.28	Sapsford *et al*., 2006
Fm B_1	Direct	600	Azcona-Olivera et *al.*, 1992
Fms	Direct	80	Iijima *et al*, 1996
Fm B_1	Indirect	76	Barna-Vetro *et al*., 2000
Fm B_1	Indirect	112	Christensen *et al*., 2000
Fms	Indirect	10	Ono *et al*, 2000
Fms	Direct	430	Savard *et al*, 2003

The biological material can be classed as affinity or biocatalytic such as antibodies, DNA, receptor protein, enzymes, tissues, whole cells or organs interacting with specific analyte. The interaction can then be transformed by the transducer into a measurable electrical signal. When applied to a variety of physical transduction techniques such as optical, electrochemical, piezoelectric, acoustic and calorimetric methods, the diversity, divisions and sub-divisions of the biosensor is vast. Usually the target for a biosensor application is to be able to detect very low concentrations of analyte with the use of high affinity and stability, sensing layers, suitable transducers and a digital electronic signal.

Figure 1.7*:* The illustration of a biosensor (Tothill and Turner, 2003).

1.5.2 Sensing Materials (Receptors)

If the biomolecule of a biosensor is an antibody or an antibody fragment such as an antigen sensing material then the sensor device is called an immunosensor. The antibody or antigen is usually immobilised at the surface and coupled to a signal transducer of the immunosensor. The affinity interaction of antibody and antigen (Ag) is very high and therefore an immuno-complex (Ab—Ag) is formed in the solution (Hock, 1997). The interaction equilibrium is illustrated as below:

$$Ab + Ag \longleftrightarrow [Ab\text{----}Ag] \quad \text{(Ab/Ag complex)} \qquad (1.4)$$

The high affinity of the immuno reaction between Ab and Ag is capable of capturing the analyte in the presence of much substance interference.

Advances in the creation of sensing material such as polyclonal and monoclonal antibodies and recombinant antibodies have an impact on the development of immunosensor devices. The use of antibody fragments and molecularly engineered antibodies with an enhanced affinity and stability is a future area for new receptors of immunosensor application.

Antibodies specific to mycotoxins for immunoassay analysis have been generated. For example, products of both monoclonal and polyclonal antibodies specific to aflatoxins (Shapira *et al*., 1997; Devi *et al*., 1999; Wang *et al*., 2001) and fumonisins (Azcona-Olivera et al., 1992ab; Yu and Chu, 1999; Biazon *et al*., 2006) have been used for immunoassay tests. The use of synthetic antibody fragments such as a single variable chain antibody (scFv) against the highly toxic mycotoxins AFB_1 (Daly *et al*., 2002) and FmB_1 (Lauer *et al*., 2005) and their application as recognition materials has become a new research in the area.

1.5.3 Immobilisation (Coating)

Since the focus is on immunosensing, this section will discuss mainly the immobilisation methods used for antibodies or antigens. The critical point of the sensitivity of immunosensor detection is dependent on the concentration of the antibody or antigen immobilised on the surface of a transducer (Guilbault *et al*., 2004). Therefore, the need for an immobilisation technique which can control the antibody or antigen binding on the device surface is important. Two techniques are mainly used for reagent immobilisation in biosensor devices and these are either physical (passive adsorption, electrostatic entrapment, cross linking) or chemical (covalent binding). These are to determine the stability of the binding orientation, the specificity, the reproducibility and the sensitivity of the affinity sensor development (Wang *et al*., 2006).

Physical immobilisation basically refers to non-covalent interaction such as hydrogen bonding, hydrophobic interaction, electrostatic interaction, or van der Waals force, depending on the substrates of the transducer. For the non-polarity-sensing substrate, the antibody or antigen molecule can be adsorbed through the hydrophobic interaction

and van der Waals force, while for the charge substrates, the non-covalent interactions are mainly associated with the electrostatic interactions. Moreover, antibodies or antigens may be physically entrapped into the films of organic high polymer or inorganic materials such as sol-gel and graphite powder. These kinds of immobilization have the ability to encapsulate the biomolecule, as well as physical tenability, optical transparency, mechanical rigidity, and low chemical reactivity (Wang *et al*., 1993; Wang *et al*., 1998).

Physical immobilization is the simplest technique used for immobilising the antibody/antigen in a common immunoassay method. Not only is it simple, but this immobilisation technique has the prospect of higher commercial use, reduces time and cost and is still able to increase catalytic activity (Gao *et al*., 2009). The antibodies can be coated on a variety of support materials such as plastic, carbon and diamond paste. The adsorption technique need to be carefully managed and normally is suitable for use for disposable materials such as one short device. In the case of immunosensing applications, many researchers have investigated the immobilization of antibody/antigen procedures onto electrodes for an optical immunosensor (Wang *et al*., 1993) and for an electrochemical immunosensor (Wang *et al*., 1998).

Through a direct passive immobilization of the antibody onto the sensor surface, the sensor may have problems with a reduction of sensitivity, low level of detection affinity and less reproducibility of the sensor (Akdogan *et al.,* 2006). This is because some factors such as lack of mechanical strength (Mulchandani, 2008) and leakage of adsorbed proteins (Wang *et al*., 2006) from the surface of the sensor have been found. It is important to minimise or avoid non-specific proteins binding on the surface, and eliminate steric hindrance and therefore the covalent immobilisation method on a chemically modified sensor surface is an alternative technique (Akdogan *et al.,* 2006). Using this technique, Patel and co- workers have found that better binding orientation or coverage and good sensitivity and reproducibility of the sensor can result from covalent immobilisation (Patel *et al*., 1997).

Since gold working electrode is a material for immunosensor devices, the development of covalent immobilisation for antibodies or antigens is conducted by the use of self-assembled monolayers (SAMs). The choice of SAM technique is important, as it differs depending on the type of transducer to be used. Self-assembled monolayers can be prepared using different types of molecules such as alkylsiloxane monolayers, fatty acids on oxidic materials, alkanethiolate monolayers and disulfides. Indeed, the formation provides the functional groups like –SH, –CN, –COOH, $–NH_2$ and silanes on the surface. This thesis has focused on the use of SAMs for functionalized alkanethiols such as long or short chain thiols and their derivatives on gold surfaces. One of the reasons is the spontaneous adsorption of thiol groups on gold surfaces at room temperature without the need for long incubation (Li *et al*., 2008).

1.5.4 Transducers used in biosensor devices

A transducer is an electrical transduction device for converting one type of energy to another form. In the case of the immunosensor transducer, it is used for converting the specific interaction between the antibody/analyte into a measurement signal. There are four main classes of transducers used in immunosensor application: electrochemical (electrode), optical (optrodes), calorimetric (thermistor or heat sensitive sensor) and mass (piezoelectric or surface acoustic wave devices (SAW)) (Tothill & Turner, 2003). The most widely used transduction modes of measurement for the highly toxic mycotoxins include: optical (fluorescence, reflection) (Daly *et al*., 2000; Maragos, 2001; Gaag *et al*., 2003) and electrochemical (potentiometry and amperometry) (Elizalde-Gonzalez *et al*., 1998; Ammida *et al*., 2004; Pemberton *et al*., 2006; Piermarini *et al*., 2006, Parker and Tothill, 2009).

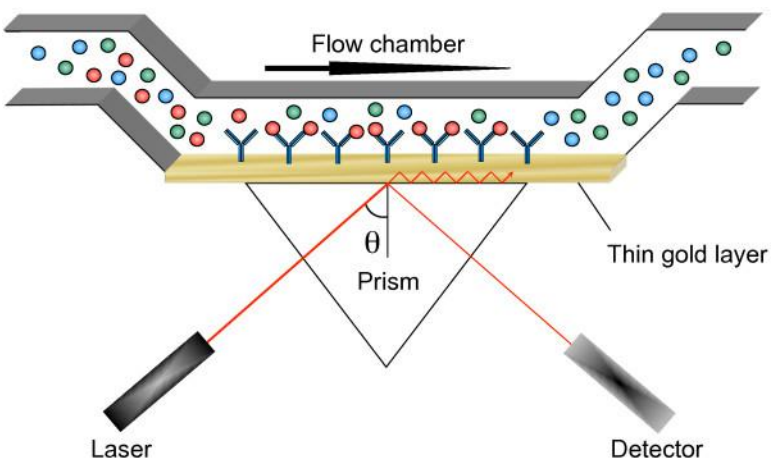
In the case of mycotoxin analysis, it has been mostly dominated by research into optical technologies such as surface plasmon resonance (SPR), and the fibre optics and analytes detected are mainly of aflatoxin B_1 (Boiarski *et al*., 1996; Carlson *et al*., 2000; Gaag *et al*., 2003; Dunne *et al*., 2005). The detection of mycotoxin on a biological system using colour and light intensity can be performed on an optical transducer to produce an optical biosensor. The physical phenomenon of the

measurement includes: surface plasmon resonance (SPR), waveguides, resonant mirrors, grating couplers, total internal reflection fluorescence, ellipsometry and interferometry (Homola, 2003).

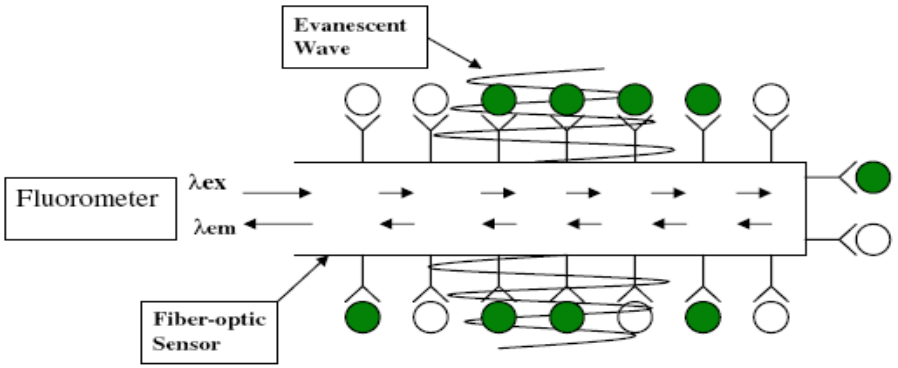
Surface Plasmon Resonance (SPR) is one of the techniques to measure biospecific interactions in real-time, which does not require the use of labels. The formation is based on the immobilization of one of the interactants onto the sensor surface, then the others are free in solution and passed over the surface. It continuously detects changes in the refractive index due to the sensing receptor interaction with the target analyte (Tothill and Turner, 2003). *In principle, the technology is based on excitation of the electron plasma of the thin metal layer covering the surface of the waveguide* (Figure 1.8a) ((Richens *et al.*, 2009).

For example, the use of SPR biosensor technology for the detection of mycotoxins such as aflatoxin B_1, fumonisin B_1, ochratoxin A, zearalenone, and deoxynivalenol has been reported (Mullet *et al*., 1998; Daly *et al*., 2000; Gaag *et al*., 2003; Dunne *et al*., 2005). SPR optical immunoassay has also been investigated for determination of aflatoxin B_1 using two different antibodies (Daly *et al*., 2000) and single chain antibody fragments (Dunne *et al*., 2005). In this case, the ranges of detection limit obtained were between 3-90 ng mL^{-1} and 390-12000 pg mL^{-1}, respectively.

The SPR biosensor has also been used for detection of fumonisin B_1. FmB_1 was measured by SPR equipment (BIACORE 2000) based on changes occurring at the sensor surface due to binding of the immobiliser antigen (Fumonisin) with the specific antibody (interacting molecules) (Mullet *et al*., 1998; Daly *et al*., 2000; Gaag *et al*., 2003). The detection limit of FmB_1 using polyclonal (Mullet *et al*., 1998) and monoclonal (Gaag *et al*., 2003) antibodies produced against FmB_1 onto a thin gold film substrate, coupled to a glass prism was 50 ng mL^{-1} in buffer.



(a)



(b)

Figure 1.8: (a) Schematic of the principle of SPR utilising a Kretschman-Raethen configuration (● analyte, Y antibody (Richens *et al.*, 2009). (b) Principle of fiber optic immunosensor (○ analyte, ● fluorescence labelled analyte , Y antibody) (Zheng *et al.*, 2006).

Fiber-optics is an optical technique and has been concentrated on the use of evanescent wave technology. The principle of a utilized evanescent wave through a fiber-optic sensor based on light intensity has been described (Maragos and Thompson, 1999; Zheng *et al*., 2006). *An evanescent wave is generated at the interface between an optical fiber and an outside lower refractive index material* (e.g. liquid or cladding) (Figure 1.8b) (Zheng *et al*., 2006). In this case the fluorescent analytes can absorb the light (energy) from the evanescent wave and fluoresce. A portion of the fluorescence emitted can be directed back into the fiber and can be detected.

Using fiber-optics has great potential: for example, the sensor uses monoclonal antibodies produced against FmB_1 attached to heterobifunctional silanes of optical fiber (800 µm core) based on competition of FmB_1 and FB_1-FITC (labelled with fluorescein). This immunosensor has achieved limits of detection of 10 ng mL^{-1} of FmB_1 in buffer (Thompson and Maragos, 1999).

Other than optical, electrochemical techniques are powerful methods with very sensitive detection (providing a low detection limit and a wide dynamic range), a simple system with very good compatibility (miniaturized and portable), require the use of a small volume of samples, and are very selective. These instruments based on electrochemical transduction usually have the potential to be simple, reproducible and low cost devices.

1.6 Electrochemical Immunosensor

An electrochemical system deals with a chemical reaction by interplay between chemicals and electricity for measurement of electrical current, potential or charge (Wang, 2006). This combination electrochemical immunosensor system has often been used for the quantitative determination of a binding event which uses an electrical signal.

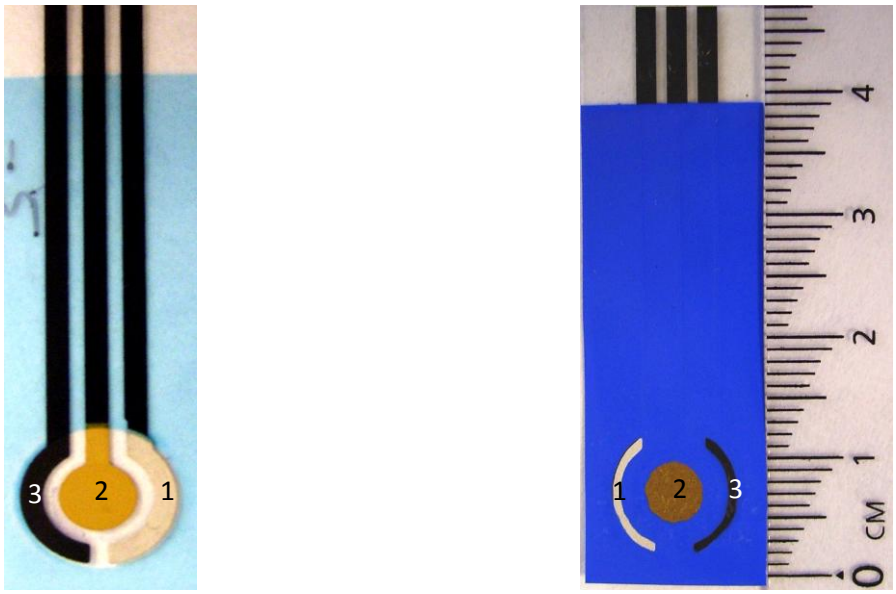
Electrochemical detection techniques are often used in conjunction with immunoassay systems to produce immunosensors, and these can be divided into: potentiometry,

amperometry and voltametry systems. The unique advantages in using electrochemical devices are that they are simple, portable systems, have low cost instrumentation, can measure low concentrations of analytes, and are compatible with being miniaturized (Huang *et al.*, 2008).

1.6.1 Electrode systems used for immunosensors fabrication

The support system of electrochemical immunosensors for electron flow measurement normally uses a three electrodes system, which is comprised of a working electrode, a reference electrode and counter (auxiliary) electrodes. In principle, by applying a constant potential between the working and reference electrodes, the desired redox reaction in which the analyte of choice is involved gives an increase to a current proportional to its concentration which is measured between the working and counter electrodes (Wang, 2006). The current signal at the surface is produced by the transfer of electrons from the electrode to the oxidation reduction (redox) species.

A variety of electrodes have been used for immunosensor fabrication; for instance, using conductive material paste such as gold electrode, platinum electrode, graphite electrode and carbon electrode (Rao *et al*., 2006). In this case, and applied in this thesis, a screen-printed electrode (SPE) was used in the development of an immunosensor and designed with a three electrodes system (working, reference and counter). Screen-printed electrodes (SPEs) can be produced by thick-film printing technologies such as screen-printing and ink–jet printing. The design, ink property and order in which they are applied result in small, highly reproducible conductive electrodes and are electrochemically inert. The common use of screen-printed electrodes rely on planar carbon or gold working electrodes and a silver/silver chloride reference electrode which are printed on a plastic or ceramic support (Ramirez *et al.,* 2009) (Figure 1.9). The SPEs have been broadly used because they are economical (easy to fabricate in bulk and disposable), easy to handle, have high sensitivity and a miniaturized portable system.



a b

Figure 1.9: Example of Gold SPE used in this work a) Screen-printed gold electrode fabricated using Du Pont screen-printing facilities, b) Screen-printed gold electrode printed at Cranfield University. Label 1 is the reference electrode (silver/silver chloride), 2 is the working electrode (gold ink) and 3 is the counter electrode (carbon ink).

Currently, electrochemical immunosensors using disposable screen-printed carbon electrode SPCEs are widely used for the measurement of mycotoxins. For example, Micheli *et al*. (2005) have developed a screen-printed carbon electrode (SPCE) for the detection of aflatoxin M_1 as an analyte in milk. Peirmarini *et al*., (2006) designed an SPCE incorporating a multichannel electrochemical plate (96 well screen-printed microplates) for multichannel detection of AFB_1 in corn samples using an intermittent pulse amperometry (IPA) technique. Another disposable SPCE immunosensor for aflatoxin B_1 was developed by Ammida *et al.*, (2004) based on an indirect competitive ELISA format using a differential pulse voltammetry (DPV) detector, and achieved a 20-30 pg mL^{-1}. Using the SPCE, Pemberton *et al.* (2006) have also used linear sweep voltammetry for measuring AFB_1 with a competitive format of immunoassay technique based on the competition of a free antigen (AFB_1) with a biotinylated AFB_1 conjugate. They also generated multiple carbon SPEs for multichannel array, similar

to the Peirmarini *et al*. concept. However, the detection limit for AFB_1 is higher than Peirmarini's study, which is 0.15 ng mL^{-1} in buffer solution (Pemberton *et al*., 2006).

There are many publications that have reported about the electrochemical characteristics of thick-film carbon and gold electrode. However, fewer reported on the detection of mycotoxins using an electrochemical gold electrode immunosensor. Using the gold working electrode (conductive material) in this study may provide better conductivity compared to carbon. The gold also *stable against oxidation and their chemical functionalization for immobilization events* (Ulman, 1996). According to Rao *et al.*, different types of ink pastes of working electrodes of SPE can influence the performance of immunosensing. The sensitivity of the electrode depends on the amount of antibody/antigen adsorbing on the surface, which is part of good electrochemical properties (Rao *et al*., 2006).

1.6.2 Cyclic Voltammetry (CV)

Cyclic voltammetry were utilised to investigate the electrochemical properties of molecules in solution (Jurgen, 1984; Faulkner**,** 2000; Wang, 2006). This is relatively simply and can provide high qualitative information based on electrochemical reactions through the accompanying electron transfer on the interface. For example, the illustration of the possible redox (oxidation/reduction) reaction of molecules such as ferrocyanide ion $[Fe(CN)_6]^{-4}$ / ferricyanide ion $[Fe(CN)6]^{-3}$ in electrolyte solution is shown in the equation below:

$$[Fe(II)(CN)_6]^{-4} \longleftrightarrow [Fe\ (III)(CN)_6]^{-3} + e^- \quad \text{.....(1.5)}$$

Figure 1.10 show the important peak parameters of oxidation reduction of molecule. The reversible redox couple reaction forms the oxidation of Fe^{2+} to produce Fe^{3+} on the forward scan in the anodic site, while the reverse scan reaction occurs (Fe^{3+} is reduced to Fe^{2+}) in the cathodic site.

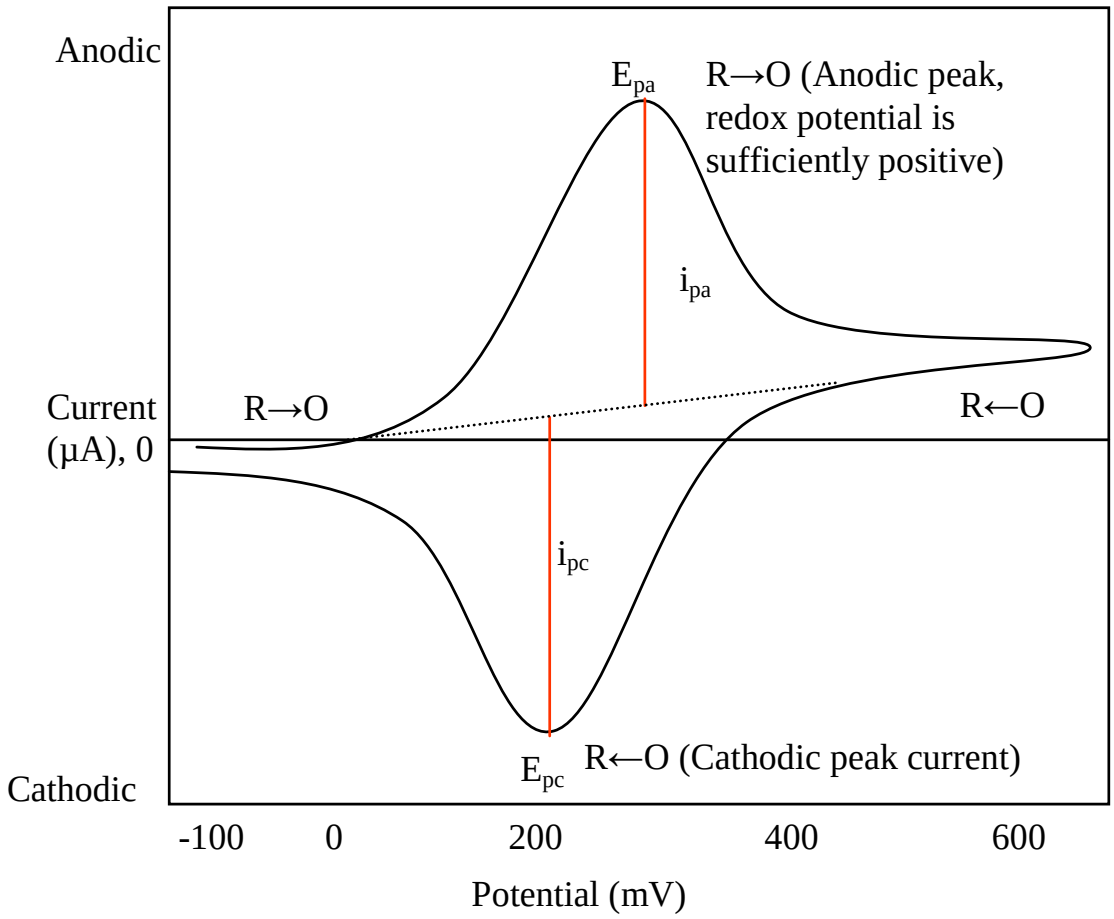


Figure 1.10: A typical cyclic voltammogram of species in solution for the important peak parameter. A reversible reaction of cathodic (c) and anodic (a), E_p refers to peak potential and i_p refers to peak current (Wang, 2006; Zoski, 2007).

From Figure 1.10, the following parameter values are applied to characterise the CV of a reversible process:

E_p (peak potential separation) = $E_{pa} - E_{pc}$ = 59/n mV …….....(1.6)

i_p (peak current ratio) = i_{pa}/i_{pc}= 1 at all scan rates ……(1.7)

Peak current function $i_p/v^{1/2}$ (v = scan rate) is independent of v (see equation for peak current)

For a charge transfer process under the same reversible conditions, as in Equation 6, the peak current density i_p is known by the Randles-Sevcik equation below:

$$i_p = 2.69 \times 10^5 n^{3/2} A C D^{1/2} v^{1/2} \quad \text{.....(1.8)}$$

where i_p is the current at the peak maxima (Amp cm^{-2}), n is the number of electrons transferred, A is the electrode surface area (cm^2), D is the diffusion coefficient ($cm^2 s^{-1}$), C is the concentration of the bulk solution (mol cm^{-3}) and v is the scan rate (Volt s^{-1}). In this study, a cyclic voltammetry will apply for the electrochemical immunosensor characterisation. The investigation will illustrate the surface characterisation of the gold working electrode (Wang, 2006; Zoski, 2007).

1.6.3 Chronoamperometry (CA)

Chronoamperometry is an electrochemical system which is commonly used for evaluating the diffusion coefficient of electroactive molecules or the surface area of the working electrode. It is an electrochemical technique for applying a step potential and the resulting current (i) is recorded as a function of time (t). This i-t response involves two elements: the current due to charging on the object surface that placed into a liquid (double layer) and the other one due to the electron transfer reaction with the electroactive species. Both current-time responses will occur simultaneously depending on the initial and final value of the potential E_i and E_f, respectively.

The signal is generally easy to interpret when a planar electrode is in an unstirred solution. The interpretation of the signal also depends on the applied potential which is sufficient to oxidize and reduce the electroactive species reaction to the electrode surface (Heineman, 1996; Bard & Larry, 2000; Zoski, 2007).

For the data analysis of chronoamperometry, the calculation is based on the Cottrell equation (Wang, 2006) as follows:

$$i = nFACD^{½}\pi^{-½}t^{-½} \quad \text{.....(1.9)}$$

Where n and t is the number of electrons transferred/molecule and time, respectively, F is Faraday's constant (96,500 C mol^{-1}), A is electrode area (cm^2), D is diffusion coefficient ($cm^2\ s^{-1}$) and C is concentration (mol cm^{-3}).

In the case of amperometric sensors, the current may be measured as a function of time that is proportional to the analyte concentration. As compared to other amperometric techniques, chronoamperometry provides a good signal to noise because *the current is integrated over relatively longer time intervals* (Heineman, 1996; Zoski, 2007)

1.6.4 Potentiometric detection

The current example of potentiometric immunosensor is the measuring of enzyme-labelled immunocomplexes at the surface of screen-printed electrodes, as carried out by Purvis and co-workers (Purvis *et al*., 2003). It relies upon a change in potential that occurs between the working electrode and a reference electrode as a result of specific interaction between an antibody and its antigen by measuring the product (H^+, CO_2 and NH_3) generated by the enzyme-labelled antigen (Marco & Barcelo, 1996). The operation is under condition of zero current flow. Mostly used and currently available ion selective electrodes can detect ions such as K^+, Na^+, NH_4^+, H^+ and Cl^- (Tothill and Turner, 2003). The use of the potentiometric immunoassay for mycotoxins is not yet available in the literature.

1.6.5 Amperometric detection

Recently, the literature survey on electrochemical immunosensors has indicated a common trend toward current detection which is amperometric immunosensors

development (Wang and Pamidi, 1998; Campanella *et al*., 1999; Liu *et al*., 2001; Darain *et al*., 2003; Sadik *et al*., 2009; Parker *et al*., 2009). This amperometry operates by applying fixed potential and then measuring the current based on the redox of an electroactive species on the electrode surface. In the amperometric sensor, a voltage is applied between the working and reference electrodes (for example, Ag/AgCl). This involves potential that encourages the electron transfer to produce causing a net current flow. The signal produced (current generated) is linearly proportional to the amount of analyte or to the specific binding reaction (Bannister *et al*., 1991). According to Sadik *et al.,* this device is more attractive and interesting because of its versatility, highly sensitivity, fast response and wide linear range (Sadik *et al*., 2009).

The amperometric enzyme electrode is the first type of transducer used as a biosensor. This analytical approach is a combination between the enzymatic reaction and the analytical device of an electrochemical detection. The function of the enzyme is to produce an electroactive species in a stoichiometric association with its target analyte. The amperometric transducer allows the electrochemical activity (oxidation or reduction) to keep on at the electrode surface and give an increase to a current.

Currently, amperometric immunosensors based on enzyme labelled techniques continue to be developed for a range of food and environmental analytes. This technique has an advantage as it can detect a current signal from the catalysis of a small amount of enzyme labelled reaction with the substrate (Lu *et al*., 1997). The example of the principle of amperometric immunosensor detection is shown in Figure 1.11, where the signal is detected by monitoring the specific binding of the enzyme labelled antibody to the concentration of analyte on the electrode. It is mostly based on the catalysis of substrates (H_2O_2) that produces products such electrons.

1.6.5.1 Hydrogen peroxide and mediators

The predominant electrochemical analysis system for biosensors involves hydrogen peroxide (H_2O_2) (Lu *et al.,* 1997; Ruo Yuan *et al.,* 2007; Salam and Tothill, 2009).

Hydrogen peroxide can be detected by both oxidation and reduction based amperometric analysis and is particularly important, because it is highly sensitive .

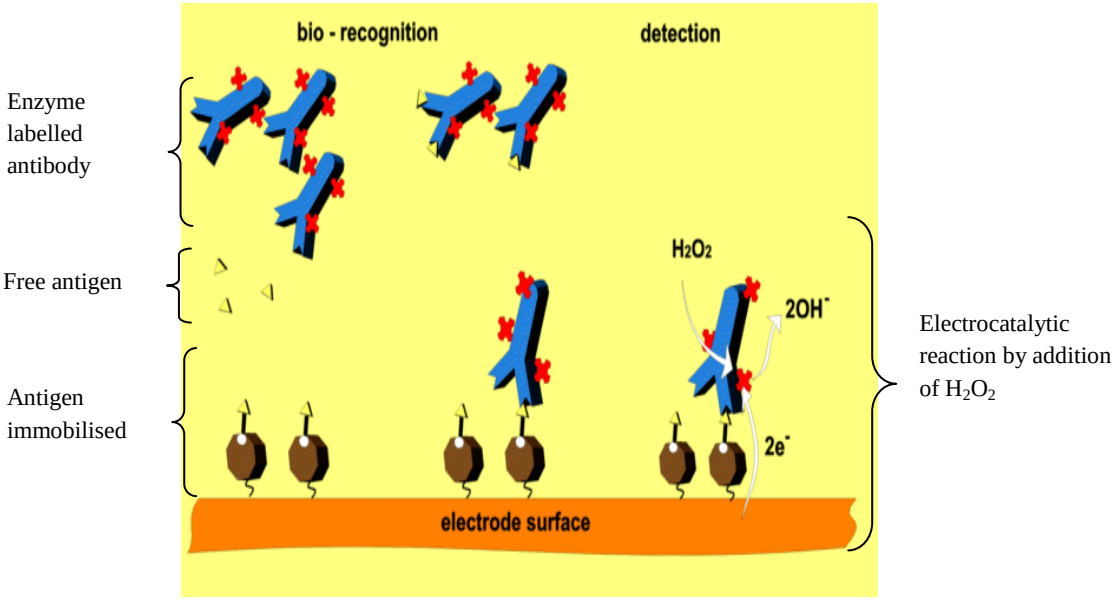


Figure 1.11: Concept of amperometric immunosensor reaction for generating electron to electrical signal using enzyme labelled antibody by the addition of H_2O_2 based on competitive assay format (Padeste *et al*., 1998).

With the combination of mediator-immobilization and potential control for amperometric H_2O_2 detection, this is an attractive option for H_2O_2 determination (Morales *et al*., 1996). This is because the mediators are generally low molecular weight species that act as intermediate electron acceptors at low potentials shuttling electrons from H_2O_2 to the electron surface. The mechanism of the redox reaction is shown below:

$$H_2O_2 + \text{mediator}_{(\text{oxidised})} \rightarrow O_2 + 2H^+ \text{ mediator}_{(\text{reduced})} \quad \text{.....(1.10)}$$

$$\text{Mediator}_{(\text{reduced})} \rightarrow \text{Mediator}_{(\text{oxidised})} + ne^- \quad \text{.....(1.11)}$$

From the equation, electrons are transferred from the H_2O_2 and reduce the mediator, which is subsequently re-oxidised electrochemically producing a current at the electrode. The most widely used mediators are molecules such as potassium ferricyanide, tetrathiafulvalene (TTF), tetracyanoquinodimethane (TCNQ), and the ferrocenes (Tothill and Turner, 2003).

For amperometric H_2O_2 biosensors, the immobilization of enzymes such as horseradish peroxidase (HRP) on the sensing interface is the key factor. Ruo Yuan and his group have immobilised HRP onto modified glassy carbon electrode by amperometric detection to show that HRP retained a high bioactivity (Ruo Yuan *et al*., 2007), while a low detection limit can be reached when the combination of a mediator, such as hydroquinone (Santandreu et al., 1999) or *o*-aminophenol (Liu et al., 2001), or 3,5,3',5'-tetrametilbenzidine TMB (Salam & Tothill, 2009) is used with HRP in combination with H_2O_2 as the substrate.

In the case of amperometric enzyme immunosensor detection, the method has been developed based on affinity interaction of an antigen or antibody labelled with an enzyme on the electrode surface and measuring the enzyme activity as a final step (Volpe *et al*., 1998). Through different approaches of immunoassay, the amount of target analyte bound to a specific antibody can be detected by measuring labelled enzyme catalysing of reaction of substrate and mediator (Lu *et al*., 1997; Salam & Tothill, 2009; Parker & Tothill, 2009). Enzymes such as horseradish peroxidase (HRP) are widely used as enzymes labelled for immunological detection and employed to construct the redox substrate (H_2O_2), thus enabling the reaction to be monitored electrochemically. Normally, a diffusion electron mediator such as 3,5,3',5'-tetrametilbenzidine (TMB) is used to transfer the electron between the redox centre of the labelled enzyme and the electrode surface (Salam & Tothill, 2009; Parker & Tothill, 2009).

1.6.5.2 A mediator of 3,5,3',5'-tetrametilbenzidine (TMB)

The TMB / substrate system has been used in commercial ELISA test kits with HRP assays where the colour change is read with a spectrophotometer (Liem *et al*., 1979,

Baldrich *et al*., 2009). TMB has also been used for the detection of hydrogen peroxide in samples as an electrochemical mediator (Compagnone *et al*., 1998). The TMB chromogenic substrate is the most common one used for HRP reaction because it is less toxic (noncarcinogenic) (Cattaneo & Luong, 1996) and more sensitive (quickly oxidised - faster reaction) (Liem *et al*., 1979; Volpe *et al*., 1998) than o-phenylenediamine (OPD) and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Fanjul-Bolado *et al*., 2005).

A well-known process of the oxidation of TMB by peroxidase reaction is a system illustrated by Josephy *et al.* (1982). The reaction scheme of the mechanism for HRP and TMB mediator is shown in the reaction below:

$$TMB_{(red)} + H_2O_2 \xrightarrow{HRP} TMB_{(ox)} + 2H_2O \quad \text{.....(1.12)}$$

$$TMB_{(ox)} + 2H^+ + 2e^- \longrightarrow TMB_{(red)} \quad \text{.....(1.13)}$$

In the presence of HRP, the catalysis shown above takes place. This equation shows that the reaction is an electroactive process, allowing an electrochemical analysis to be used.

Volpe and co-workers have described the application of TMB and H_2O_2 as a mediator/substrate in an HRP activity based immunoassay with amperometric detection (Volpe *et al*., 1998). According to them, TMB/H_2O_2 is a good substrate for the detection of a low level of HRP. This thesis will also describe and report on the use of TMB/H_2O_2 mediator as a substrate for measuring enzyme activity.

1.7 Microsensors

In principle, a microsensor is a miniature transducer fabricated by using conventional thin and thick film technologies. It is used for devices that convert either a non-

electrical physical or chemical quantity into an electronic signal (Gardner, 1994). Miniaturization of devices is a growing industry and it is expanding especially in the scope of analytical chemistry detection systems.

Microsensors based on microelectrode applications can be classified as a small electrode in *which at least one dimension is in µm range and not greater than 25 µm* (Amatore, 1995; Wang, 2006). Advances in microfabrication technology have seen the emergence of a variety of microelectrode devices that are reduced in size (Buttner *et al.*, 1990). A reduction in the size of a sensor has considerable increase in its applicability through being more sensitive, having a faster response of signal detection, and being more accurate and more economical (Gardner, 1994). This is allowed to replace conventional bulky electrodes.

1.7.1 Microelectrode arrays design

Several different materials and procedures have been applied to fabricate microelectrodes and microelectrode arrays (Sreenivas *et al.,* 1996; Buss *et al.,* 1999). Different materials such as iridium, platinum, gold and carbon on silicon wafers are normally used especially in the fabrication of micron dimension electrodes. A common microelectrode preparation involves the use of encapsulation of a cylindrical microfiber into Pasteur pipettes or glass or Teflon capillaries (Zhang & Ogorevc, 1998; Clark & Ewing, 1998). Microelectrode arrays (MEA) have been also fabricated to produce miniaturized electrochemical sensors (Berduque *et al*., 2007; Huang *et al*., 2009). Various types of MEA have been prepared including a microdisk electrode (Wittkampf *et al*., 1997; Aguiar *et al*., 2007), a microband electrode (Ordeig *et al*., 2008), interdigitated MEA (Fiaccabrino *et al.,* 1996) and three dimensional MEA (Xu *et al*., 2008) by standard deposition, etching, lithographic and photolithographic techniques (Figure 1:12). For the electrochemical application, this could include an on chip reference and counter electrodes.

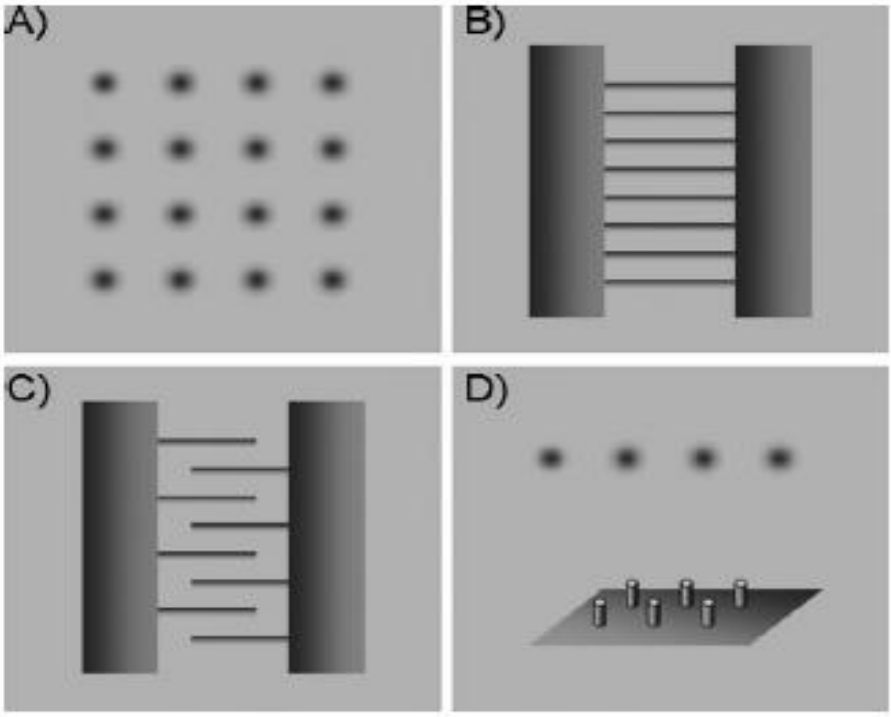


Figure 1.12: Types of microelectrode arrays. A) Microdisk electrode, B) Microband electrode, C) Interdigiated microelectrode array and D) Three dimensional microelectrode array (Huang *et al*., 2009).

1.7.2 Electrochemical microelectrode arrays application

In comparison to a traditionally sized electrode, microelectrode arrays have unique properties as electrochemical sensors (Bond, 1994). These include: small capacitive-charging current, faster diffusion of electroactive reaction and resulting in sigmoidal (or steady-state) diffusion current (Berduque *et al.*, 2007). Therefore, the final result for the sensor is an improved response time and greater sensitivity. In recent years, electrochemical polymerization techniques with a simple step immobilization to co-immobilize biomolecule and electropolymerized films have received much attention. The electrochemical polymerization technique was involved with conducting polymers such as polypyrrole, polythiophene and polyindole as well as with non-conducting polymers such as poly *o*-phenylenediamine, polyphenol and

polyethacridine. Polymer materials have been attached to substrates by covalent binding, physical mixing, or electrodeposition. It is the latest of mechanisms techniques of attachment. In comparison with conducting polymers and non-conducting polymers have a fast amperometric response to substrates and a high selectivity to eliminate possible electrochemical interference in samples (Bian *et al*., 2005). Furthermore, it is thermodynamically possible that dissolved redox species may be oxidised or reduced by the oxidised or reduced form of the polymer film. This means that the film may behave as an electron-transfer mediating catalyst (Yano *et al*., 1992; Gornall *et al*., 2009). Through this method, Gornall and co-workers evaluated the suitability of the screen-printed carbon electrode as a host for the enhanced production of sonochemically fabricated microelectrode array sensors, for use as electrochemical sensors and biosensors (Gornall *et al*., 2009). This is an important technique in order to increase the sensitivity and exclude the interference ion at the microelectrode surface and to facilitate an enhanced sensor response.

Different biosensor design based on microamperometric devices have been reported in the literature for medical applications, such as to immobilise antibodies for Human immunoglobulin G (HIgG) detection (Bian *et al*., 2005) and to immobilize enzymes for the assay of glucose, lactate, alanine, etc (Gue *et al*., 2002; Miao *et al*., 2004). Several studies on the development and application of electrochemical microsensors have also been reported such as detection of O_2 and pH (Revsbech *et al.*, 1999), toxin hydrazine (Wei Mo *et al*., 2000), bacteria pathogen (Li *et al*., 2004), and aflatoxin M_1 (Parker *et al*., 2009). A microelectrode array immunosensor for aflatoxin M_1 detection has been developed using a standard photolithographic method. The MEA fabricated contains the working electrode, reference electrode and counter electrode which are necessary for electrochemical detection. The competitive immunoassay on the modified MEA surface has been measured using the chronoamperometry technique (Parker *et al*., 2009). However, no report has been found for other mycotoxins such as AFB_1 and fumonisin detection.

In the case of pathogen detection, several examples have been given in the literature. Li and co-workers have developed an impedance immunosensor based on an interdigitated microelectrode array (IDA) that had a gold surface electrode on a borosilicate glass substrate for the detection of *E.coli* O157:H7 in food (Li *et al*.,

2004). Anti-*E.coli* O157:H7 polyclonal antibodies labelled with biotin were immobilised on the MEA surface with a self assembled monolayer of streptavidin. The measurement is based on electrochemical impedance in the presence of the electrolyte as a redox probe and showed higher sensitivity due to enhanced flux patterns and mass transport rates, when compared to bulky electrodes.

1.8 The functional of nano-particles for a biosensing application

A highly sensitive analysis of analyte is commonly accomplished through antibody-antigen interaction (immunoassay). However, recent efforts have led to the development of nano-particles for immunoassay application (Wang, 2007). The unique physical and chemical characteristics of nano-particles such as gold colloid provide excellent prospects for biosensing application (Rosi and Mirkin, 2005). Generally, based on their unique characteristics, the main functions can be classified as: *1) immobilization of biomolecule; 2) catalysis of electrochemical reactions; 3) enhancement electron transfer; 4) labelling biomolecule and 5) acting as reactant* (Luo *et al*., 2005).

These nano-particles can also present a good system for interacting biomolecules recognition events with electrochemical signal transduction. This can be done either by using the colloid along with an inert, planar electrode or by deposition of nano-particles onto a conducting matrix. In the sections below, the use of nano-particles for electrochemical imunosensor application is reviewed.

1.8.1 Nano-particles for electrochemical immunosensors

Several novel strategies to functionalize gold nano-particles have been proposed for developing an electrochemical immunosensor with enhanced detection sensitivity. However, the use of functionalized nano-particles on the electrode surface to achieve better sensitivity compared to conventional electrochemical immunosensors such as passive adsorption and SAM modified surface is still under investigation until today.

Some of the examples of using gold nano-particles in immunosensor applications are reported in the literature. Dequaire and co-workers have developed an electrochemical immunosensor for immunoglobulin G (IgG) detection using a gold colloid label via anodic stripping voltammetry technology and obtained a low detection limit (Dequaire *et al*., 2000). The electrochemical immunosensor was based on the non-competitive heterogeneous immunoassay where a primary antibody was used for the specific capture of the goat IgG and then sandwiched by the gold colloidal-labelled antibody (secondary antibody labelled). Other examples used an enzyme label electrochemical immunosensor. Das *et al*. have developed a new immunoassay for mouse IgG or prostate specific antigen (PSA) detection with specific binding to IgG−nanocatalyst (gold particles) conjugate (Das *et al.*, 2006). The gold-nanocatalyst label generates p-aminophenol (AP) by catalytic reduction. This reaction is very fast, and its kinetic values (k_{cat} and k_{cat}/K_M) are large and not decreased significantly even after IgG conjugation to the gold nanocatalyst.

The application of nano-particles in assay development for mycotoxin has also been reported recently. Xiulan and co workers have developed an immunochromatographic method for AFB_1 detection using a combination of an antibody with a nanogold conjugate probe (Xiulan *et al.*, 2005). The transfer in this system is induced by the capillary action of aqueous medium through porous membrane to separate the unbound reactant from the bound complex at the liquid–solid interface. The antibody was directly conjugated on the surface of the colloidal gold particle. The mechanism of the antibody was adsorbed on the surfaces mainly by hydrophobic and van der Waals force interaction. The limit of detection was found to be twice that achieved using an ELISA. Another method, bio-electrocatalytic reaction on a micro-comb electrode by means of self-assembling horseradish peroxidase (HRP) and AFB_1 antibody molecules onto gold nano-particles functionalized biorecognition surfaces has been developed by Liu *et al.*, (2006). The formation of immunoreaction of the antibody–antigen assay by a one-step procedure between the immobilised anti-AFB_1 and AFB_1 in the sample solution was developed. Then direct electrical contact was introduced between the immobilised HRP and the electrode surface, thus the signal was then detected by the HRP bio-electrocatalytic reaction.

To the best of our knowledge, there are limited reports focusing on electrochemical systems studying the antibody-antigen interaction on screen-printed electrodes and microarray electrodes in combination with a nano-particles modified complex for the determination of mycotoxins.

1.9 Immunosensor for aflatoxin B_1 and fumonisins analysis

Many immunosensors have been reported for aflatoxin B_1 using optical and electrochemical sensing (Table 1.6). In the case of nano-particles involved in electrochemical immunoassay, several works on AFB_1 detection with different approaches for the assay have also been reported in Table 1.6. However, in the case of fumonisins determination, no reports on electrochemical sensor detection have been used. This table is important as a guideline for new immunosensor research on aflatoxin and fumonisin determination. A different approach of immunoassay for AFB_1 and fumonisins detection based on electrochemical immunosensors, either direct immunoassay application or using nano-particle on the working gold electrode, will be developed in our work. This is in order to achieve a low detection limit that is easy to use for on-site analysis and rapid detection of these two toxins.

The possible advantages of using immunosensors over conventional immunoanalysis methods are: an increase in sensitivity and decrease in low detection limit; decrease of analysis time; decrease in the amount of expensive reagents; simplification of the analysis procedure (fewer stages); miniaturisation of equipment and automation (Tothill, 2001). Although immunosensors have the potential for diverse analyte detection, often they are limited by the availability of the antibody required.

Table 1.6: Immunosensor studies of AFB_1 and fumonisins electrode by previous researchers.

Mycotoxins	Sensing methods	Electrodes	Range detection (μg L^{-1})	References
Aflatoxin				
B_1	Optical	NR	NR	Boiarski *et al*., 1996
Total AFs	Electrochemical		7-10	Elizalde-Gonzalez *et al*., 1998
B_1	Optical		2	Maragos & Thompson, 1999
B_1	Optical	SPR	3-90	Daly *et al*., 2000
B_1	Electrochemical	SPCE	0.03-0.09	Ammida *et al*., 2004
B_1	Electrochemical	SPCE	0.15	Pemberton *et al*.,2006
B_1	Optical	OWLS	0.5-10	Adanyi *et al*., 2006
B_1	Optical	Array	0.5	Sapsford *et al*., 2006
B_1	Electrochemical	Au /ME/NG	0.1	Liu *et al*., 2007
B_1	Electrochemical	SPCE	0.05-2	Piermarini *et al*, 2007
B_1	Electrochemical	GCE/NG	0.05	Sun *et al*., 2008
B_1	Piezoelectric	PQC/NG	0.01	Jin *et al*., 2009
B_1	Piezoelectric	QCM	0.3-7	Wang & Gan, 2009
Fumonisin				
B_1	Optical	SPR	50	Mullet *et al*, 1998
B_1	Optical	fiber optic	10-1000	Thompson & Maragos, 1996
B_1	Optical	SPR	NR	Daly *et al*, 2000
B_1	Optical	SPR	50	Gaag *et al*., 2003
Total Fms	Optical	Array	0.5	Sapsford *et al*., 2006

NR: Not recorded; SPR: Surface plasmon resonance; SPCE: Screen-printed carbon electrode; OWLS: Optical waveguide lightmode spectroscopy; IME: Interdigitated microelectrode; NG: Nanogold; GCE: Glassy carbon electrode; PQC: Piezoeletric quarzt crystal, QCM: Quarzt crystal microbalance.

1.10 Aim and objectives

Mycotoxin contaminations in food and feed processing, especially aflatoxin and fumonisin, are harmful to human and animal health and have economic implications. Monitoring and controlling the contamination is a major priority for food safety and it is of increasing importance to consumers, the food industry and regulatory authorities. Many analytical methods for mycotoxin detection have been developed such as TLC, HPLC, ELISA and biosensors. However, several methods are very difficult to handle (need expertise), are expensive, have a slow output result, need a long procedure for extraction and clean-up, and use hazardous chemicals.

The aim of this study is to develop a rapid and sensitive affinity sensor for AFB_1 and fumonisins analysis in food samples at the required legislative limit. In order to achieve these objectives the work is focused on selecting the optimal antibody system, optimising the assay procedure, and selecting the transducer and finally the amplification/enhanced detection system and optimisation for real food samples analysis. The first stage of the biosensor development comprised the construction of an immunoassay to optimise and characterise the assay format. An indirect and direct competitive immunoassay format was chosen to apply in the immunosensor system.

The second stage comprises the preparation of the transducer component used for development of the immunosensor system. The transducer relates the biological signal (as a result of a binding interaction) via a detector towards an electronic data output. Electrochemical detection was chosen using an electrode as a transducer. The proposed sensor format was a mediated amperometric immunosensor using screen-printed gold electrodes directly transducing the current response arising from an indirect and direct competitive enzyme-linked immunoassay. The use of a microelectrode array in the immunosensor system as compared to a screen-printed gold electrode is a platform in the context of miniaturisation and portability of the sensor device.

The third stage involves the effect of nanogold in developing a competitive immunosensor assay using gold working electrodes for aflatoxin B_1. Gold nano-particles were coated with an antibody and a horseradish peroxidase for the

electrochemical detection using modified screen-printed gold electrode. Furthermore, the performance criteria of the amperometric immunosensor with specifically adapted characteristics are discussed.

The proposed immunosensor will be used to detect and monitor AFB_1 and fumonisins contamination in peanut and corn samples, respectively. In this case it involves the extraction and clean-up process in real samples (peanut and corn) which have to be considered for achieving interference-free and sensitive detection. In order to complete this study the following steps will be taken.

For the ELISA development:

i. Sourcing a suitable primary antibody and secondary antibody.
ii. Immunoassay format design
iii. Design of ELISA protocol.
iv. Optimisation of ELISA protocol.
v. Evaluate assay, characterisation, response and sensitivity.

For the Screen-printed gold Electrode (SPGE)

i. Fabrication and characteristics of SPGE.
ii. Transfer of ELISA method onto an electrochemical SPGE.
iii. Optimisation of immuno-reagents concentrations using the developed electrochemical screen-printed gold electrode. Study surface activation, immobilisation of antibody by passive adsorption and modified working electrode SPGE.
iv. Study of the application of nano-particle interaction.
v. Response, reproducibility, sensitivity, etc.

For Microarray electrode

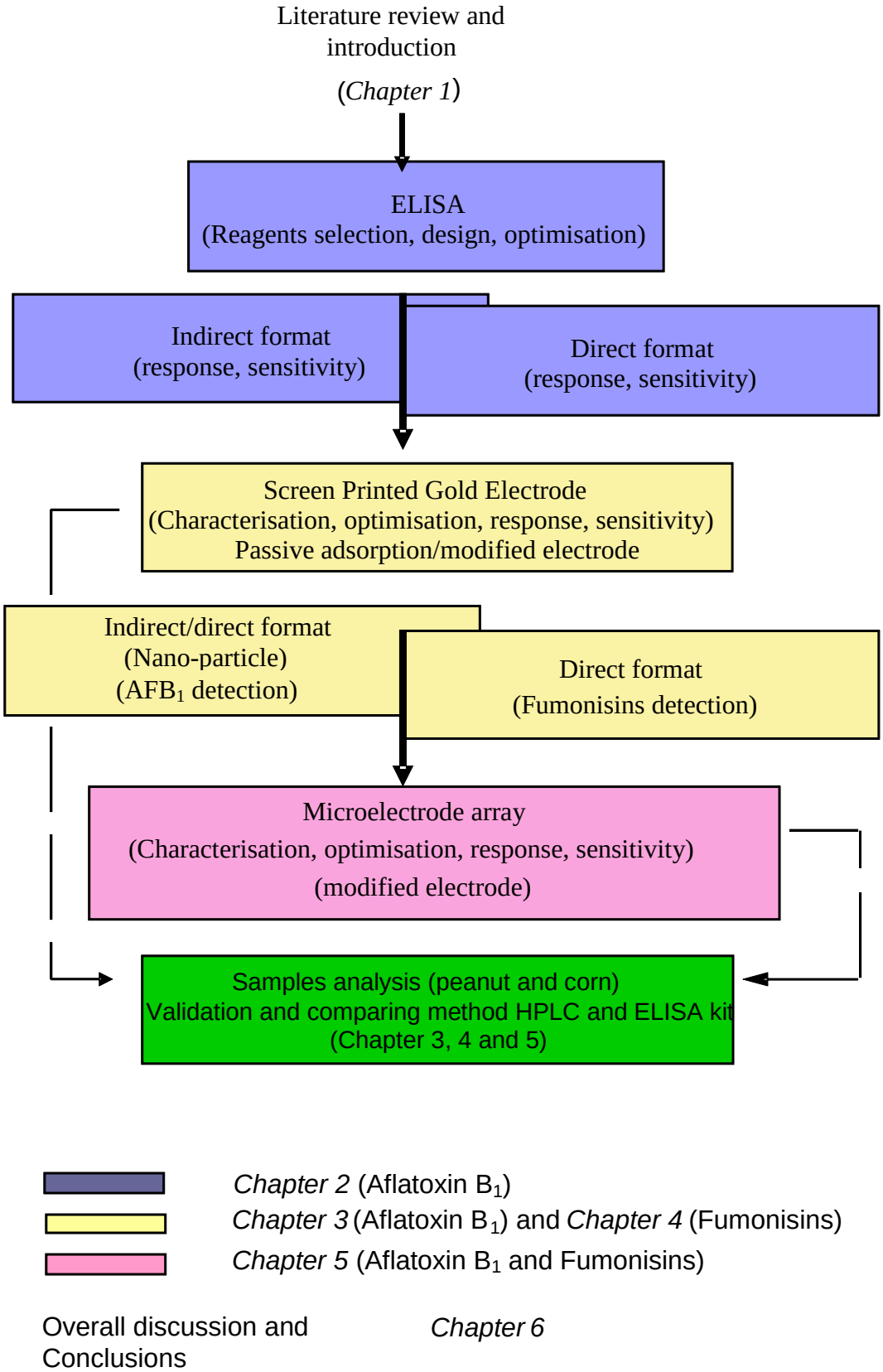
i. Characterise the microelectrode sensor.
ii. Transfer the assay from SPGE to the modified microarray electrode.
iii. Optimisation, response, reproducibility, sensitivity, etc.

For Samples Analysis and Validation Method

i. Sampling, extraction, cleanup, separation and detection, apply to the sensor.

ii. Comparing result against HPLC and ELISA kit using real samples (food samples).

1.11 Overall activities for Aflatoxin B_1 and Fumonisin detection



CHAPTER 2

DEVELOPMENT OF ELISA FOR AFLATOXIN B_1

2.1 Introduction

The initial stage for the development of the immunosensor is to develop an Enzyme-Linked Immunosorbent Assay (ELISA) technique. The ELISA methods of analysis address two analytical concepts. First, the antibodies used as a recognition element are highly specific to their antigen (analyte). Secondly, the assay format will produce a more sensitive result for the analyte of interest. This is an important technique for optimisation and validation of the commercially available reagents on the microplate.

In this work different type of tests were examined, both direct and indirect competitive assay format. The assay format for direct and indirect competitive assay was described in Section 1.4.3.1 of Chapter 1. Optimisation of the assay is accomplished by changing the concentrations (reagents) and assay conditions (times and temperature), as well as other experimental parameters such as blocking agents selection and washing reagents. This process is for the purpose of achieving maximum response for optimum assay performance which mean highly specific binding of the antibody-antigen and a higher signal:noise ratio.

Different concentration or dilution ranges of reagents should first be investigated on the microplate of ELISA for the optimisation assay. The checkerboard titration assay was used for the optimisation of reagents concentration. In this system, two different reagents in varying concentrations or dilution were investigated in one experiment. For example, the serial concentrations of the primary antibody were applied across the plate, and the other serial concentrations of reagent (enzyme-labelled secondary antibody) were used down the plate. This assay design obtained the signal:noise ratio in each well and set the optimal concentration of the two reagents which is the best signal:noise ratio. In this case, the performance of the assay is critically dependent on the concentration of the primary antibody. The background reading will increase if too large an amount (high concentration) of the primary antibody is used. However, the use of a smaller amount (too low concentration) of the primary antibody will result in a low signal or none. Therefore, the optimal concentration of the primary antibody needs to be determined practically.

In competitive ELISA, the sensitivity of the assay also depends on the concentrations of the antibody or antigen coating on the plate. Normally the antibody or antigen coating is in a coating buffer which is prepared for adsorptive immobilisation on the support surfaces (for example plastic microtitre plates) or other protein binding surfaces. The investigation of the coating buffer for ELISA development should be at the beginning of the assay. The common applied coating buffer are carbonate buffer (pH 9.6), Tris HCl buffer (pH 8.5) and PBS buffer (pH 7.4) (Crowther, 2001; Zhao *et al*., 2005). The pH-values can affect the antibody/antigen coating because of the steric composition of the antibodies or the antigens influence.

Blocking agents have been used in most cases of ELISA system developments in order to minimise or avoid non-specific binding of proteins and to increase the sensitivity of the assay. For example, a variety of blocking buffers ranging from synthetic polymers to protein polymers have been applied to block unreacted binding sites on the solid surface. The most efficient blockers are Bovine Serum Albumin (BSA), casein, gelatine, polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP). These have been examined and found to be capable of reduce non specific binding from 75 to 92% (Pemberton *et al*, 2006).

Many researchers (Gascon *et al*., 1997; Oubina *et al.*, 1999; Grabowska *et al*., 2002; Zhao *et al.*, 2005) also studied the effect of the incubation conditions such as temperatures and times and their influence on the antibody or antigen immobilization activity using a competitive immunoassay. The correct times and temperatures of incubation will achieve optimum coating and also increase the specific binding activity. The incubation steps of coating, binding and detecting are dependent on how many steps of assay are used. For example, in the case of the direct competitive immunoassay method for detecting AFM_1, used by Micheli and co-workers, three steps of incubation were applied. The first coating of the secondary antibody obtained no difference between either incubation for two hours at 37ºC or overnight at 4ºC (Micheli *et al*., 2005). In the case of the competition step, to achieve high performance of specific binding, about 15 to 120 minute incubations are tested. However, if too long an incubation of the detection step using the enzyme labelling method, then a high background signal will be found which can increase the non-specific binding. This will affect the sensitivity of the analytes detection limit. In

other cases, Ammida *et al*. also developed three steps of indirect competitive immunoassay format for detecting AFB_1 that involves three times incubation to complete the assay (Ammida *et al*., 2004).

In this work, ELISA tests were developed using a direct or indirect immunoassay format and commercially available reagents based on achieving lower detection limits, a wider dynamic range, and a higher signal:noise ratio and sensitivity. For the development of AFB_1 ELISA tests, a monoclonal antibody against aflatoxin B_1 ($MAbAFB_1$) was used. The direct format was based on competition of unlabelled and labelled AFB_1 binding to the $MAbAFB_1$ site immobilised on the well, similar to the procedure describe by Pemberton *et al.* (2006), while an indirect format was carried out through the competition between the proteins-conjugated AFB_1 immobilised and the free AFB_1 in the sample for the binding sites of $MAbAFB_1$ (Ammida *et al,* 2004). The last stage is introducing the enzyme substrate to react with the enzyme labelled (indicating the presence of antigens) for the colour development. The absorbance reading is inversely proportional to the concentration of the toxin. The limit of detection (LOD) regarding the direct and indirect competitive detection format is then compared.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

The following is a description of the materials that were utilised during indirect and direct competitive immunoassay experiments. All of the chemicals used were of analytical grade.

Protein-toxin conjugates, Aflatoxin B_1-BSA (AFB_1-BSA) and Aflatoxin B_1 (AFB_1) standard solutions, were obtained from Sigma-Aldrich Co. Ltd (Gillingham, United Kingdom (UK)). The monoclonal antibody against AFB_1 (primary antibody) ($MAbAFB_1$), and rabbit anti-mouse IgG (H+L) labelled with horseradish peroxidase (HRP) (secondary antibody) were purchased from Abcam Ltd. (Cambridge, UK). Affinity purified anti-antibody unconjugated, anti-mouse IgG (H+L) from goat for the

direct format was obtained from Pierce (U.K) Ltd, and RIDASCREEN AFB_1 assay kit with AFB_1 labelled with HRP (AFB_1-HRP) was purchased from R-Biopharm (Glasgow, UK).

Other reagents, polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), gelatine, potassium chloride and polyethylene glycol sorbitan monolaurate (Tween 20) were purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). Concentrated milk diluents (blocking solution) were found from KPL Ltd (UK). A substrate of 3,3',5,5'-Tetramethylbenzidine (TMB) solution was purchased from Insight Biotechnology (UK).

2.2.2 Instrumentations

The micro well polystyrene plates, MaxiSorp^{TM} (Nunc Immuno) were purchased from Fisher Scientific (Loughborough, UK). Incubations for every step of reactions were carried out by Labsystem iEMS incubator/shaker HT (UK). Spectrophotometric analysis of colour developed was performed by a BMG Fluostar galaxy ELISA plate reader (Aylesbury, UK).

2.2.3 Buffer Solutions

A 0.1 M carbonate buffer, pH 9.6 was prepared for the immobilization of AFB_1-BSA and anti-IgG unconjugated (precoating) on microplates following the recipe:
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
This was dissolved in 1 L water and the pH was adjusted to 9.6.

A 0.01 M phosphate buffered saline (PBS), pH 7.4 used for the preparation of AFB_1 standard, blocking solution, dilution of antibodies and washing solution was purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). The washing solution was prepared by adding 0.05% Tween 20 to the PBS (PBS-T). The AFB_1 standard solution (in acetonitrile) was prepared by diluting the stock solution (in PBS) with

PBS. A 0.05 M phosphate-citrate buffer, pH 5.0 used in the preparation of TMB solution was obtained from Sigma-Aldrich Co. Ltd (Gillingham, UK).

2.2.4 Blocking solutions

A chemical polymer (1% PVA and 1% PVP) and protein polymer (1% gelatine and 1:20 milk diluents) in PBS, pH 7.4 were used as blocking reagents.

2.2.5 Standard solutions

The AFB_1 standard solution in acetonitrile was diluted using PBS, pH 7.4 for stock solution (1 mg mL^{-1}) and stored at -18ºC in an amber bottle (dark) and tightly capped. From the stock, about 0.001 to 100 µg L^{-1} of standard solution concentrations were prepared in PBS for standard curves of the ELISA system. Safety measures were applied such as wearing gloves, protection glasses and a mask when handling the chemical due to the potentially carcinogenic properties of aflatoxin. The toxin solution in acetonitrile was generally diluted in buffer upon arrival and labelled as a stock toxic reagent before being stored in a locked fridge.

2.2.6 Indirect competitive ELISA

In initial experiments, the different concentration range of AFB_1-BSA conjugate, anti-aflatoxin B_1 antibody (monoclonal antibody) and anti-antibody labelled with HRP was determined by performing a checkerboard assay as detailed by Crowther (2001) (Figure 2.1). The optimization of different concentrations of reagents in different type of buffers, the condition of incubation times and the use of blocking agents in this experiment was followed as described by Ammida *et al*. (2004). The different volumes of reagents solution for well plates coated were also investigated.

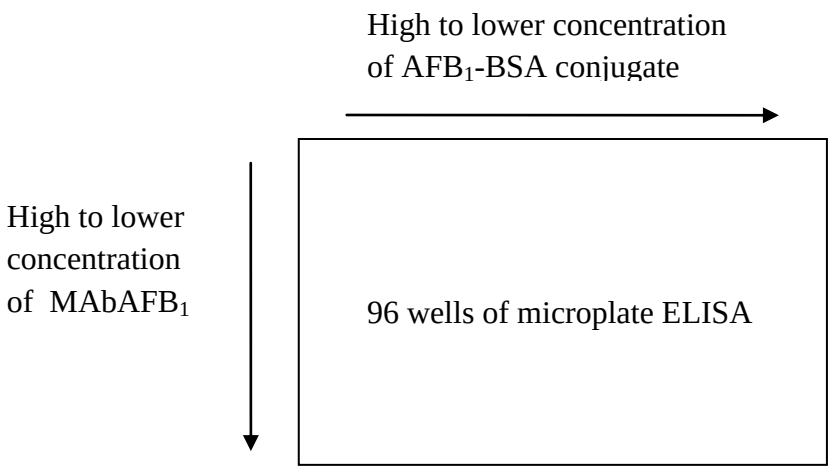


Figure 2.1: Example schematic diagram of checkerboard design for optimisation of reagents.

The ELISA system was also optimised by producing calibration curves using a series of standards of AFB_1 ranging from 0 to 100 µg L^{-1}. Three experiments were done: the effect of incubation time during the competitive step from 0 to 120 minutes, the concentration of monoclonal antibody ($MAbAFB_1$) was varied from 5, 10 and 50 µg mL^{-1} concentrations, and the concentration of the HRP-labelled antibody was varied from 0.5, 1 and 5 µg mL^{-1}.

2.2.6.1 Optimisation of reagents

By using the checkerboard method in the microwell plates, all the different concentrations of reagents and conditions parameters as present in Table 2.1 were performed.

The formation of the non competitive assay was followed by immobilisation of AFB_1-BSA first, then blocking before coating with anti-aflatoxin B_1 antibody and labelling with anti-antibody labelled with HRP. In each step of the assay, a 50 µl/well of reagents was added; the washing procedure was performed twice with a 150µl/well phosphate buffered saline containing Tween 20 (PBST) and once with PBS alone. The colour development was initiated by addition of a TMB/substrate solution (50

µl/well) to each well and incubation at room temperature for 15 minutes before measuring the absorbance at 450 nm in a plate reader.

Table 2.1: The parameters for optimisation of the ELISA test with different concentrations of reagents and conditions.

Reagents/buffers	Concentrations	Conditions/Incubation
AFB_1-BSA conjugate in 0.1M carbonate buffer pH 9.6	0 to 50 µg mL^{-1}	4°C (18 hours)
Blocking with PVA	1%	37ºC (1 hour)
Anti-aflatoxin B_1 antibody in 0.01 M PBS buffer pH 7.4	0 to 10 µg mL^{-1}	37ºC (2 hours)
Anti-antibody labelled with HRP 0.01 M PBS buffer pH 7.4	0 to 10 µg mL^{-1}	37ºC (1 hour)

2.2.6.2 Effect of coating buffers and blocking agents

First, a coating of AFB_1-BSA conjugate in two different buffers (0.1 M carbonate buffer pH 9.6 and 0.01 M PBS buffer pH 7.4) was added on the well microtitre plate. To reduce the non-specific binding of the ELISA assay, four difference blocking agents were tested. About 50 µl/well of 1 to 3 % PVA, 1 to 3 % PVPP, 1:10 to 1:40 milk diluents and 1 to 3% gelatine was added on the well after first coating the plates with the antibody. This experiment was performed before the competitive assay was running.

2.2.6.3 Competition assay preparation

A 50 µl/well of AFB_1-BSA conjugate (1 µg mL^{-1}) in a coating buffer (0.1 M carbonate buffer pH 9.6) was added to the wells of a microtitre plate and incubated at

4°C overnight (18 hours), then followed by washing steps (same washing procedure as above). The assay was then blocked with a 1% PVA blocking solution (50 µl/well) before being incubated at 37°C for 1 hour, followed by washing. Competition reaction involved the addition of various AFB_1 standard solutions (0-100 µg L^{-1}) with a fixed anti-aflatoxin antibody ($MAbAFB_1$) (10 µg mL^{-1}) in PBS for 30 minutes at room temperature in an eppendorf tube (pre-incubation) before 50 µl/well of mixture was transferred to the microtitre plate for 1.5 hours at 37ºC incubation. After the washing step, a 50 µl of anti-antibody labelled with HRP (anti-IgG-HRP) (1.0 µg mL^{-1}) in PBS was then added to the microtitre plate and incubated for 1 hour at 37ºC, after which the unbound material was then washed off. The colorimetric reaction was initiated by the addition of a TMB substrate solution (50 µl/well) to each well and incubated at room temperature for 15 minutes and stopped the reaction with H_2SO_4 (25 µl/well) before measuring the absorbance at 450 nm in a plate reader. The illustration of the schematic assay of the indirect competitive method used is shown in Figure 2.2.

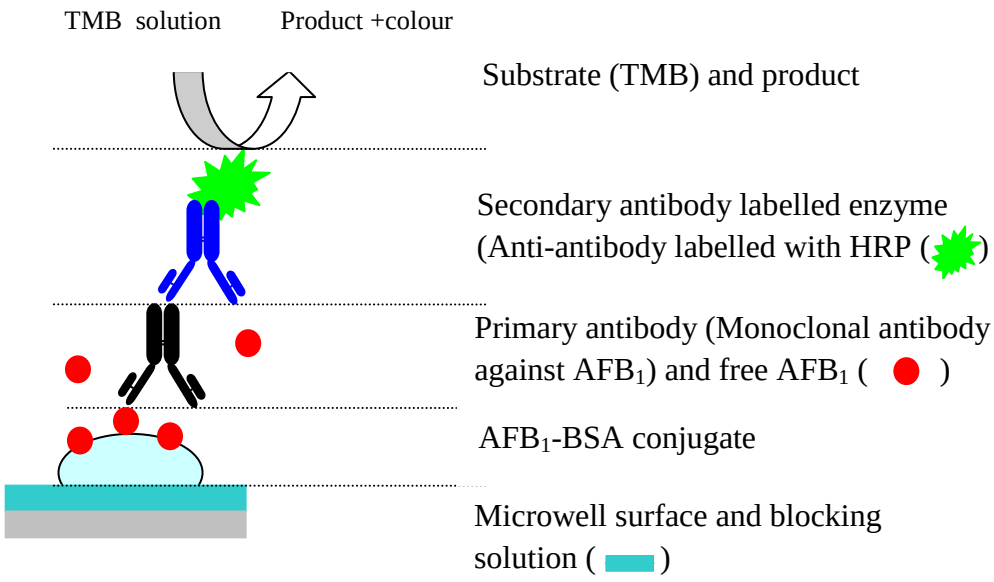


Figure 2.2: Schematic diagram of indirect competitive format used in this study.

2.2.6.4 Effect of concentrations of reagents and incubation time for competitive assay development

These experiments were performed based on the indirect competitive format assay to investigate the sensitivity of the competitive ELISA. The assay was tested with the different concentrations of reagents and incubation time as listed in Table 2.2.

Table 2.2: Different concentration reagents and different times of incubation used to test the sensitivity of the indirect competitive ELISA format.

Concentrations/Conditions	Components
1, 5, 10 and 50 μg mL^{-1}	$MAbAFB_1$
0.1, 0.5, 1 and 10 μg mL^{-1}	anti-IgG HRP
1, 30, 60 and 90 minutes	incubation times of competition reaction

2.2.7 Direct competitive ELISA

2.2.7.1 Optimisation of reagents

The first step of this work was to evaluate and optimise the antibody (primary and secondary) range (0.1 to 50 μg mL^{-1}) and commercial AFB_1 labelled with Peroxidase (AFB_1-HRP) range (1:30 to 1 dilution). The tests were performed in a 96-well microplate in accordance with a direct ELISA format based on the method described by Micheli *et al*., (2005). The illustration of the direct competitive method is shown in Figure 2.3.

2.2.7.2 Competition assay preparation

Experiments were based on a competition between AFB_1 labelled with HRP and unlabelled AFB_1 in the sample or standards for binding sites of antibodies, immobilised on the 96 well microtitre plate support. As in the indirect method, the optimum antibodies, conjugate enzyme, buffers, incubation times and blocking reagents were used in producing a standard curve of AFB_1 ranging from 0 to 100 µg L^{-1}.

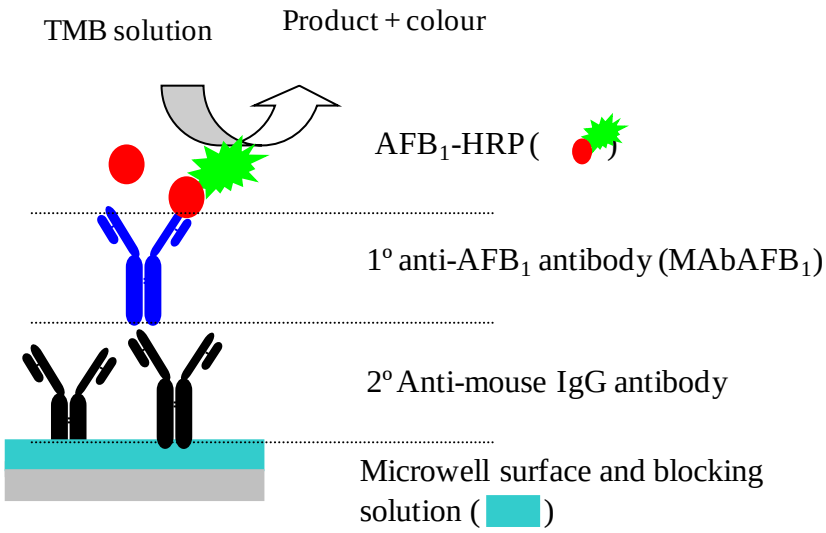


Figure 2.3. Schematic diagram of direct format. Precoating the wells with anti-antibody (anti-IgG) unconjugated before coating the wells with anti-aflatoxin B_1 antibody ($MAbAFB_1$).

The assay was done by following this procedure: The plate was precoated with anti-mouse IgG antibody (10 µg mL^{-1}, 50 µl/well) in an 0.1 M carbonate buffer pH 9.6, for 18 hours (overnight) at 4ºC, followed by washing twice (150µl/well) with a phosphate buffered saline containing Tween 20 (PBST) and once with PBS alone. Then, the unspecific binding was blocked by adding 1% PVA (50 µl/well) and then incubated for 1 hour at 37ºC. After washing, the plate was coated with an anti-AFB_1 antibody (monoclonal antibody) (20 µg mL^{-1}, 50 µl/well) in PBS for 2 hours incubation at 37 ºC, followed by washing. The competition solution was prepared by mixing (50 µl/well) the free AFB_1 (0-100 µg L^{-1}) in PBS with a fixed dilution of a

solution of AFB_1-HRP conjugate (1:5) in PBS. The competition reaction was allowed to proceed for 2 hours at 37 ºC and then rinsed with PBS-T (twice), followed by PBS (once). Finally, the absorbance was measured by the addition of a TMB substrate solution (50 µl/well) to each well and incubated at room temperature for 15 minutes and stopped the reaction with H_2SO_4 (25 µl/well) before measuring at 450 nm in a plate reader.

2.2.8 Calculation for the sensitivity of the assay

The calculation of % relative binding of competitors value was as follows:

$$\% \text{ relative binding} = B/B_0 \times 100 \quad \text{.....(2.1)}$$

where B is the absorbance value of competitors and B_0 is the absorbance of non-competition analyte (maximum binding).

Calibration curves were fitted by non-linear regression using the following four parameter logistic functions (Warwick, 1996):

$$y = (a - d)/[1+(x/c)^b]+ d \quad \text{.....(2.2)}$$

Where parameter *a* and *d* are the asymptotic maximum and minimum value of the calibration curves, respectively, *x* is the concentration at the EC_{50} value, *c* is the analyte concentration and *b* is the hill slope. EC_{50} is an effective concentration for 50% value.

The limit of detection (LOD) was calculated as well as shown in the equation:

$$LOD = x\,[(a - d/\,(a - d) - 3\sigma)-1]^{-1/b} \quad \text{.....(2.3)}$$

where σ is the standard deviation of the zero value.

2.3 Results and Discussion

In this study, the spectrophotometric competitive enzyme-linked immunosorbent assay (ELISA) for AFB_1 detection was developed before the electrochemical study was carried out. The tests were performed in a microwell titre plate in two different formats which were indirect and direct competitive formats.

2.3.1 Indirect ELISA

The first step in the optimisation assay development was to determine the optimal concentration of different reagents, selection of the buffer (carbonate and phosphate saline), blocking reagent and incubation times.

2.3.1.1 Optimisation of anti-AFB_1 antibody (monoclonal antibody against AFB_1) and AFB_1-BSA conjugate

The performance of the specificity binding of the monoclonal anti-AFB_1 antibody ($MAbAFB_1$) to the binding site of the AFB_1-BSA conjugate was tested through the checkerboard titration method. The result in Figure 2.4 was obtained by different concentrations of AFB_1-BSA (0 to 50 µg mL^{-1}) immobilised and varying the $MAbAFB_1$ (0 to 10 µg mL^{-1}). The different absorbance reading in each microwell plate was plotted. Each curve contained the signal for a different concentration of AFB_1-BSA conjugate, and the increasing of the signal was observed with the increase of $MAbAFB_1$ concentration. However a difference signal reading was clearly obtained between a 1 to 10 µg mL^{-1} of $MAbAFB_1$ in every treatment of different concentration of AFB_1-BSA. This indicates the binding interaction of AFB_1-BSA for the $MAbAFB_1$ is in the range of 1 to 10 µg mL^{-1}.

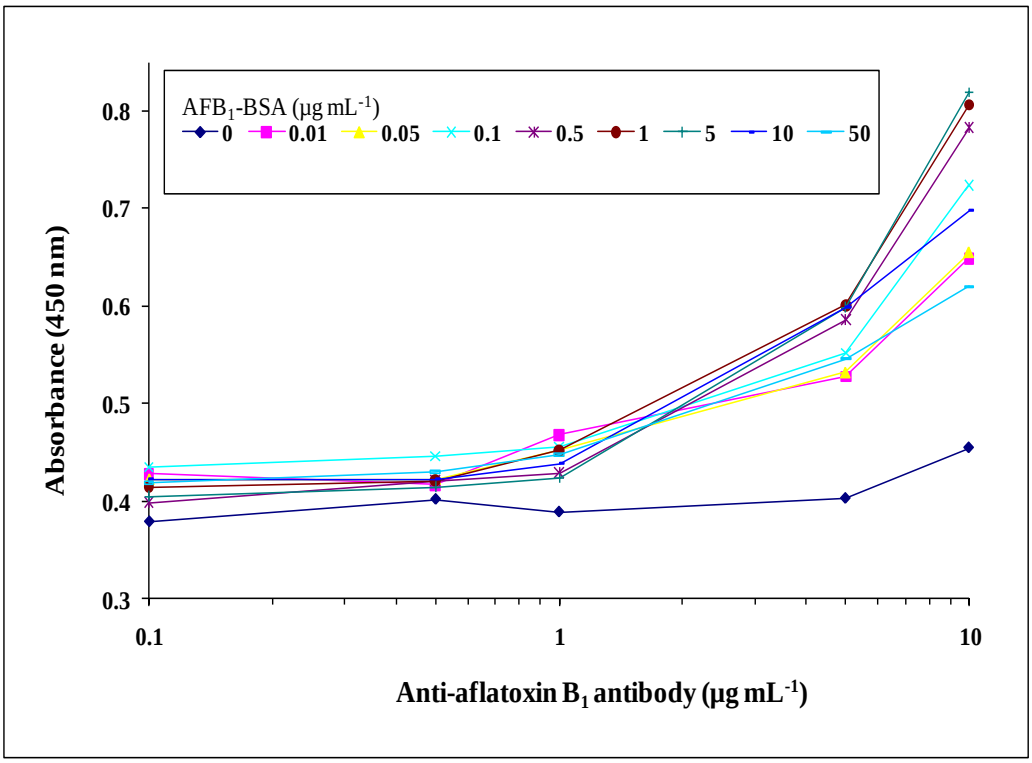


Figure 2.4: Optimisation of plate immobilisation with different concentrations of AFB_1-BSA conjugate and different concentrations of anti-aflatoxin B_1 antibody ($MAbAFB_1$) in an indirect format without using free AFB_1 (non-competitive).

In the range of 5 to 10 µg mL^{-1} of $MAbAFB_1$, a high absorbance reading was achieved by 1 and 5 µg mL^{-1} of AFB_1-BSA. The result indicates that the optimal concentration for AFB_1-BSA for the specific binding of the antibody is in the range of 1 to 5 µg mL^{-1}. Hence, the smallest value (1 µg mL^{-1}) that still produces optima binding ability was chosen for economical reasons and ensures that the test cost will be competitive. Based on these results, a plate coating concentration of 1 µg mL^{-1} AFB_1-BSA and monoclonal antibody concentration of 10 µg mL^{-1} will be used for further experiments. In this experiment, standard deviations cannot be generated since the experiment is unrepeated; therefore error bars are not produced. Once the system was optimised then the experiment was repeated in order to assess the standard deviation and hence the repeatability.

The checkerboard titration for the optimisation of two reagents was suggested by Crowther (2001). First, there was the direct adsorption of an AFB_1-BSA to the support surface (96 well), and this approach to the immobilisation was followed by

other authors such as Ammida and co-workers (Butler, 2000; Ammida *et al*., 2004). Then the captured antibody of the monoclonal of anti-aflatoxin B_1 antibody (mouse monoclonal antibody) for the binding interaction from commercial site was selected. This is because using monoclonal antibodies (MAbs) enables the recognition of a specific epitope and there are no other antibodies present, specific or cross-reactive, to compete for binding to the adsorbed antigen and influence the measured antibody binding profiles (Giardina *et al*., 2003).

The parameters used as a standard method for ELISA development were also followed by Ammida *et al*, in their method. However, when the optimal concentrations of two reagents (AFB_1-BSA and $MAbAFB_1$) were achieved, an anti-IgG labelled enzyme, coating buffer, blocking agents and different conditions of assay were then optimised in the next set of experiments.

2.3.1.2 Optimisation of anti-mouse IgG-Horseradish peroxidase conjugates (anti-IgG-HRP)

In the second experiment, based on the indirect assay format, different concentrations of anti-mouse IgG-Horseradish peroxidase (anti-IgG-HRP) conjugate were optimised. The enzyme labelled (horseradish peroxidase) is commonly used for catalytic reaction and produces a stable and sensitive product for measurement (Collona *et al*., 1999). The solution of anti-IgG-HRP conjugate was used as a tracer, and the TMB solution as the enzyme substrate. The concentration of the anti-IgG-HRP conjugate used in the immunoassay was optimized by performing a series of assays utilizing varying amounts of anti-IgG-HRP (0–10 μg mL^{-1}) with a 1 hour incubation time.

The optimisation range of anti-IgG-HRP is shown in Figure 2.5. The trend is expected, that is the absorbance reading increases with the amount of anti-IgG-HRP used. This is because the enzyme reaction measured is proportional to the amount of specific binding of anti-IgG-HRP to the fixed amount of $MAbAFB_1$ in the test solution.

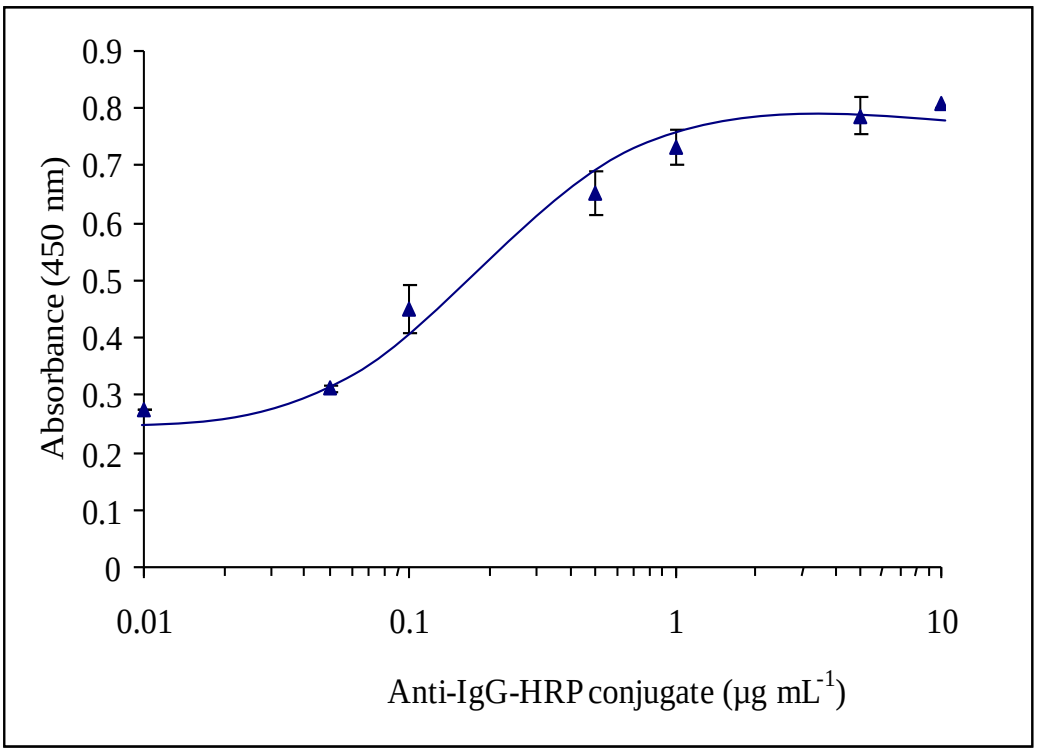


Figure 2.5: Optimisation of anti-mouse IgG-HRP conjugate. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (10 µg mL^{-1}) with an amount of anti-IgG-HRP (0-10 µg mL^{-1}). TMB solution as a substrate and H_2SO_4 was used to stop the reaction. Error bars indicate=SD, n=3.

The absorbance response was dramatically increased between 0.05 to 1 µg mL^{-1} concentrations and was saturated after the maximum binding of 5 µg mL^{-1}. However, 1.0 µg mL^{-1} of anti-IgG-HRP was selected for future immunoassay tests. This is because at a concentration of 1.0 µg mL^{-1} a high signal was still obtained, which refers to the specific recognition capabilities of 10 µg mL^{-1} of $MAbAFB_1$ concentration for the corresponding 1 µg mL^{-1} AFB_1-BSA conjugate. The result still showed a high value of signal: background, which indicates that this may produce a high dynamic range in calibration curves of the competitive assay. The use of a low concentration of reagents signal also can reduce the cost of the analytical method developed.

2.3.1.3 Effect of coating buffer in indirect non-competitive method

The adsorptive immobilisation of AFB_1-BSA to the plastic surface is dependent on the pH value of the coating buffer. Two different coating buffers (carbonate buffer, pH 9.6 and PBS, pH 7.4) were used for testing on the effect of immobilisation. These buffers with different pH value are able to influence the steric structure of protein which can influence the immobilisation system. The influence of the buffer at pH 9.6 and 7.4 response signal is shown in Figure 2.6. The adsorption of AFB_1-BSA on the plastic surface using the carbonate buffer pH 9.6 produced a higher reading compared to that of the phosphate buffer at pH 7.4. This result also shows the high ability for binding of the $MabAFB_1$ with AFB_1-BSA conjugate starting from 1 to 50 µg mL^{-1} before saturation occurs.

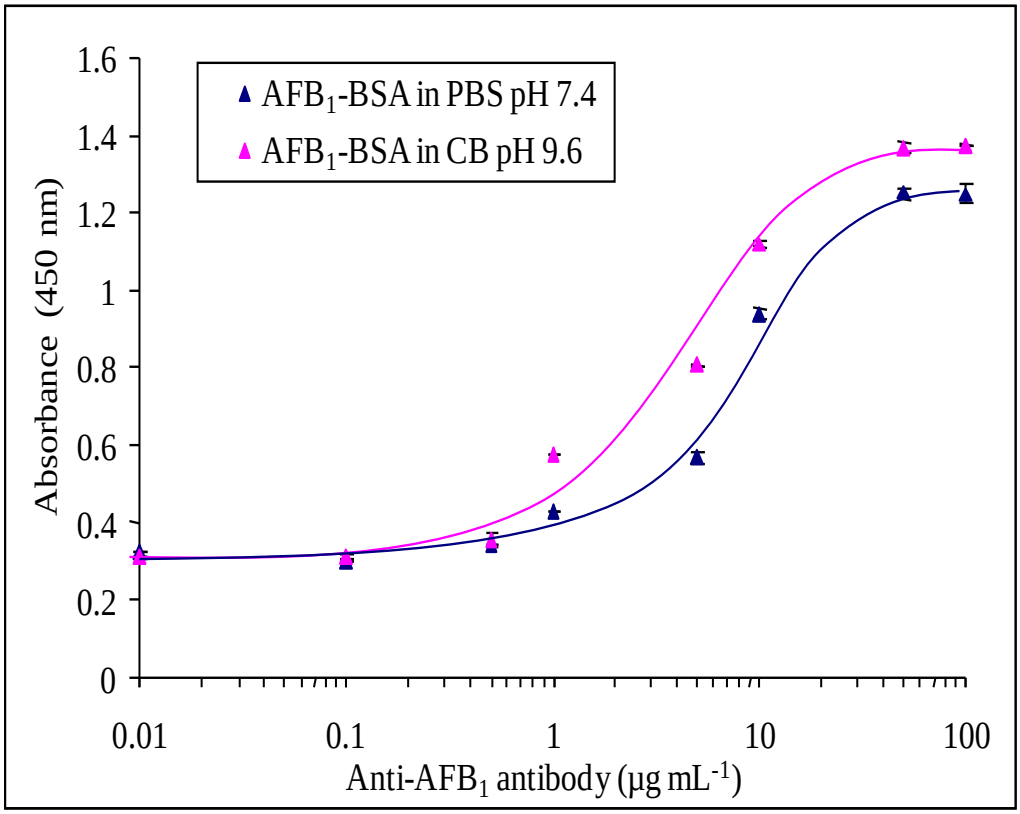


Figure 2.6: Anti-AFB_1 antibody ($MAbAFB_1$) binding of AFB_1-BSA conjugate in two different coating buffers 0.01 M PBS pH 7.4 and 0.1 M CB pH 9.6. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA, followed by $MAbAFB_1$ (10 µg mL^{-1}) and continued with amount of anti-IgG-HRP (1 µg mL^{-1}). TMB solution as a substrate and H_2SO_4 was used to stop the reaction. Error bars indicate =SD, n=3.

The result was suggested that the carbonate buffer in alkaline pH binding enhances the immobilising adsorption of AFB_1-BSA on the polysterene plates. So the carbonate buffer pH 9.6 was chosen as the coating buffer for this format and continues to be widely used in ELISAs. Previous ELISA studies for mycotoxin detection using direct and indirect assays have also proposed the use of a carbonate buffer (pH 9.6) for antibody or protein conjugated coating onto microwells as this produce a higher response (Ammida *et al.*, 2004; Alarcon *et al*., 2004; Michell *et al*., 2005; Piermarini *et al*., 2007). There is evidence that proteins conjugated and exposed to high pH prior to adsorption, display higher activity than when neutral pH is used.

2.3.1.4 Blocking agents

The use of blocking agents in an AFB_1 ELISA system is very important for investigating the blocking reaction on the surface of the wells. The main purpose is to reduce the amount of the non-specific binding of proteins as well as to produce low background readings in the system. Four types of blocking agents were chosen for testing in this experiment which used chemical polymers (PVA and PVP) and protein polymers (gelatin and milk diluents). These four types of blockers are frequently used as blocking agents in the ELISA system. Following the study of Ammida *et al,* and Micheli *et al.*, 1% PVA was used (Ammida *et al.*, 2004; Micheli *et al*., 2005). In another case, PVP polymer has been used and obtained a low background result from the assay (Studentsov *et al*., 2002; Parker & Tothill, 2009). As compared with protein polymer, milk diluents are a common blocker applied in many ELISA test kits. In the past, gelatine has been applied as a blocking agent (Crowther, 2001; Lee *et al.*, 2004). Thus the two polymers and two proteinaceous blocking agents were performed and compared to each other to provide low background readings in this experiment.

For each blocker with a concentration of 1% of PVA, 1% of PVP and 1% of gelatin as well as 1:20 milk diluent in PBS pH 7.4 were performed for replicate wells in the absence of free AFB_1. Figure 2.7 shows the background readings obtained for each agent.

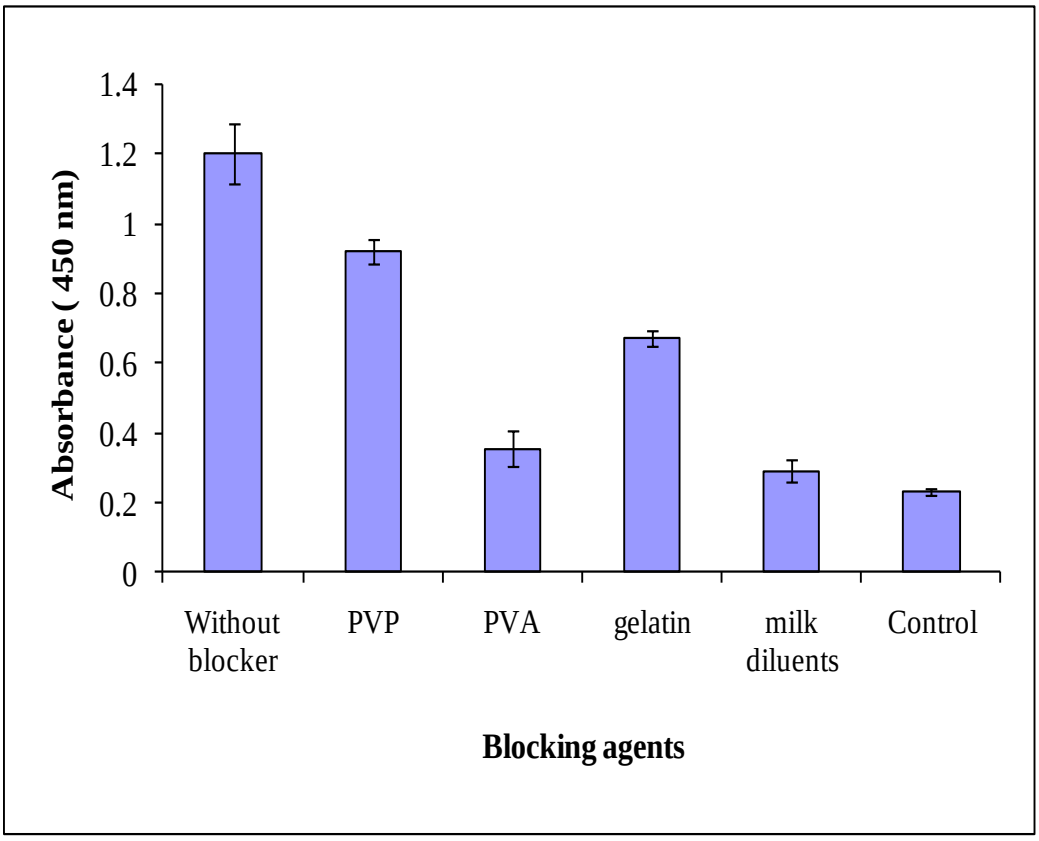


Figure 2.7: Effect of various blocking agents on absorbance reading by 1% PVP, 1% PVA, 1% gelatin and 1:20 milk diluents. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked, and followed by anti-IgG-HRP (1 µg mL^{-1}). The control was coated with AFB_1-BSA (1 µg mL^{-1}), without blocker and without anti-IgG-HRP. Error bars indicate =SD, n=3.

Figure 2.7 show that a high absorbance reading was obtained in the absence of a blocker, using PVP and gelatin. The presence of a high signal indicates a high amount of non-specific binding of the antibody or enzyme labelled conjugate on the surface as well as providing a high background reading in the system. According to Chen *et al*., (2006) high background readings may possibly be caused by the stickiness of the antibodies to the well surface. Chen *et al* also suggested that the addition of a blocking step after the first coating can eliminate or reduce non-specific binding on the surface (Chen *et al.*, 2006).

Figure 2.7 also shows that a low background reading similar to the control was also achieved by adding the milk diluents and PVA in the assay. This indicates that the

most efficient blockers are milk diluents and PVA in this assay. These results would provide an indication of the ability of the blocker to reduce non-specific binding of antibodies to the surface. The reducing of non specific binding on the surface will provide low background readings that enhance the signal/background (S/B) ratio for the assays. This phenomenon will present better specificity and sensitivity for the ELISA test developed in this work.

Milk diluents and PVA were chosen for further work to investigate which one is better as a blocking agent to the unoccupied sites on the well surface. The test was carried out to determine the ability of blocking agents in the competition immunoassay which is exposed to 0 and 100 ng mL^{-1} of free AFB_1 concentration. The results in Figure 2.8 show that at zero toxin concentration, a higher signal was obtained in the absence of a blocker and in the presence of PVA compared to milk diluents added. However, the results also found a high background reading in treatments of without blocker as compared to the presence of blockers, PVA and milk diluents. So, the different value of signal (zero toxin) over background (S/B) of each treatment of without blocker, by the adding of PVA and by the presence of milk diluents was 1.6, 4.06, and 2, respectively.

A high S/B value in the presence of PVA would give an indication of the ability to prevent non-specific protein adsorption, which may enhance the sensitivity of the assay. The use of milk diluents in this assay had an interference on the surface and this indicated that it may reduce the specific binding of antigen to the antibody site. The use of the milk diluents may have a tendency to deteriorate rapidly if not properly prepared and stored (unstable blocking agent) (Gibb, 1996). According to Gibb, milk diluents may also exhibit little cross reactivity with typical immunoassay components such as antibodies and enzymes (Gibb, 1996). In this case also, milk may absorb mycotoxins, which is why it is not suitable for mycotoxin assays (Parker, 2008).

From the two assayed blockers, PVA showed better results than milk diluents in use for this developed immunoassay. This is because PVA did not interfere in a great manner with the antigen–antibody reaction and, at the same time, did not increase the background signal. The addition of PVA as a blocking solution in competitive immunoassay reduced the non-specific protein adsorption on the micro-plate surface.

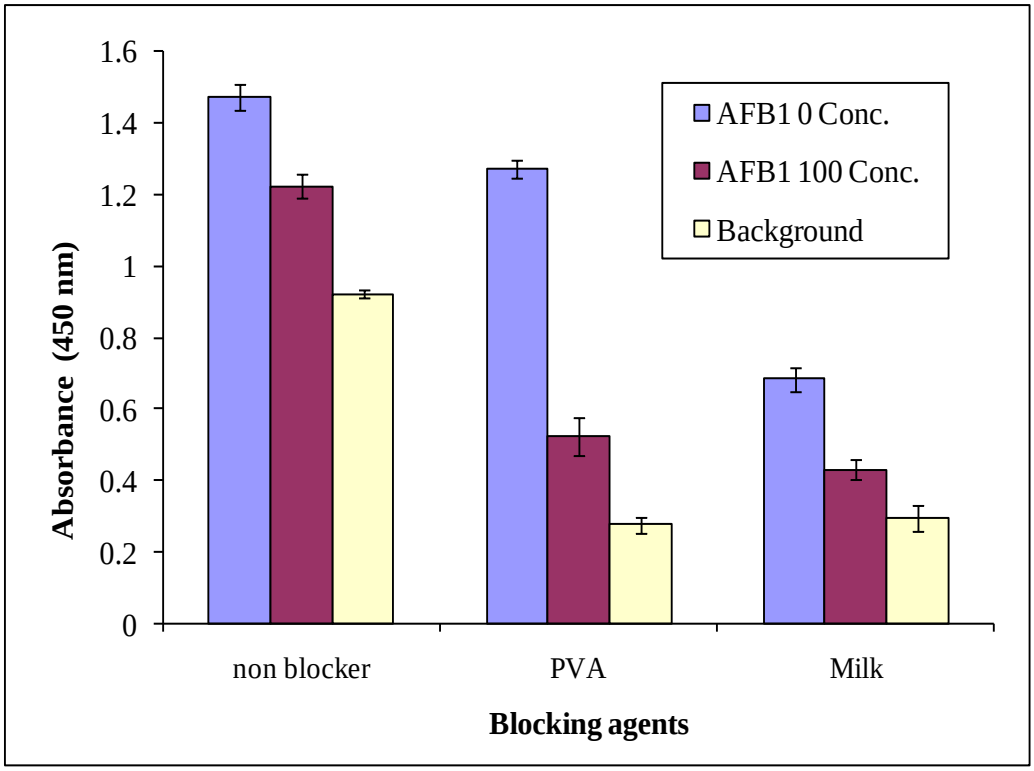


Figure 2.8: Effect of different blocking agents in the competitive method by 1% PVA and 1:20 milk diluents. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked, and followed by $MAbAFB_1$ (10 µg mL^{-1}) and free AFB_1 before continuing with anti-IgG-HRP (1 µg mL^{-1}). Background was conducted using AFB_1-BSA (1 µg mL^{-1}), blocked then with anti-IgG-HRP (1 µg mL^{-1}). Error bars indicate =SD, n=3.

The results also gave a wide signal difference between zero and 100 µg L^{-1} of AFB_1 concentration when the assay was treated with PVA compared to the others. These results suggest that a high dynamic range of calibration curve can be produced using this system.

2.3.2 Assay optimisation with free AFB_1

2.3.2.1 Effect of the sample volume on the assay

The next experiment with the optimal parameters was to create a calibration curve of AFB_1 concentrations from 0 to 100 µg L^{-1} with different volumes of reagents in the 96 Nunc Maxsorp microwell plate. In this competition step, the free AFB_1 and the

MAbAFB$_1$ was premixed in eppendorf tubes for 30 minutes at room temperature (25ºC) then transferred to the ELISA plate. This suggests that the specific binding of MAbAFB$_1$ and free AFB$_1$ will react first before it is added to the wells coated with AFB$_1$ conjugate. A volume of 100 and 50 µl of reagents in each step of the procedure was used to compare the sensitivity of the result. Normally, these volumes added on the ELISA microplate are commonly used in an ELISA system.

The result for both volumes used is presented in Figure 2.9. From the result, the absorbance reading was decreased with the increasing of the AFB$_1$ concentration for both effects. The response also showed a low background reading was found. This showed that with 100µl and 50µl volumes of reagents, a high affinity of specific binding interaction with a low one of non-specific binding on the well surface was achieved. The percentage of relative binding (%B/B$_0$) (based on absorbance reading) of low to high AFB$_1$ concentration was found slightly similar for both volumes by 97% (highest) to 30 % (lowest), respectively. This observation suggests that 50µl could be used for further experiments and can be applied in 96 Nunc Maxsorp microwell plates that may be allowing less use of expensive reagents.

The maximum permissible limit for AFB$_1$ in foodstuffs is 1–20 µg L^{-1} in more than 50 countries including the EU. The ranges of detection limit for the calibration curve have to include that level. To obtain a better calibration curve, additional work was carried out as described below.

2.3.2.2 Effect of incubation times in the competition steps

One of the most important parameters to influence the sensitivity of the assay is the incubation times. After a 30 minute premix of the competition step, the plate was incubated at different times by 1, 30, 60, and 90 minutes at 37ºC. The purpose of this experiment was to investigate the optimal time needed to complete the competition reaction to achieve a sensitivity of the assay.

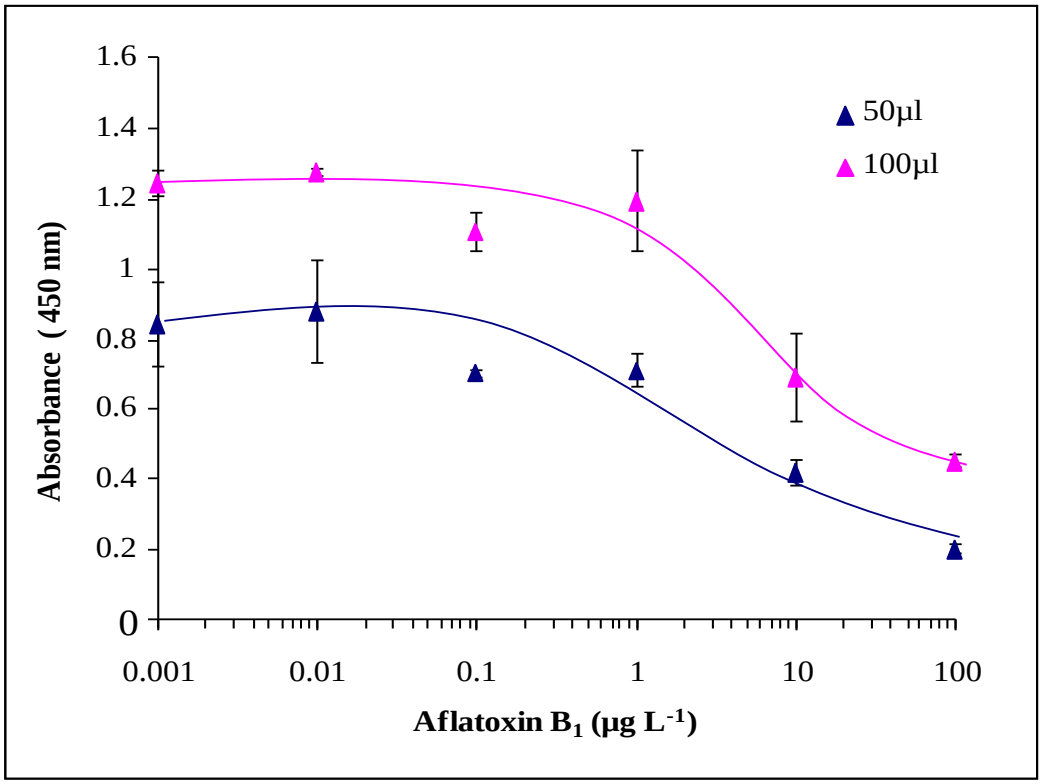


Figure 2.9. Calibration curves by competitive ELISA for AFB_1 with different volumes of reagent in the well plates. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (1 µg mL^{-1}). Error bars indicate=SD, n=3.

Figure 2.10 shows that the signal obtained increased with the increase in the incubation time. However, the noise with high SD also increased with the increase in the incubation time. The data, from 30 to 90 minutes show that there is a detectable difference in absorbance reading between low and high concentration of AFB_1. For these values, 30 minutes produced a low signal compared to 60 and 90 minutes. A high background reading was obtained at 90 minutes incubation and the signals became unstable and flattened. The sensitivity of the assay may be increased with a longer incubation time at 37°C, but the top of the standard curve may flatten out and become unstable, limiting the assay range and may increase the background reading. As an experiment the best range was achieved at 60 minutes of incubation and this was therefore chosen to continue the investigation.

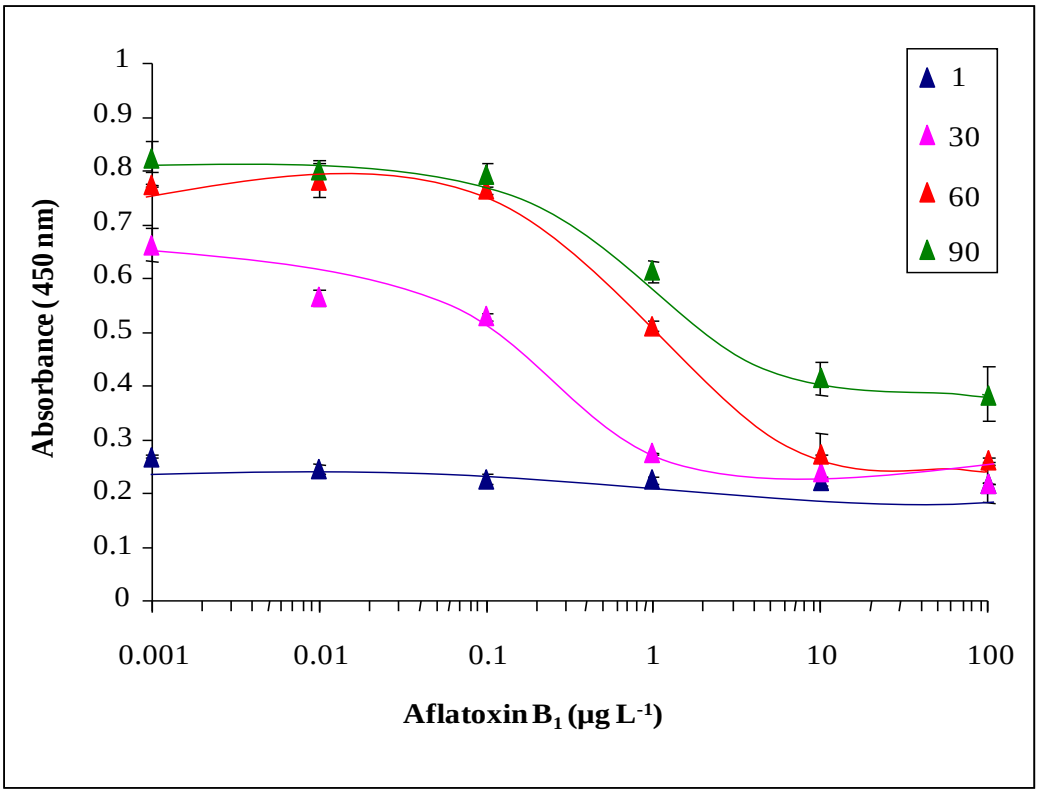


Figure 2.10. Effect of incubation times from 1 to 90 minutes at 37ºC in competition step of indirect format for AFB_1 detection. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (1 µg mL^{-1}). Error bars indicate=SD, n=3.

2.3.2.3 Effect of different concentrations of anti-aflatoxin B_1 antibody ($MAbAFB_1$)

Three different concentrations (5, 10 and 50 µg mL^{-1}) of a specific antibody against the AFB_1 ($MAbAFB_1$) were tested in the indirect competitive assay to determine the sensitivity of the assay. The competition tested was determined in the presence of a fixed concentration of AFB_1-BSA (1 µg mL^{-1}) immobilised and anti-IgG-HRP (1 µg mL^{-1}) labelled. The $MAbAFB_1$ dilution on the titration curve was selected as the concentration, which induced approximately 100% (50 µg mL^{-1}), 75% (10 µg mL^{-1}) and 50% (5 µg mL^{-1}) of the A_{450} of plateau (Figure. 2.6), using a similar method to the one described elsewhere by Singh *et al.*, (2004). The result of the studies is presented

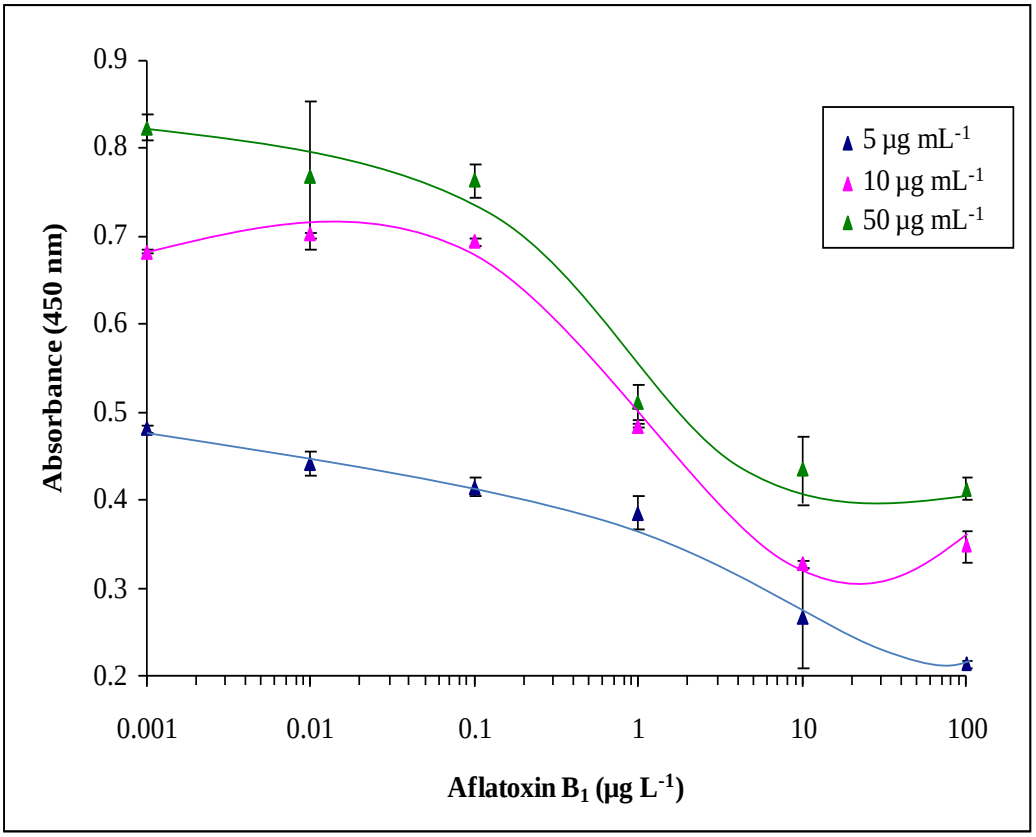


Figure 2.11. Optimising the concentration of 50, 10 and 5 µg mL^{-1} anti-aflatoxin antibody ($MAbAFB_1$) in the presence of the free aflatoxin B_1 for AFB_1 calibration curve. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (5 to 50 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (1 µg mL^{-1}). Error bars indicates=SD, n=3.

in Figure 2.11. For the detection of AFB_1 in the range of 1 to 20 µg L^{-1} (range limit from legislative), the graph must be able to distinguish between 1 µg L^{-1} or lower concentrations.

The result for all of the plots in Figure 2.11 showed that the range level present was from 0.1 to 10 µg L^{-1}. This shows that the use of 5 to 50 µg mL^{-1} concentration of $MAbAFB_1$ in this system can provide a detection limit of less than 1 µg L^{-1}.

On the basis of the understanding that a lower amount of antibody may cause a greater competition between the free AFB_1 and the AFB_1-BSA conjugate as observed by Sapsford (2006), however, the signal for 5 µg mL^{-1} of $MAbAFB_1$ shows low absorbance reading and this may provide less sensitivity. However, a 50 µg mL^{-1} was

observed to increase the signal and also increase the background reading. The use of a concentration of 10 µg mL^{-1} $MAbAFB_1$ in this system still shows a high signal, a high dynamic range with the low non-specific binding to reach a detection range of 0.1 to 10 µg L^{-1}. In terms of cost, 50 µg mL^{-1} would mean a high cost in the assay system. Therefore, a 10 µg mL^{-1} was chosen for further investigation.

2.3.2.4 Effect of different concentrations of anti-IgG-HRP

A 10 µg mL^{-1} was used for competitive studies with varying concentrations of anti-IgG-HRP (5, 1 and 0.5 µg mL^{-1}) present in the labelling step. This study investigates the optimal amount of this antibody binding in the competitive assay. As shown in Figure 2.12, the signal increased when increasing the concentration of anti-IgG-HRP. The plots produced similar results in the range detection of AFB_1 concentration: whenever 5 µg mL^{-1} of the enzyme label was used, the signal obtained was higher compared to 1 and 0.5 µg mL^{-1}. However, using a concentration of 0.5 and 1.0 µg mL^{-1} for the antibody would seem preferable with a low background reading.

The optimal amount of specific anti-IgG-HRP to the $MAbAFB_1$ bound AFB_1 during the competition and detection step was 1.0 µg mL^{-1}. The concentration was chosen based on providing a standard curve of a high signal, high dynamic range with the low non-specific binding to reach a detection range of 0.1 to 10 µg L^{-1}.

The data from Figures 2.10, 2.11 and 2.12 were observed and summarised in Table 2.3. The summary shows the selected concentration of reagents after optimisation in the presence of AFB_1 and using AFB_1-BSA, anti-aflatoxinB_1 antibody (mouse monoclonal antibody against AFB_1), anti-antibody labelled with HRP (goat anti-mouse IgG conjugate with horseradish peroxidase). Another factor, incubation times, was also found to have an effect on the sensitivity of spectrophotometric assay. The optimal incubation time in the competition step was attained after 60 minutes incubation at 37ºC.

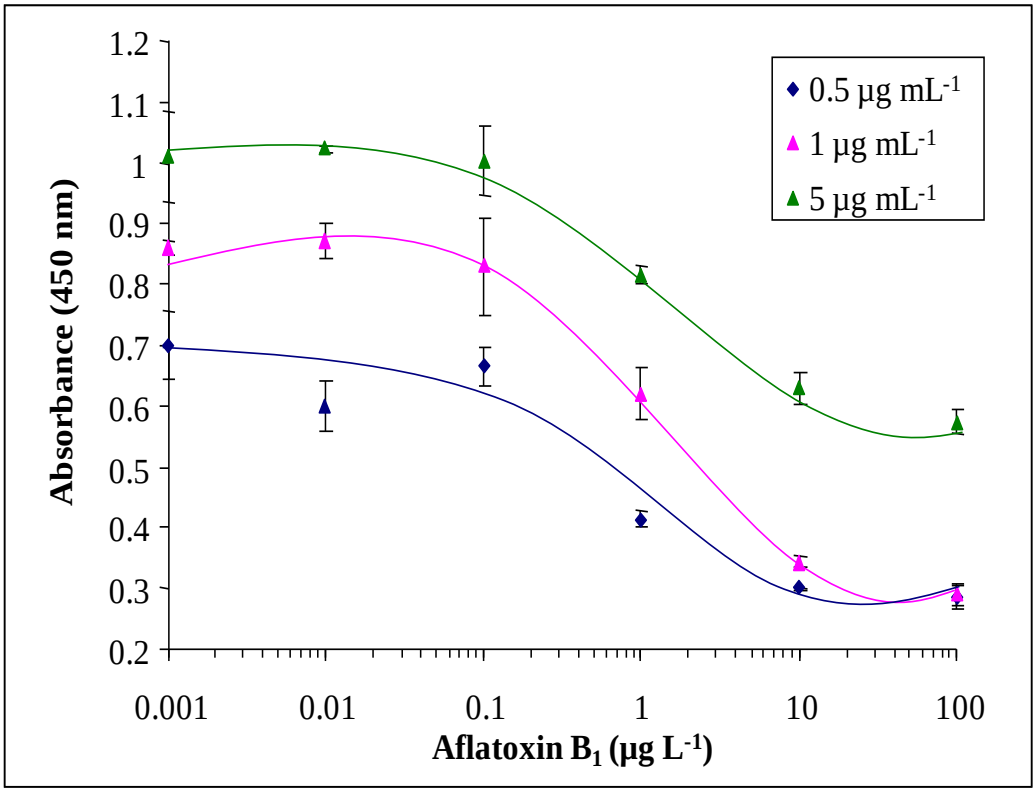


Figure 2.12: The effect of different concentrations of anti-IgG-HRP in the presence of free AFB_1. Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (0.5 to 5 µg mL^{-1}). Error bars indicate=SD, n=3.

Table 2.3. Selected concentration of the different reagents used in the indirect format of the spectrophotometric immunoassay for aflatoxin B_1

Reagents	Concentrations	Incubation time
AFB_1-BSA conjugate	1.0 µg mL^{-1}	Overnight (12 h), 4 ºC
Anti-aflatoxin B_1 antibody	10 µg mL^{-1}	1.5 h, 37 ºC
Anti-antibody-HRP	1.0 µg mL^{-1}	1.0 h, 37 ºC

One of the critical parts that may cause a high background reading in the assay is high non-specific binding on the wells surface, which may be from antibody conjugated enzyme binding. With a minor modification, the buffer solution of anti-IgG-HRP was mixed with 1% of PVA to avoid high non-specific binding in the wells. The best

result of the calibration curve of AFB_1 was achieved following the procedure described above, as shown in Figures 2.10, 2.11 and 2.12. Based on the assay sensitivity, stability of reagents and optimum conditions, the indirect ELISA assay will be transferred to the electrochemical immunosensor that considerable potential in sensitive detection to achieve better sensitivity and lower detection limit (Huang *et al*., 2008).

2.3.3 Direct ELISA format

The development of the spectrophotometric ELISA using the direct competitive method was also studied to compare the sensitivity of the detection limit for AFB_1 analysis. The experiment was performed based on a competition between added free AFB_1 and AFB_1 labelled with the enzyme for the binding sites of antibodies coated on the wells. The checkerboard system was not applied in this experiment because of the small volume (limited) of reagents (AFB_1-HRP conjugate from Ridascreen kit) supplied to be used in the test. The assay set up and development were carried out as described by Kolosova *et al.,* (2006) and Lee and Rachmawati (2006).

2.3.3.1 Optimisation of $MAbAFB_1$ and AFB_1-HRP without free AFB_1

The mouse anti-aflatoxin B_1 antibody (monoclonal antibody against AFB_1) from Abcam (UK) was selected for incorporation and development into an ELISA. This specific antibody was performed similarly in the indirect competitive method as described above. The first step in the assay development was the selection of the immobilisation reagent ($MAbAFB_1$) by the incubation of increasing concentrations of $MAbAFB_1$ in the carbonate buffer pH 9.6 for 2 hours at 37ºC. A fixed amount of AFB_1 labelled with enzyme (AFB_1-HRP) was used (from ELISA kit, Ridascreen). The dilution ratio of AFB_1-HRP is 1:3, followed the Ridascreen kit method. The results show a very low signal response. However, the signal alteration was dramatically increased from 5 to 50 µg mL^{-1} (Figure 2.13). From the graph, the optimal signal was obtained for the range 10 to 50 µg mL^{-1}. This show that 20 µg mL^{-1} is the best optimal response chosen for further experiments.

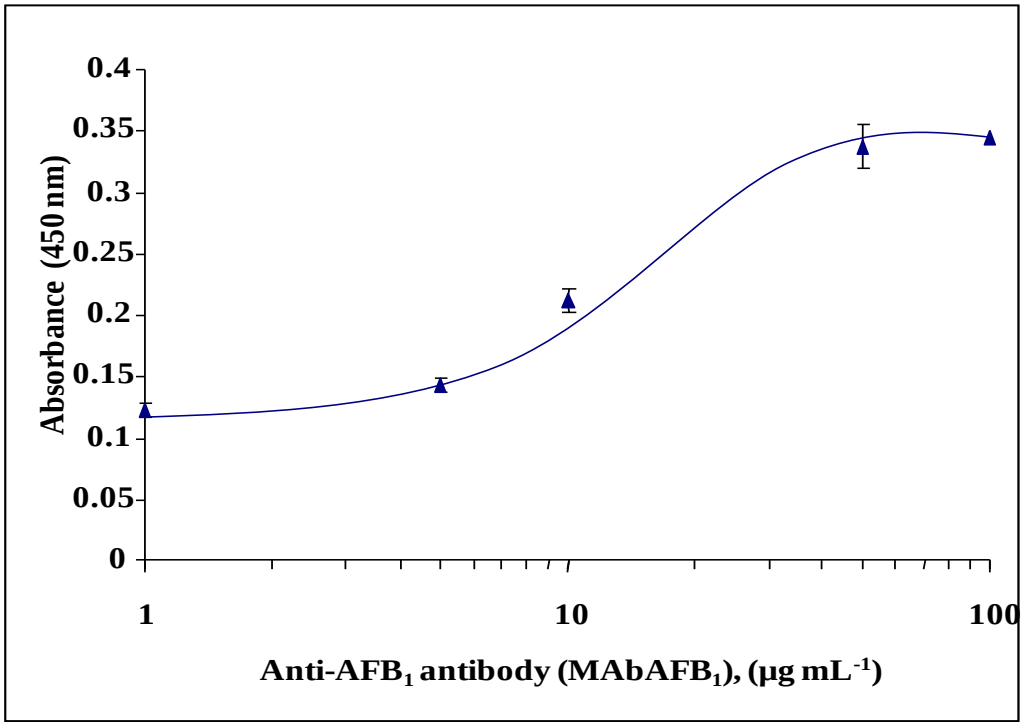


Figure 2.13: Optimisation of a different concentration of anti-AFB_1 antibody (monoclonal antibody) coating. Well plates were coated with anti-aflatoxin B_1 ($MabAFB_1$) (0 to 50 µg mL^{-1}), blocked with 1% PVA then aflatoxin B_1-HRP from Ridascreen kit (1:3). Error bars indicates=SD, n=3.

With the same application of an enzyme labelled antigen from the Ridascreen kit, Micheli *et al*., (2005) also found a very low absorbance reading, in the range of 0.06 to 0.25 OD. In particular, changing the coating buffer with PBS and the incubation time to overnight at 4ºC produced no difference in the signal reading and alteration (data not shown).

The optimisation of the binding assay was continued by investigating several dilutions of AFB_1-HRP conjugated with a fixed immobilised $MAbAFB_1$ (20 µg mL^{-1}) into the microplate well for 2 hours at 37ºC. Figure 2.14 shows the signal response of different dilution concentrations of AFB_1-HRP binding to the immobilised $MAbAFB_1$. The signal was slightly increased with the increasing of dilution factors. The increasing of dilution factors may reduce the interference or preventing of specific binding antibody and antigen. This phenomenon may be due to the use of an unknown solution of AFB_1-HRP from the kit that may give a high interference of specific binding.

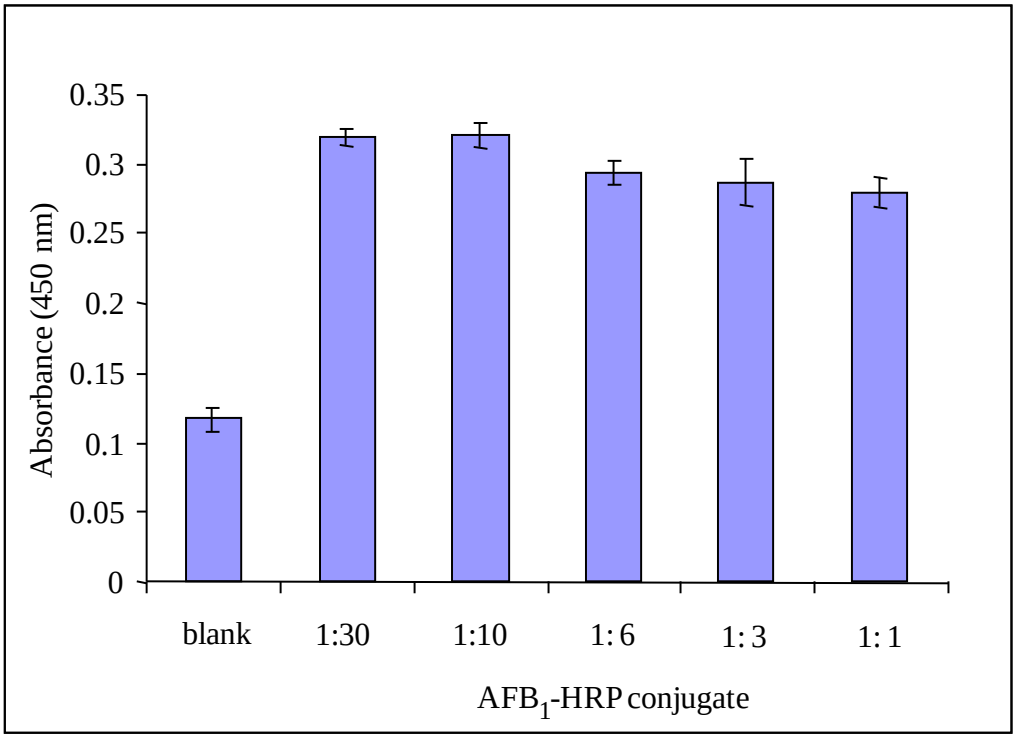


Figure 2.14. Optimisation of decreasing dilution of enzyme labelled toxin (AFB_1-HRP). Well plates were coated with anti-aflatoxin B_1 antibody ($MAbAFB_1$) (20 µg mL^{-1}), blocked with 1% PVA then AFB_1-HRP from Ridascreen kit (1:30 to 1). Blank was using anti-aflatoxin B_1 ($MAbAFB_1$) (20 µg mL^{-1}), blocked with 1% PVA without AFB_1-HRP. Error bars indicates=SD, n=3.

If the reagents solution contains additives such as the blocking reagents (PVA or BSA), the ELISA will produce a low signal. (Kolosova *et al*., 2006). In this case, a 1:10 dilution was chosen for further tests because it appeared more reliable compared to the other dilutions.

2.3.3.2 Optimisation of anti-IgG (mouse) unconjugated without free AFB_1

The different concentrations of anti-IgG were immobilised on the wells for overnight incubation at 4 ºC. After that, the assay was continued with a saturated amount of coating $MAbAFB_1$ and AFB_1-HRP for the binding site of antibodies immobilised on the wells by 20 µg mL^{-1} and 1:10 dilution, respectively.

From the results shown in Figure 2.15, the signal was raised with the increasing of anti-IgG concentration and saturated with a 50 µg mL^{-1} immobilised. A maximum reading was present at 10 µg mL^{-1} and chosen for the amount of pre-coated of assay. When compared to the control, the signal clearly showed an increase of about 35%. This suggests that with the addition of the anti-IgG pre-coating step in the system, the specific binding improved via better orientations and good coverage of the primary antibody.

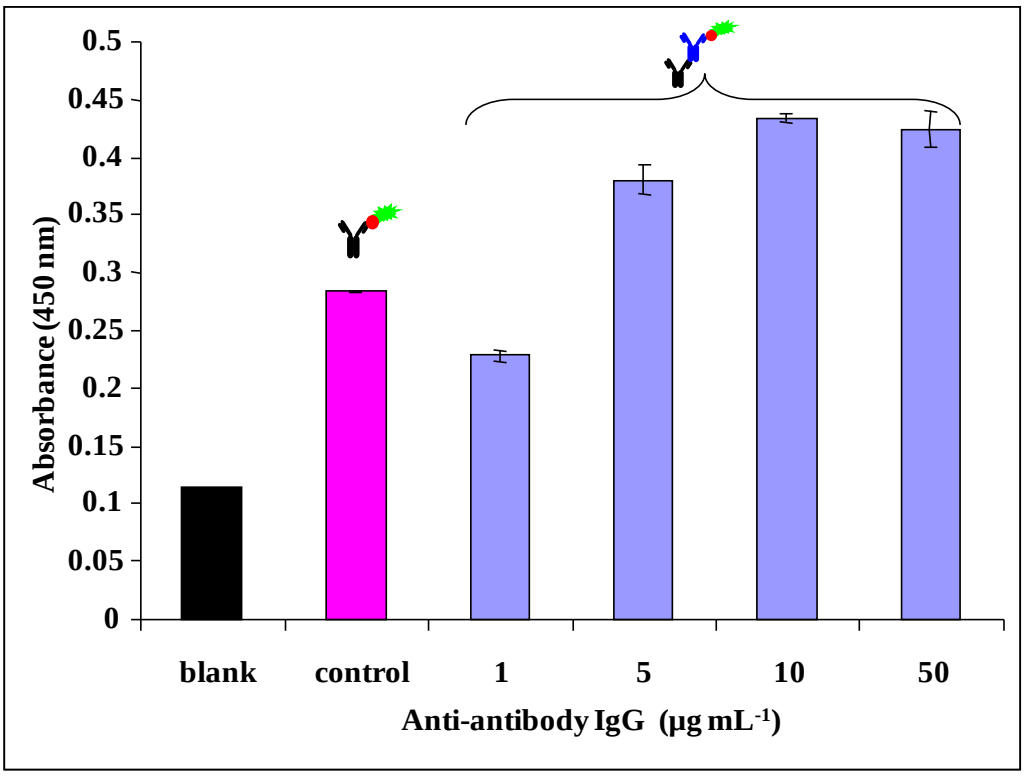


Figure 2.15. Precoating study: comparison between the results obtained with and without pre-coating and optimisation of different concentrations of anti-IgG immobilization in the wells. Well plates were coated with anti-IgG (1 to 50), blocked with 1% PVA then anti-aflatoxin B_1 antibody ($MAbAFB_1$) (20 µg mL^{-1}) before continuing with AFB_1-HRP (1: 10). Blank was only using blocking solution 1% PVA and AFB_1-HRP. Error bars indicate=SD, n=3.

Micheli and co-workers also used the anti-IgG antibody on the well surface (precoating) and produced an increment of about 60% of binding (Micheli *et al.*, 2005). However, there was only a 10 to 20% signal increase when anti-mouse IgG was first immobilised in the direct immunoassay for ochratoxin detection (Alarcon *et al.*, 2006). Therefore, pre-activation of the well surface with immobilisation of anti-

IgG will be used for further investigation. A 10 µg mL^{-1} of anti-IgG concentration was chosen because it obtained a reasonable optimal signal.

The optimal dilution/concentration and condition of difference reagents for direct format are reported in Table 2.4.

Table 2.4. Optimal dilution/ concentration and condition of the different reagents in the direct format of ELISA for aflatoxin B_1

Reagents	Dilution/Concentration	Incubation times
Anti-mouse IgG	10 µg mL^{-1}	Overnight (12 h), 4ºC
Anti-aflatoxin $_1$ antibody	20 µg mL^{-1}	2 h, 37 ºC
Aflatoxin B_1 labelled with HRP	1: 10 dilution	1 h, 37 ºC

2.3.3.3 Calibration curve of AFB_1

After optimisation of the reagents in the selected buffer and time incubation, the best assay conditions were achieved when 10 µg mL^{-1} of anti-IgG in the carbonate buffer pH 9.6 was incubated overnight at 4ºC. Furthermore, 20 µg mL^{-1} of $MAbAFB_1$ (primary monoclonal antibody) in PBS pH 7.4 was added and incubated for 2 hours at 37ºC. The competition step was carried out by adding several concentrations of free AFB_1 (0 to 100 µg L^{-1}) onto the wells for 10 minutes at room temperature (25ºC) before the addition of 1:10 of AFB_1-HRP dilution. The free AFB_1 (unlabelled) and AFB_1-HRP competes for $MAbAFB_1$ in the ELISA plate.

From Figure 2.16, a non linear calibration curve was obtained with the absorbance reading being reduced with the increasing concentration of AFB_1 standard solution in buffer. This result shows that higher signals were steadily obtained from 0 to 0.1 µg L^{-1}, then dramatically decreased and declined steadily after a 10 µg L^{-1} concentration of AFB_1.

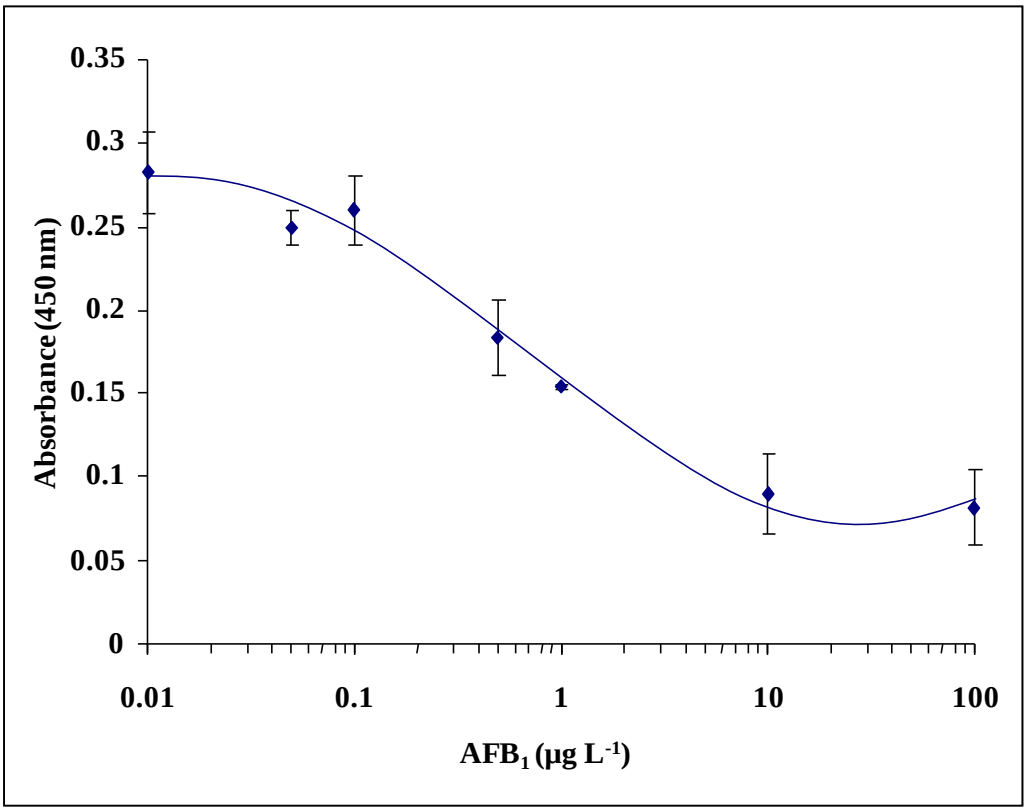


Figure 2.16. Non linear calibration curve of AFB_1 with immobilisation of pre-coating (anti-IgG) in direct competitive method. Well plates were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA then anti-aflatoxin B_1 antibody ($MAbAFB_1$) (20 µg mL^{-1}) before continuing with competition AFB_1-HRP from Ridascreen kit (1: 10) and free AFB_1 (0 to 100 µg L^{-1}). Error bars indicate=SD, n=3.

The high signal means, the high amount of labelled AFB_1 bound to $MAbAFB_1$ coated on the wells, which is without or less amount of free AFB_1 bound (0.1 µg L^{-1} below). The result also show (Figure 2.16), that the high amount of free AFB_1 standard in solution showed the low of signal present. This means, the less of labelled AFB_1 and high concentration AFB_1 bound to $MAbAFB_1$ sites on the plate. Therefore, from the observation, the working range was 0.1 to 10 µg L^{-1}, which is in the range of the maximum level of AFB_1 (1 to 20 µg L^{-1}).

2.3.4 Sensitivity of the immunoassay development

Both of the formats were further repeated using optimal concentrations and conditions as mentioned in Tables 2.1 and 2.2. All the reagents were also freshly prepared to achieve better specificity and sensitivity. The result from the calibration curves obtained is shown in Figure 2.17. The maximum absorbances were found to be at around 0.7 and 0.3 unit for indirect and direct respectively. In this case, the maximum signal of the direct assay was lower than the indirect one.

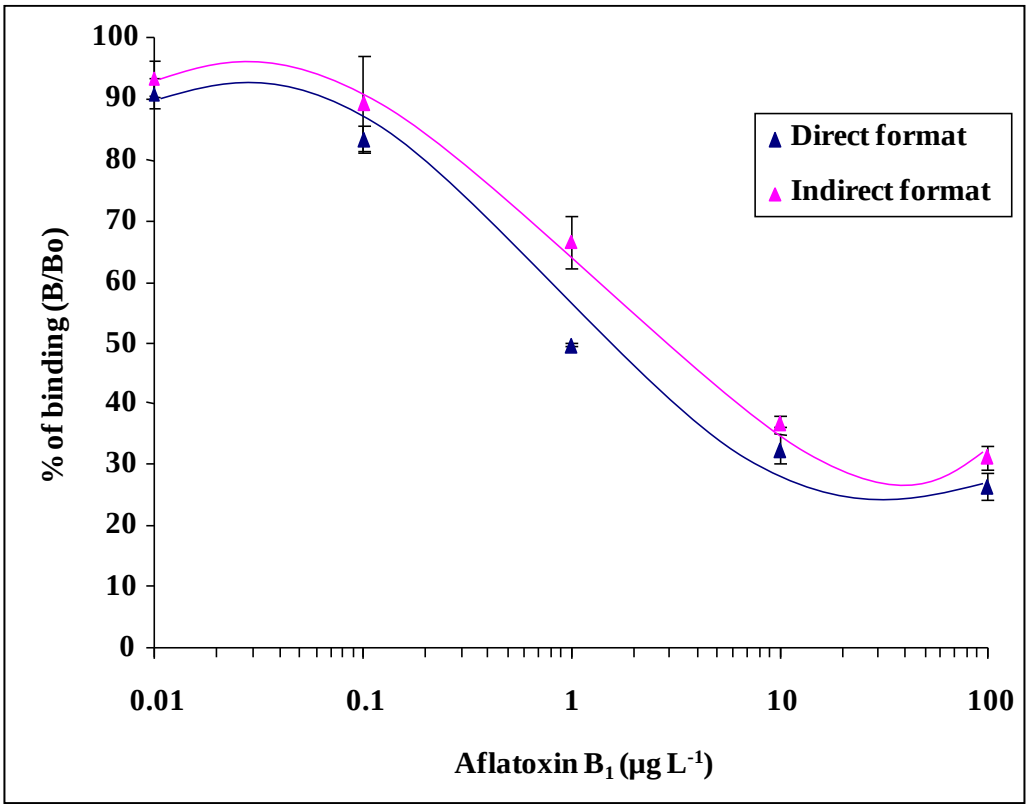


Figure 2.17: Calibration curves of AFB_1 for indirect and direct format using spectrophotometric detection. Indirect: Wells were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by $MAbAFB_1$ (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti IgG-HRP (1 µg mL^{-1}). Direct: Wells plate were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA then anti-aflatoxin B_1 antibody ($MAbAFB_1$) (20 µg mL^{-1}) before continuing with competition AFB_1-HRP from Ridascreen kit (1: 10) and free AFB_1 (0 to 100 µg L^{-1}). Error bar = standard deviation, n=3, %CV= 9 and 7.4 for indirect and direct, respectively.

However, when the absorbance values were converted to percent of relative binding (% B/B_0), they were about 95 – 35 % for indirect and 91 – 27 % for direct (Figure 2.17). For the indirect assay, a linear range from 0.1 to 10 µg L^{-1} with R^2 value of 0.9986. The EC_{50} value, the point at which 50% competition occurs (from a linear range), the value obtained was 0.97 µg L^{-1}. The limit of detection was determined to be 0.08 µg L^{-1}. For the direct method, the corresponding assay using a precoating antibody exhibited a linear range from 0.1 to 10 µg L^{-1}, where R^2 value is 0.9946 and EC_{50} and LOD of 1.07 and 0.09 µg L^{-1}, respectively. The LOD for both systems in ELISA is in the range of maximum level of AFB_1 (1 to 20 µg L^{-1}). Because of there being no significant difference obtained on the sensitivity of the assay, both systems can be used for AFB_1 analysis.

2.4 Conclusions

Indirect and direct ELISA methods using monoclonal antibodies have been developed. Both of the assays were optimised through changing reagents concentrations. Both assays were able to detect AFB_1 to less than 1.0 µg $L^{-1,}$ which meets the legislative limits imposed by the European Union and most other countries around the world. Optimal reagent concentrations and assay conditions obtained are summarised in Tables 2.1 and 2.2, for the indirect and direct competitive assay, respectively.

For the indirect method, a working range of 0.1 to 10 µg L^{-1} was achieved by optimal concentration of coating AFB_1-BSA, antibody binding $MAbAFB_1$ and followed by Anti-IgG-HRP. The choice of blocking agent is also of vital importance for the signal/background ratio, thus the PVA block generally resulted in a better S/B ratio than the milk diluent. In the case of the direct method, the application of pre-coating the adsorption surface using anti-IgG unconjugated showed an increase in the signal response of the assay. The ELISA resulted in a working range of 0.1 to 10 µg L^{-1}.

The assay developed here can now be transferred to the sensor surface. The performance on the SPGE will still need to be investigated.

CHAPTER 3

DEVELOPMENT OF ELECTROCHEMICAL IMMUNOSENSOR USING GOLD WORKING ELECTRODE FOR AFB_1 DETECTION

3.1 Introduction

In chapter 2, the development of an ELISA method for aflatoxin B_1 was reported. However, to move the method to a more portable system this method was transferred to the surface of an immunosensor. The possible advantages of an immunosensor over conventional immunoanalysis methods are: an increase in sensitivity and decrease in detection limit; a decrease in the amount of expensive reagents; simplification of the analysis procedure (fewer stages); and miniaturisation of equipment and automation (Tothill, 2003).

3.1.1 Electrochemical Immunosensor

The research in this chapter focuses on the development of AFB_1 sensing techniques based on immunosensor systems. The adopted approach is based on an electrochemical sensor platform fabricated by using screen-printed gold electrodes (SPGE) combined with specific competitive immunoassay methods. The use of electrochemical sensing presents the advantages of high sensitivity of an electrochemical transducer and selectivity inherent to the use of immunochemical interaction. In this study, the screen-printed gold electrodes fabricated from thick film technology were used as the transducer of the electrochemical sensing. Then the electrodes were characterised by the cyclic voltammetry of the electrochemical technique.

In this work, the competitive immunoassay format was applied. The detection was facilitated by labelling a secondary antibody or AFB_1 with Horseradish Peroxidase (HRP) and chronoamperometric detection was carried out by constant potential versus Ag/AgCl (reference electrode). The mediator 3,5,3',5'-tetrametilbenzidine (TMB) hydrogen peroxide (H_2O_2) substrate system was used for the amperometry determination of HRP activity to monitor the AFB_1 concentration.

3.1.2 Immuno-gold Nano-particles Application

The availability of gold nano-particles with unique properties for the construction of an immunosensor has helped in providing better ability and more chances in the orientation of the immobilised biomolecules (Mirkin *et al*., 2007, Airwal and Mitra, 2009). In recent years, gold nano-particles have been used extensively in immunoassay development and they are able to interact strongly with biomolecules (Yang and Wang, 2009). However, the improvement of functionalised nano-particles on sensor surfaces to achieve better sensitivity compared to conventional electrochemical immunosensors is still under investigation until today.

In this study, the development of an electrochemical nano-biosensor for the detection of AFB_1 was investigated. The AFB_1-BSA conjugate was immobilised on the surface of the screen-printed gold working electrodes (SPGE) by passive adsorption. Subsequently the competition between the free AFB_1 in the buffer/sample and the immobilised AFB_1 was carried out in the presence of gold nano-particles conjugated to the monoclonal anti-AFB_1 antibody and HRP on surface. While the detection is facilitated by the labelling of immuno gold nano-particles with the enzyme horseradish peroxides (HRP) the detection is then conducted using an amperometric system via a mediator.

The activity of HRP can be measured by determining the reduction current generated by $TMB_{(ox).}$ The concentration of $TMB_{(ox)}$ produced is proportional to the activity of HRP (He *et al*., 2002). This developed technique is required in order to be able to detect the toxin at low levels (ppb or ppt range) and to comply with the legislative limit analyses.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

The following is a description of the materials that were utilised during the competitive immunosensor experiments. Most of these materials used, and referred to

in this chapter, such as reagents, working solution, blocking agents, buffers, and chemicals were similar to those discussed in Chapter 2 (2.2). Other chemical reagents, which are 3,3-dithiodipropionic acid (DTDPA) and N-hydroxysuccinimide (NHS), 30% hydrogen peroxide (H_2O_2), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (powder), potassium chloride, and potassium ferrocyanide were purchased from Sigma-Aldrich Co. Ltd (Gillingham, United Kingdom (UK)). 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC) was purchased from Pierce Ltd. UK. An average diameter of 20 nm gold colloid (gold nano-particles) with the number of particles being about 7 x 10^{11} particles per mL were purchased from BBInternational, Cardiff, UK.

3.2.2 Buffer and Solution

The working buffers were the following: A 0.1 M carbonate buffer (CB), pH 9.6 was prepared for the immobilization of AFB_1-BSA and anti-IgG unconjugated (precoating) on electrodes. A 0.01 M phosphate buffered saline (PBS), pH 7.4 (used for preparation of AFB_1 standard, blocking solution, dilution of antibodies and washing solution) was purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). A phosphate citrate buffer (PCB), pH 5.0 was used in the preparation of TMB solution which was also obtained from Sigma-Aldrich.

The AFB_1 standard solution (in acetonitril) was prepared by diluting the stock solution with PBS. A 0.1 M Kalium chloride (KCl) and 0.05 M phosphate citrate buffer, pH 5.0 solution was prepared for the preparation of a substrate of 5 mM TMB solution and 0.075% Hydrogen peroxide (H_2O_2). A 3% amount of H_2O_2 was prepared in a citrate phosphate buffer, while the TMB chromogen was prepared by dissolving 1 mg in 150 µl of distilled deionised water.

A washing solution was prepared by adding 0.05% Tween-20 (v/v) to the PBS (PBS-T). All organic solvents (methanol and acetonitrile) and water for the immunoassay and immunosensor analysis used were HPLC grade and purified water (deionised) was obtained from a Millipore system.

3.2.3 Apparatus

Incubations for every step of the reactions were carried out by Labsystem iEMS incubator/shaker HT. Electrochemical measurements were performed using AUTOLAB model PGSTAT with multichannel GPES software was from Eco Chemie, Utrecht, Netherlands).

3.2.4 Screen-Printed Gold Electrode (SPGE)

Two types of SPGE were used in this experiment, and the first was fabricated in house by a multi-stage screen printing process using a DEK model 248 machine. The second was fabricated by using the printing facilities at DuPont Ltd, UK. The electrodes were screen-printed, based on a gold working electrode, a silver/silver chloride pseudo-reference electrode and a carbon counter electrode.

3.2.4.1 Preparation of SPGE fabricated in house

The three electrodes (working, reference and counter) were fabricated by printing onto polyester sheets (250 µm thick). The parameters used for printing were set at a 4 psi squeegee pressure, a carriage speed of 50 mm sec^{-1} with a 2.5 mm print gap. The Electrodag 423-SS graphite ink from Acheson Industries (UK) were first printed onto the polyester sheet surface (basal track) (Figure 3.1, A). Then the auxiliary electrode was also printed using the same ink (Figure 3.1, B). For the third step, the reference electrode was printed by Electrodag 6037-SS silver/silver chloride ink onto one of the basal tracks (right track) (Figure 3.1, C). Then gold ink R-464 (DPM-78) obtained from Ercon (USA) was deposited as the working electrode (Figure 3.1, D). The last step was printed using blue epoxy insulating ink (242-SB protective polymer) which was obtained from Agmet ESL (Reading, UK) (Figure 3.1, E). Between each layer the sheets were allowed to dry in an oven at 60ºC for 2 hours and then after that, the insulating layer electrode was cured at 120ºC for 2 hours and later left to dry. Figure 3.1 shows the completed design of the screen-printed gold electrode produced in house where the diameter size of the working electrode is 5 mm.

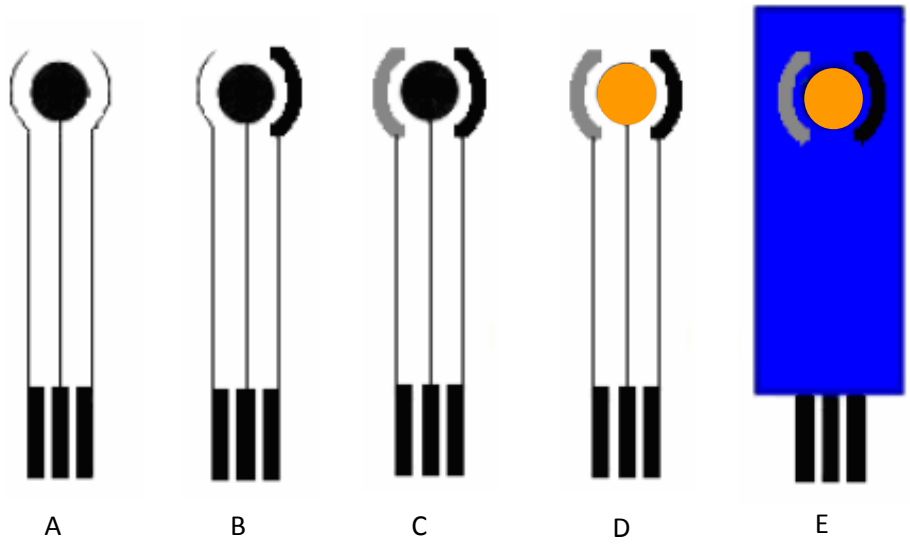


Figure 3.1: The screen-printed gold electrode fabrication process. A) The basal track, B) counter electrode, C) Ag/AgCl reference electrode, D) the gold working electrode, and E) completed screen-printed gold electrode with the final addition of the blue epoxy insulating ink.

A new electrode design was fabricated using the DuPont fabrication facilities (Bristol, UK). The printing pastes used were 107255-135E carbon, BQ331 gold, 5874 Ag/AgCl and 5036 blue encapsulant, and all inks were from DuPont Ltd. (Figure 3.2). The SPGE used in this work consisted of a gold working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area, printed on a graphite ink layer (dried at 120 °C, 30 mins) (Kadir & Tothill, 2010).

All electrodes were then tested using a multimeter before use. The sensors' edge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK). Before each experiment, electrodes were baked at 105ºC in the oven for 30 minutes and then cooled to room temperature before being cleaned with deionised water to remove any remaining interference particles.

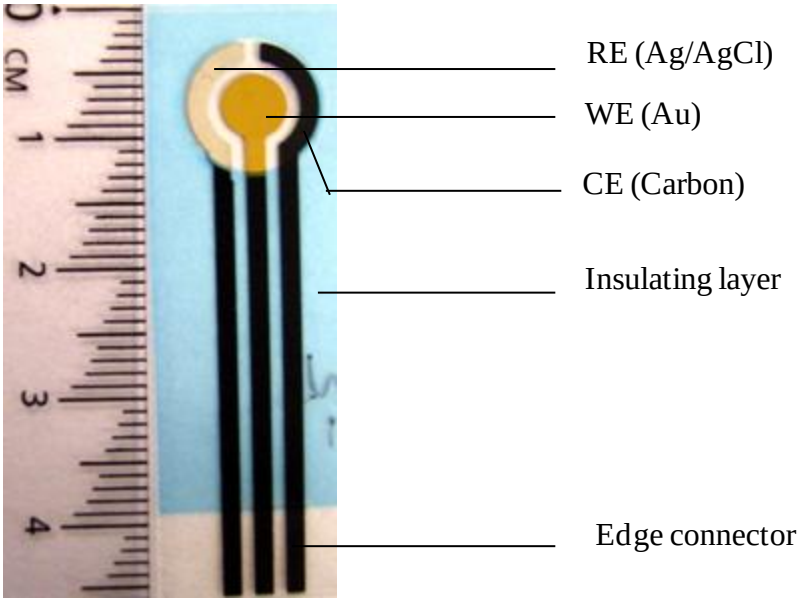


Figure 3.2: Screen-printed gold electrode fabricated using the DuPont fabrication facilities. Note: RE = reference electrode; WE = working electrode; CE = counter electrode.

3.2.5 Electrochemical Measurement

A computer was used to control the output of the Eco Chemie Autolab electrochemical analyser multipotentiostat (Eco Chemie, The Netherlands) and four connector electrodes were used for the electrochemical measurements. This machine provides four cells to connect to the 4-outlet edge connector for measuring the signal simultaneously at the same time. Then, the carbon basal track of the SPE was inserted into a 4-way edge connector. Four electrodes can be fitted into the edge connector and be monitored simultaneously. The data acquisition was recorded via the supplied GPES version 4.9 software to a PC. Figure 3.3 shows a photo of the Autolab apparatus and the electrodes connector for electrochemical measurement.

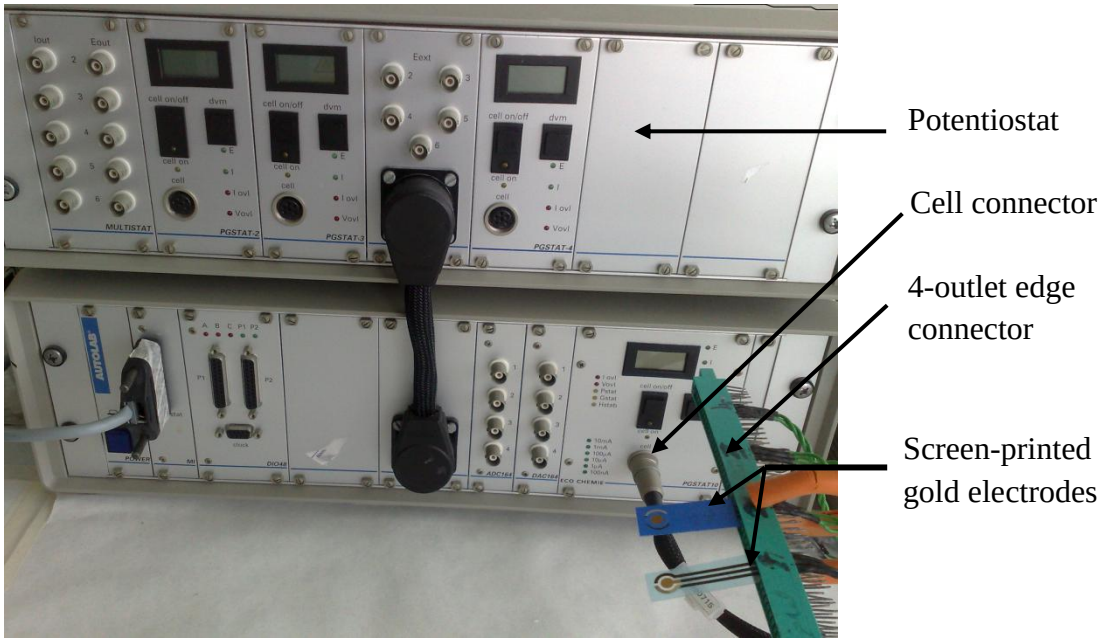


Figure 3.3: The Autolab electrochemical analyser in this work.

3.2.5.1 Voltammetric studies for characterisation of SPGE

Cyclic voltammetry (CV) and chronoamperometry were carried out using an Eco Chemie Autolab electrochemical analyser multipotentiostat at room temperature (25ºC).

Cyclic voltammetry (CV)

The characterisation of the gold working electrode surface was investigated using CV. The electrochemical behaviour of potassium ferrocyanide (5 mM) on the SPGEs was studied by varying the scan rate between 10 to 60 mV s^{-1}.

A 0.1 M KCl, 0.05 M citrate phosphate buffer pH 5.0 and a 0.05 M PBS pH 7.4 were used as the buffer solutions. This is important in order to characterise their redox reaction on SPGEs relative to an onboard Ag/AgCl reference electrode or saturated calomel electrode.

The parameters for the CV measurements were carried out by:

Number of cycles:	5
Start potential:	-0.5 V
First vertex potential:	+ 0.8 V
Second vertex potential:	-0.5V
Step rate:	0.00274
Scan rate:	0.01999

Chronoamperometry

The electrochemical method used for the measurement of the enzymatic reaction in this work was chronoamperometry. The amperometric measurement was obtained with a single fixed potential procedure with a current acquisition interval of every second. The sensitivity range was set from nAmps to 10 µAmps.

The fixed or constant potential for the amperometric method was evaluated by detecting the HRP activity of the TMB/H_2O_2 reaction on the SPGE surface. The different potential ranges from -600 to 600 mV were studied.

3.2.5.2 Surface characterisation of screen-printed gold electrodes with SEM

The characterisation of the SPGE surface was analysed using a scanning electron microscope (SEM). The SEM images and elemental scans were taken using a Philips XL30 SFEG (scanning field emission gun) (Philips UK, Guildford, UK). Analysis was performed on the basis of the XLFEG/SFEG scanning electron microscope operating instruction manual. The electrode surface element analysis was performed using energy-dispersive X-ray microanalysis (EDAX UK, Castle Camps, UK). AnalySIS 3.0 software (Soft Imaging System, Germany) was used for analysis of the metal content of the electrode surface.

3.2.5.3 Electrochemical study of TMB/ H_2O_2/ HRP

A TMB containing H_2O_2 was selected in this study, based on the performance of electrochemical reaction to produce electrons (current) by redox reaction.

Cyclic voltammetric (CV)

The CV was used to investigate the electrochemical characteristics of the TMB solution containing hydrogen peroxide (H_2O_2). The potential was scanned between -1.0 and 1.0 V at a scan rate of 20 mV s^{-1}. Cyclic voltammograms (CVs) of TMB/H_2O_2 in a phosphate citrate buffer pH 5.0 containing 0.1 M KCl were then recorded.

Chronoamperometric

The optimal concentrations of TMB and H_2O_2 were investigated by applying a reducing current of HRP activity and monitored by amperometry analysis. With the constant potential (-100 mV), the optimising of TMB (0.1 to 10 mM) and the hydrogen peroxidise (0.0015 to 15%) concentration in the phosphate citrate buffer pH 5.0 containing 0.1 M KCl were determined.

The following experiment was to monitor the baseline current and measuring the current was performed by using 5 mM TMB/ 0.075% H_2O_2 and then comparing it to an addition of HRP.

3.2.6 Electrochemical immunosensor development for competitive assay of AFB_1

The measurement of the current for the competitive immunoassay method on SPGE was performed by chronoamperometry at a constant potential -100 mV. A 5 mM TMB/ H_2O_2 solution in phosphate citrate buffer pH 5.0 containing 0.1 M KCl was used for mediated HRP enzymatic reaction. The signal current is then monitored after the baseline current was measured. The 0.01 M PBS in 0.1 M KCl was used for a baseline current measurement by about 100 seconds (Io). The current for another 100 seconds until endpoint was considered as a signal current (It). The difference between

baseline current and signal current (Io-It) was calculated and plotted against the AFB_1 concentration for a calibration curve.

3.2.6.1 Optimisation of antibody and HRP conjugate

The specific binding for the non competitive indirect and direct format was conducted for optimisation of monoclonal anti-AFB_1 antibody and HRP conjugate. A fixed amount of AFB_1-BSA (1 µg mL^{-1}), blocking with 1% PVA, followed by $MAbAFB_1$ (1-50 µg mL^{-1}) were immobilised on the gold surface before binding to a 0 to 10 µg mL^{-1} of anti-IgG-HRP concentration. In the case of the direct method, the assay was conducted by precoating a fixed amount of anti-IgG unconjugated (10 µg mL^{-1}) and blocking with 1% PVA before coated by the addition of $MAbAFB_1$ (1-50 µg mL^{-1}) on the surface. Then, the addition of AFB_1-HRP at (1:100 to 1:1 dilution) concentration. The incubation times and temperatures at each step of the assay was following the procedure at ELISA methods. During each step of the assay, washing procedures were carried out between coating, blocking, competition and detection by rinsing each electrode twice with PBS-T and once with PBS. A 5 mM TMB containing a 0.075% H_2O_2 solution was deposited onto the electrode surface for signal current development.

3.2.6.2 Physical adsorption procedure for competitive assay

The general protocol of the competitive immunoassay procedure developed in Chapter 2 was used for the SPGE in the initial investigation. The condition of the assay and concentration of reagents for both the indirect and direct competitive method were performed after optimisation on the spectrophometric study.

About 10 µl of antigen-protein conjugate (AFB_1-BSA) in a sodium carbonate buffer were immobilised on the surface of gold electrodes and then incubated overnight at 4ºC. The electrodes were then washed using 250 µl PBS-T (twice) followed by 250 µl of PBS (once) before blocking. Blocking reagent was added on the electrode surface with 10 µl of 1 % PVA in PBS before being incubated for 1 hour at 37ºC. A 10 µl of

antigen (free AFB_1 or sample) and anti-antigen antibody (monoclonal antibody) were deposited onto the surface. The different concentration AFB_1 (0 to 200 ppb) and anti-AFB_1 (2 µg mL^{-1}) antibody in PBS were premixed of 1:1 for 30 minutes at room temperature (25ºC). Incubation for the binding assay was performed for 60 minutes at 37ºC. A 10 µl secondary antibody (anti-mouse IgG labelled with HRP) was then added onto the electrode surface and later incubated for 60 minutes at 37ºC. During each step of the assay, washing procedures were carried out between coating, blocking, competition and detection by rinsing each electrode twice with PBS-T and once with PBS. A 5 mM TMB containing a 0.075% H_2O_2 solution was deposited onto the electrode surface for signal current development.

Different competitive formats were also performed to compare the sensitivity of detection. The protocol of the direct competitive assay was examined. About 10 µl of anti-antibody (anti-mouse IgG (H+L)) primary layer (capture species) in a sodium carbonate buffer pH 9.6 were immobilised on the surface of the gold electrodes then incubated overnight at 4ºC. The electrodes were then washed using PBS-T (twice) followed by PBS (once) before blocking. Blocking reagent was added onto the electrode surface with 1 % PVA in PBS before being incubated for 1 hour at 37ºC. After that, 10 µl of 20 µg mL^{-1} anti-antigen antibody $MAbAFB_1$) capture was coated on the electrode. Incubation for both formats were performed for 2 hours at 37ºC. Then, pre-incubation time for 30 minutes of a 5 µl of 1:5 antigen labelled HRP (AFB_1-HRP) before the addition of a 5 µl of 0 to 200 µg L^{-1} antigen (AFB_1) concentration on the electrode. Then, the electrode was incubated for another 30 minutes at 37ºC. During each step of the assay, washing procedures were carried out between precoating/coating, blocking, competition and detection by rinsing each electrode twice with PBS-T and once with PBS. A 5 mM TMB containing a 0.075% H_2O_2 solution was added onto the electrode surface for signal current development. All incubations were performed at 37ºC in a humidity chamber to avoid evaporation.

3.2.6.3 Thiol self assembled monolayer (SAM) modification of the gold surface

For the thiol modified surface, a monolayer procedure was described in the literature by Park *et al*. (2004). The cleaned gold surface was immersed into the 5 mM solution

of 3,3-dithiodipropionic acid (DTDPA) in absolute ethanol and left in the dark for 24 hours at room temperature. The electrode was then washed with ethanol and ultra-pure water to remove the excess thiol. The layer is very stable, and can be kept dry for several weeks under silica gel and argon gas (Mrksich & Whitesides, 1995). Protein immobilization on the modified surface was carried out by first activating the carboxylic acid groups of the thiol monolayer by adding 20 µL of EDC-NHS mixture (0.4M EDC and 0.2M NHS in HPLC grade ultra-pure water and the incubating for 1 hour at room temperature (25±2 ºC). After rinsing with water, 10 µL of the protein solution in 0.05M acetic/acetate buffer pH 5.0 was applied to the gold surface and was incubated for another 2 hours at 37^{o}C.

3.2.7 Electrochemical immuno gold nano-particle sensor detection

3.2.7.1 Self Assembled Monolayer (SAM) on gold working electrode (SPGE)

The modification of the gold working electrode using 5 mM of DTDPA in absolute ethanol for 24 h was similar to that described by Park and co-workers (Park *et al*, 2004). To activate the modified surface, 20 µl of EDC/NHS mixture (0.4 M EDC and 0.2 NHS solutions was prepared in HPLC grade ultra-pure water for 1 hour incubation). After rinsing the electrode, the immunoreagents were immobilised and incubated overnight at 4°C.

3.2.7.2 Optimisation and preparation of immuno gold nano-particle conjugated HRP

The conjugation of the antibody and enzyme to the gold colloid was prepared according to modifications by Chen *et al*., (2007). The first conjugation of a different concentration of colloidal gold (dilution) with a fixed antibody was prepared by adding 10 µL of 100 µg mL^{-1} mouse monoclonal anti-AFB$_1$ antibody to 990 µL (10 µg mL^{-1}) of colloidal gold solution and pH adjusted to 9.0, followed by slowly shaking for 1 hour at room temperature for the antibody to become absorbed into the gold nano-particles (immuno gold nano-particles). The second part of the conjugation

is with the HRP enzyme. This was prepared by adding a 50 µL of 0 to10 mg of HRP solution in 950 µL (0–1000 µg mL^{-1}) to the immuno gold nano-particles solution. The mixture was then incubated by shaking at room temperature for another 1 hour. The conjugate solution was then centrifuged at 10,000 rpm for 30 min. After being centrifuged, the supernatant content unbound protein was discarded, leaving a dark red pellet which is the sediment of the bonding of immuno gold nano-particles with HRP (Figure 3.4). The sediment of immuno-gold labelled HRP was dissolved in 70 µL of 0.01M PBS and 20 µL of 2.5M of NaCl. The conjugates can be stored at 4°C before use.

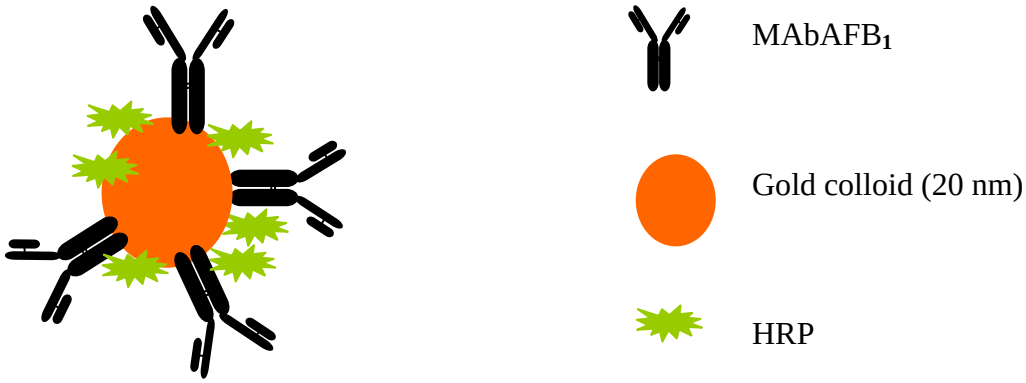


Figure 3.4: Schematic diagram of anti-AFB_1 antibody ($MAbAFB_1$) and HRP conjugated with gold colloid.

3.2.7.3 Indirect competitive assay using immuno gold nano-particles-HRP

A volume of 10 µl of AFB_1-BSA conjugate (1 µg mL^{-1}) in a 0.1 M acetic/acetate buffer (pH 5.0) (v/v) and a 0.1 M carbonate buffer (pH 9.6) was adsorbed on a modified surface and a clean surface (passive adsorption) on SPGE, respectively. Incubation was performed at 4°C overnight (18 hours), followed by washing twice with a phosphate buffered saline containing Tween 20 (PBST) and once with PBS alone. The blocking step with a 1% (w/v) PVA solution was performed and incubated at 37°C for 1 hour, followed by washing. A 10 µl of immuno-gold colloid conjugated

HRP was then added and incubated on the surface of AFB_1-BSA electrodes for 2 hours' incubation at 37ºC.

The competition between the free AFB_1 and the immobilised AFB_1 was carried out in the presence of gold nano-particles conjugated to a monoclonal anti-AFB_1 antibody and HRP. Competition reaction involved the addition of various AFB_1 standard solutions (0-1 µg L^{-1}) with the immuno-gold colloid conjugated HRP for 30 minutes at room temperature in an eppendorf tube (pre-incubation) before 10 µl of mixture was transferred to the SPGE plate for 1.5 hours at 37ºC incubation. After the washing step, the current signal was initiated by the addition of TMB/H_2O_2 before being recorded at -100mV constant potential.

3.2.8 Sample analysis

Peanut samples were collected from the United Kingdom (TESCO and Open market) and Malaysia (supermarket and open market). Samples were then ground, using a food processor or blender at medium speed for 3-5 minutes until all the peanuts were blended. Samples were then packed properly in plastic bags and stored in a cool place (0-4ºC) before use (avoiding contamination). For the spiked sample, an aflatoxin B_1 was spiked on the ground peanut samples at concentrations of 4, 40, 100 and 250 µg Kg^{-1}. The mixture was manually shaken for 30 seconds and then kept overnight to obtain a homogeneous mixture.

3.2.8.1 Sample extraction for immunoassay analysis

A 5 g amount of ground samples (spiked or blank) were placed into a 150 mL flask and 25 mL of 70% methanol was added. The samples were then shaken vigorously for 3 to 5 minutes manually (Ridascreen Kit method). Extract samples were then filtered through filter paper (Whatman No.1 or equivalent). After that, 1 mL of the filtered sample was diluted with 1 mL of PBS. About 5 µl diluted samples were used for testing on the SPGE surface. If the AB_1 concentration is expected to be higher, the sample must be diluted again.

3.2.8.2 Sample extraction for immunoassay and HPLC

The procedure was based on the 'Trucksess' method (Trucksess *et al*., 1991), modified as follows: 50 g of ground sample and 5 g of NaCl were extracted by mechanical stirring with 100 ml of methanol–water (70:30) for 15 minutes followed by filtration through pre-folded paper. Samples filtered were stored in a cool condition at 4ºC before use.

Clean-up

The filtrate was then applied to the SPE column (C_{18} minicolumn) at a flow-rate of 2–3 ml/min based on the 'Pena' technique (Pena *et al*., 2002). The C_{18} minicolumn was preconditioned first by passing 2 ml water through it, then 2 ml methanol before 2 ml water followed by a 1-min drying time with air passing through the tube via aspiration. A 2 ml samples were then passed through to the column and followed by 2 min drying before analytes were eluted by 1 ml of methanol. The extracted sample was evaporated using N_2 at room temperature.

For the immunosensor calibration curve analysis, the eluted fraction from the SPE column solvent was redisolved with 1 ml of PBS pH 7.4 before use, for the preparation of immunoassay calibration curve. Then, 5 µl of the sample was mixed with 5 µl of MAbAFB_1 before transferred onto the gold working electrode for the detection of AFB_1.

Derivatization for HPLC determination

About 1 ml volume of the eluted fraction from the SPE column was evaporated under nitrogen before the derivatization process was performed. Samples were derivatized using the trifluoroacetic acid (TFA) method as described. The dried sample was reconstituted with 200 µl of hexane then mixed with 50 µl of trifluoroacetic acid (TFA). The mixture was then vortexed for 30 seconds and left to stand for 5 minutes. About 950 µl of acetonitril/water (9 + 1) was added and then vortexed for 30 sec and let layer separate 10 min. Samples were then filtered and kept into the amber vials.

About 50 µl of filtered samples were injected into the HPLC (Waters -600E) by the loop auto sampler injection system (Waters, 712 WISP). HPLC parameters for AFB_1 measurement are described below using the isocratic system:

Mobile phase: Acetonitril/Methanol/Water (10:30:60)
Column: C18 (Phenomenex, Luna 5 micron 150 x 4.6 mm)
Flow rate: 1.0 ml/min
Detector: Fluorescence detector with Ex: 360 nm and Em. 440 nm

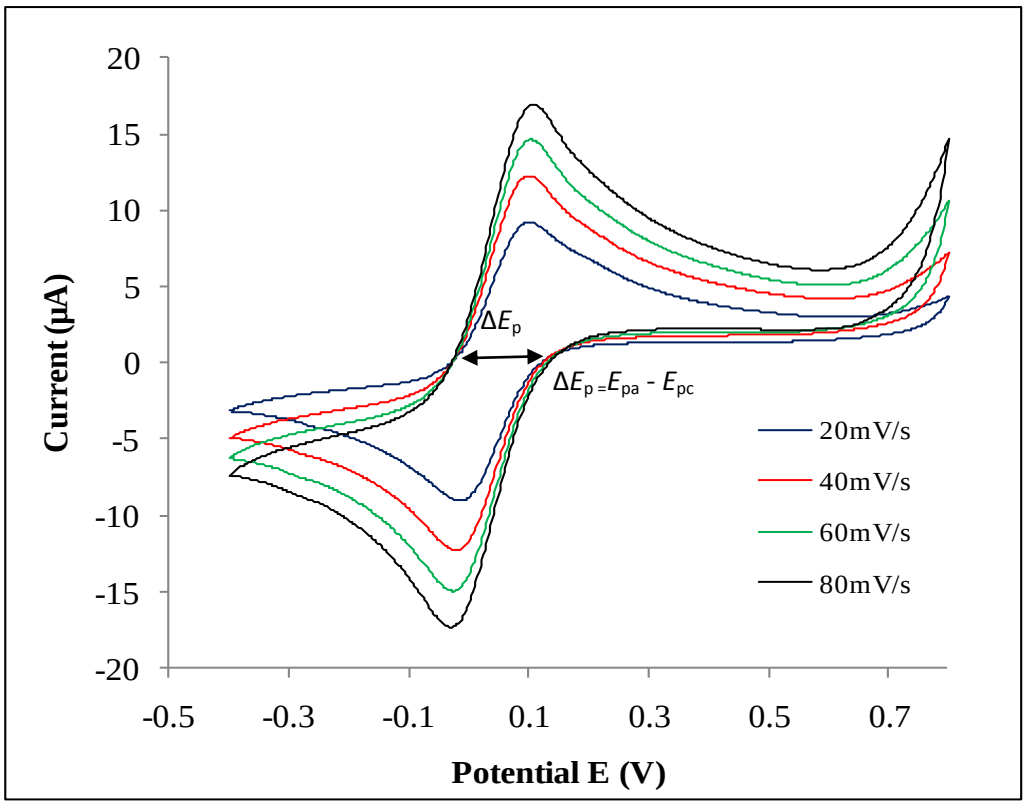
Each experiment was carried out in triplicate and each value was the mean of three determinations.

3.3 Results and Discussion

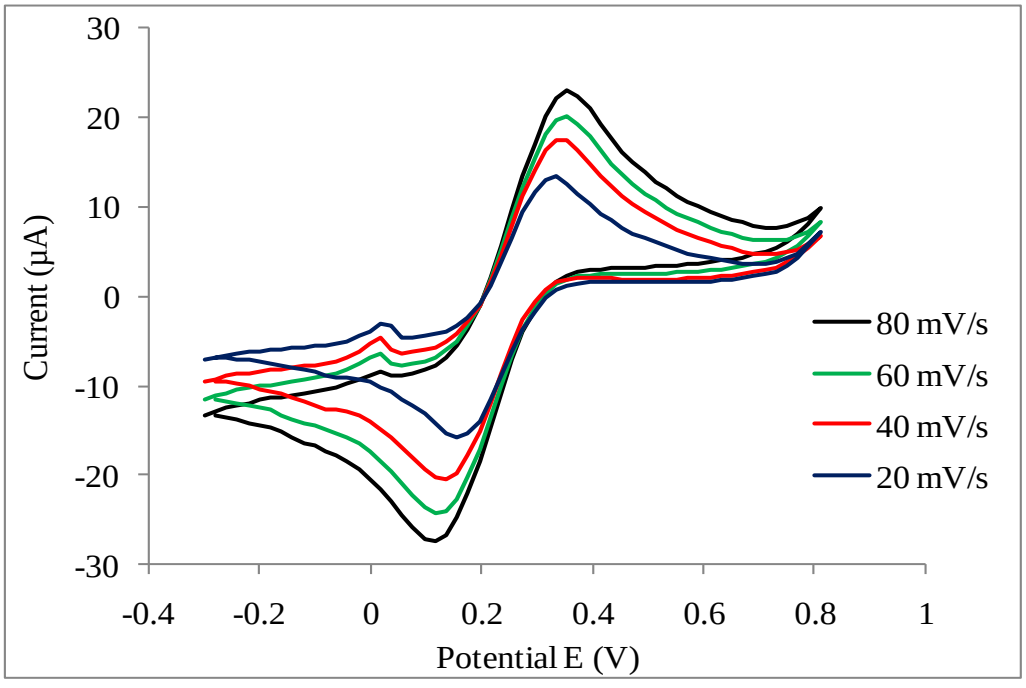
3.3.1 Characterisation of screen-printed gold electrode by cyclic voltammetry

The gold working electrode on the screen-printed sensor platform as described in section 3.2.4.1 was used. Gold inks are attractive for sensing applications because they are relatively good conductors and have a wide range of potential. Due to its versatility and relative ease of measurement, cyclic voltammetry (CV) was the initial electroanalytical technique used for the electrochemical characterisation of the SPGE Ercon and DuPont surfaces. In order to characterise the electrodes, the electrochemical behaviour of potassium ferrocyanide was studied since its electrochemical behaviour is well known (Md Noh and Tothill, 2006).

The CV was employed to determine the electroactive surface area of the working electrode (WE) as well as the electrochemical reversibility of potassium ferrocyanide on the electrode. Figure 3.5 (a) and (b) shows cyclic voltammograms of peak current and potential obtained at the working electrode for SPGE Ercon and DuPont immersed in 5 mM potassium ferrocyanide at four different scan rates.



a



b

Figure 3.5: Cyclic voltammograms of 5 mM potassium ferrocyanide in 0.1 M KCl at different scan rate of screen-printed gold electrode, a: SPGE Ercon and b: SPGE DuPont.

During the voltammetric scan at -0.5 to 0.8 V relative to the Ag/AgCl reference electrode, the potential applied to the working electrode becomes sufficiently positive at a certain value and causes an anodic current which is observed at that point. This will increase rapidly until the oxidized species fall to zero. During the reduction of the electrode immersed in a solution, the potential scan is switched to negative values and the accumulated oxidized species on the electrode surface are reduced. One cycle is complete when the reduced species are depleted in order to cause the current to peak and then to decrease.

Increasing the scan rate shows an increase in the peak potential oxidation and reduction. At the scan rate of 20 mV s^{-1}, the peak-to-peak separation ($\Delta E_p = E_{pa}-E_{pc}$) for SPGE-Ercon and Dupont was in the area of ~100 mV and ~120 mV, respectively, which is more than 59 mV value. This indicates that the redox reaction to this gold electrode is quasi-reversible. According to Wang (2006), for the quasi-reversible systes the current is controlled by both charge transfer and mass transport. The smallest peak to peak separation was also found at a scan rate of 20 mV s^{-1} which indicates a fast electrone transfer rate. The lower peak to peak separation, the lower the mass transfer influence on the diffusion controlled redox reaction of ferro. The oxidation/reduction peaks showed more defined when the lower scan rates are used. Therefore, the scan rate of 20 mV s^{-1} was chosen as the standard scan rate for further measurement.

At 20 mV s^{-1} scan rate also, the peak at anodic and cathodic surface present a high signal current -16.8 µA and 11.8 µA for SPGE DuPont compared to -8.7 and 8.4 µA for Ercon, respectively. Increasing the scan rate shows an increase in the peak current This shows that each of the two electrodes has a different characterisation of the electron kinetic transfer ($Fe^{3+} + {}^{e}/Fe^{2+}$) that may provide different sensitivity when used for calibration and concentration determination. In view of the proprietary composition of the DuPont ink, it is difficult to explain why the resulting electrode displays the most favourable redox behaviour.

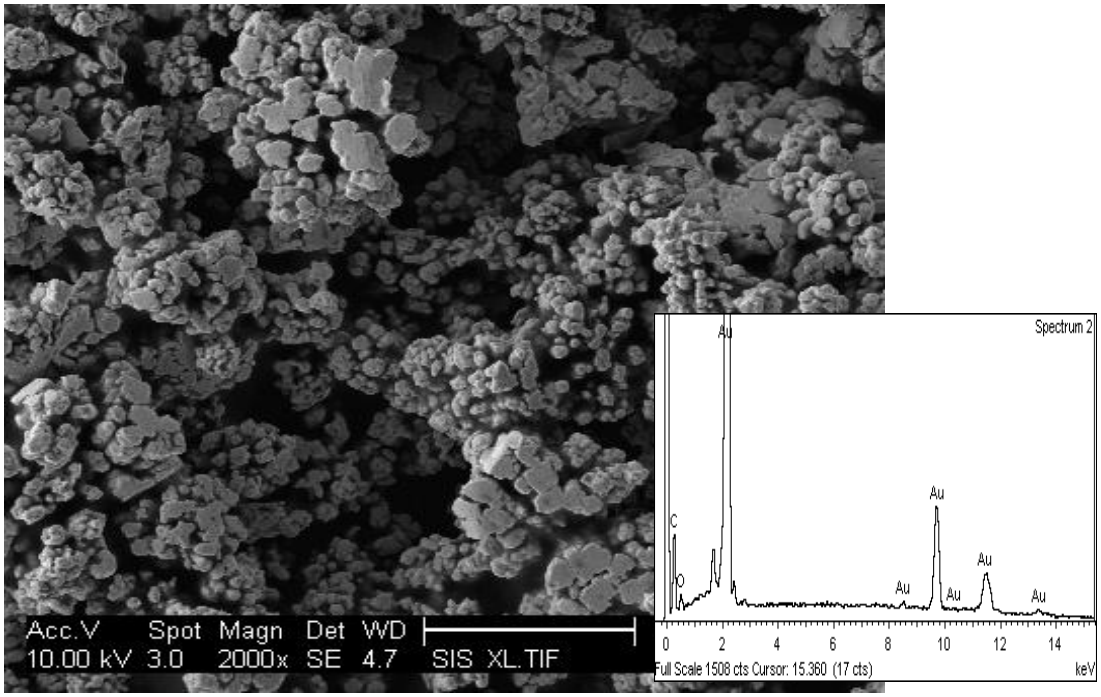
3.3.2 Surface characterisation of SPGE using SEM

Differences in physical properties may contribute to the observed behaviour. For example, microscopy imaging indicated that the DuPont electrode is characterised by a highly dense surface with small and uniform particles as compared to the Ercon one. Figure 3.6 shows the different roughness and distribution of gold particles on the gold working electrode observed by SEM at 800 magnification.

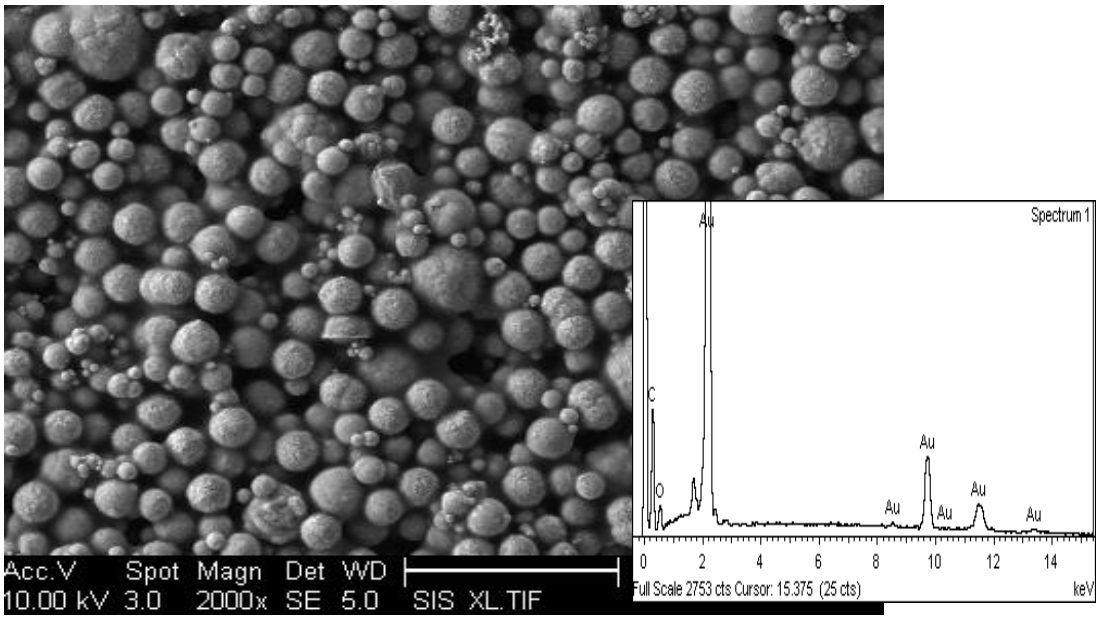
Surface roughness and the presence of clusters on the electrode surface are known to result in an increase in the active surface area. An electrode surface element analysis of the gold working electrode shows that the main component of the surface is gold (Au), with traces of carbon and oxygen. The element content of Au in Ercon is 77.54 % in weight and 65.19 % for DuPont. According to Stiene and Bilitewski (2002), when using different electrode materials they differ strongly in their electrochemical properties.

3.3.3 Cyclic voltammetry of 3,3'5,5'-tetramethylbenzidine (TMB) as an electron shuttle

With the assay transferred onto the SPGE-Ercon surface, the system could then be incorporated into an immunosensor format. Depending on the assay format, the horseradish peroxidase (HRP) labelled antigen or antibody activity measurement is performed as a final step of the assay. This was enabled by the use of a mediator such as TMB for the HRP label catalyzed reaction with hydrogen peroxide to be monitored. Therefore, the electro activity of TMB/H_2O_2 in an 0.05 M Phosphate Citrate buffer (PCB), pH 5.0 in 0.1 M KCl was first investigated by cyclic voltammetry (CV) to determine the redox profile. The voltammogram of TMB reaction compared to the 0.05 M phosphate citrate buffer (PCB), 0.1 M KCl and PCB containing 0.1 M KCl, are shown in Figure 3.7.



a



b

Figure 3.6: Typical scanning electron micrographs (800 x magnification) showing the surface features of a screen-printed electrode and analytical result for surface composition. (a) Ercon (b) DuPont.

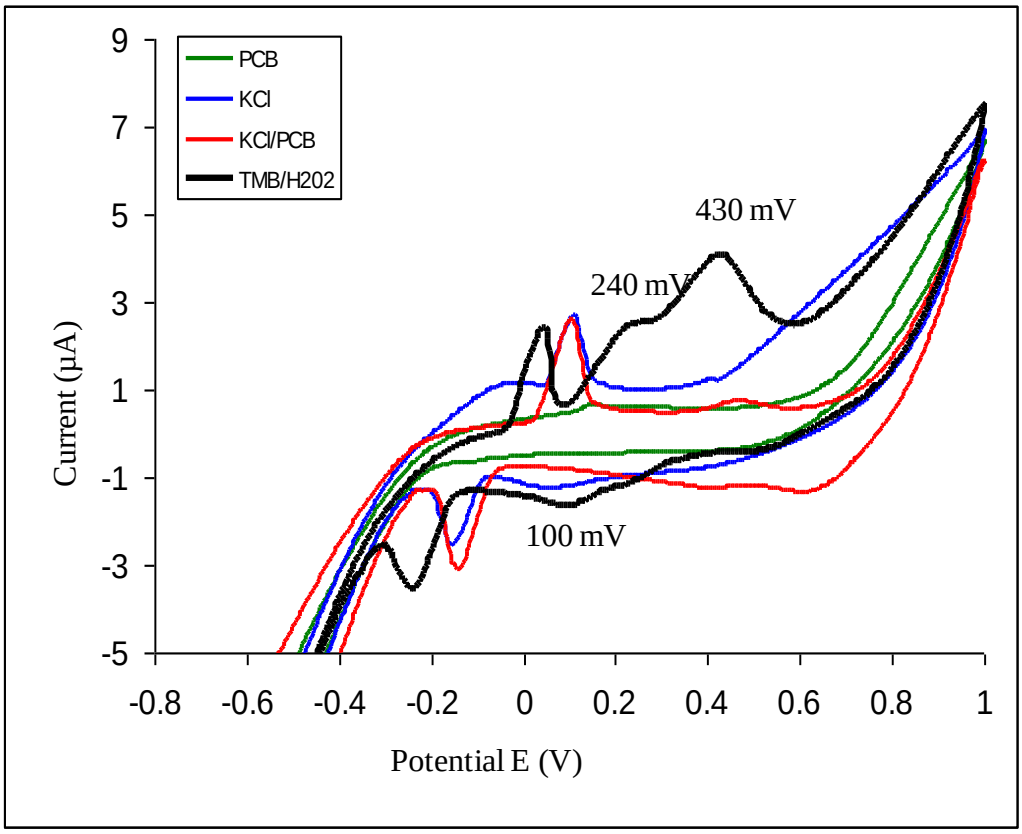


Figure 3.7: Cyclic voltammograms of buffer and TMB/H_2O_2 in 0.05 M PCB, pH 5.0 containing 0.1 M KCl on the bare SPGE at a scan rate of 20 mV s^{-1}. Scan range: -1 to 1 V.

Two oxidation peaks of TMB/H_2O_2 on the bare SPGE Ercon were seen at +240 and +430 mV. One reduction peak appears at ~ +100 mV. In this case, the electrochemical behaviour of TMB on SPGE Ercon is based on the following: $TMB_{(red)}$ can be oxidized with the oxidation peak at +240 and +430 mV. Its product, $TMB_{(ox)}$, can be reduced on this electrode at +100 mV. Heurich (2008) studied the electrochemical behaviour of $TMB_{(red)}$ and $TMB_{(ox)}$ on gold electrode and found two oxidation peak at +290 mV and +550 mV and one reduction peak at -250 mV. Volpe *et al*., (1998) also found the similar behaviour of the $TMB_{(red)}$ and $TMB_{(ox)}$ on glassy carbon electrode when used the same substrate-enzyme system.

The cyclic voltammogram of PCB containing 0.1 M KCl and 0.1 M KCl alone used as a solution of TMB/H_2O_2 also found sharp oxidation and reduction peaks at 110 and -150 mV. There is no peak oxidation and reduction of PCB solution on the bare gold

electrode. The graph shows that a sharp peak appeared in each voltammogram that involved KCl (blue, red and black) in the solution.

With the addition of 1 µg mL^{-1} anti-IgG-HRP conjugate to the TMB/H_2O_2 substrate solution, the voltammogram presented an increase in the reduction current, only one reduction peak remains (Figure 3.8). This indicates that the HRP reaction product can be reduced on the SPGE Ercon. In a report from He *et al*., (2002), the potential for HRP activity can be determined at a reduction current that is generated by $TMB_{(ox)}$. Base on this situation, the suitable detection potential for the HRP activity used on the working electrode can be measured. The amperometric voltammetry measurement of HRP activity was further carried out.

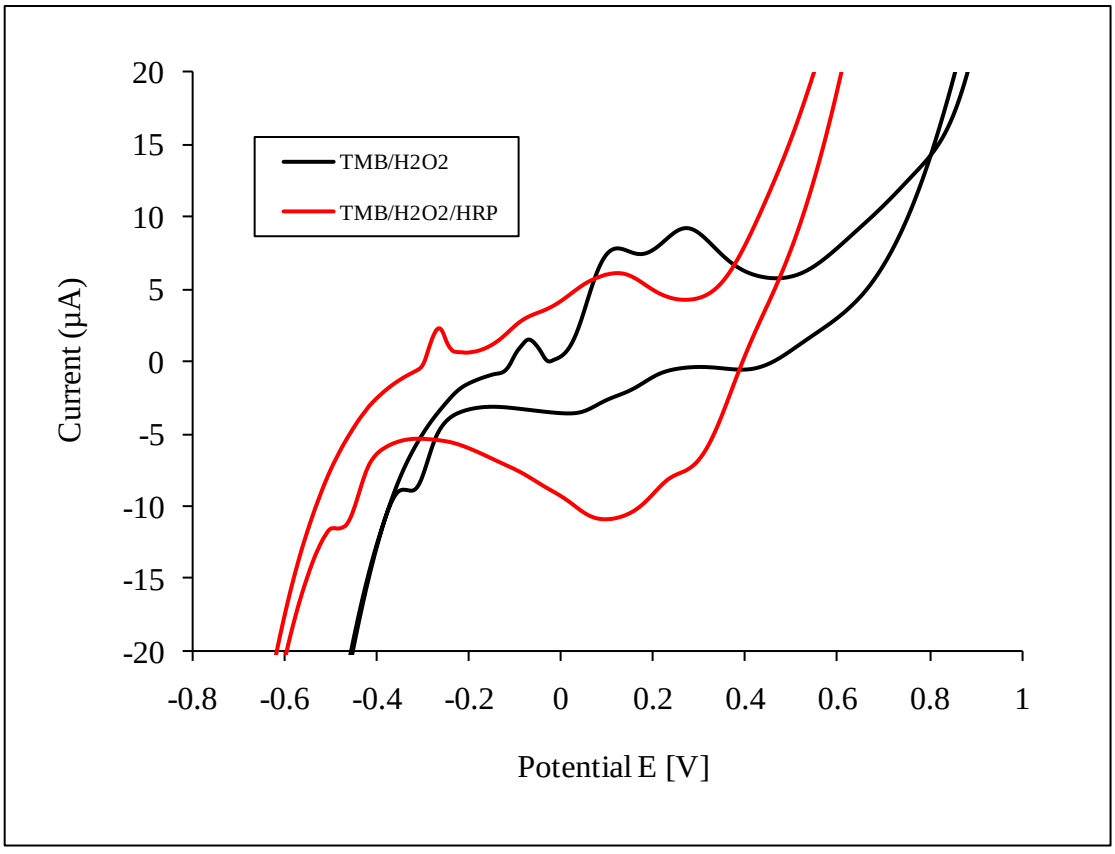


Figure 3.8 Cyclic voltammetry of 5 mM TMB/H_2O_2 in 0.05 M PCB, pH 5.0 in 0.1 M KCl. and 5 minutes after the addition of 1 µg mL^{-1} HRP in solution. Scan rate, 20 mV s^{-1}. Scan range, -1 to 1 V

3.3.4 Chronoamperometry study of enzyme activity using TMB/H_2O_2

All the experiments involved in any optimisation of TMB enzyme substrate and reagents by chronoamperometry were investigated using SPGE DuPont. This was due to the very limited number of SPGEs fabricated in house (Ercon). This in turn was because the printing machine was unusable for sensor production.

Chronoamperometry was used to characterise the change in current with the addition of H_2O_2 and TMB substrate for the enzyme activity on the gold working electrode. Figure 3.9 shows the different current responses of TMB and H_2O_2 with the addition of 1 µg mL^{-1} HRP on a bare SPGE at a constant potential of -100 mV. The detection is based on the reduction current of the HRP towards TMB/H_2O_2. From the Figure, it becomes clear there is a visible change in current, which decreases even further as a result of the catalysed reduction of H_2O_2. The resulting electrons were shuttled to the electrode via TMB. Therefore, a decrease in current indicates a high enzyme response.

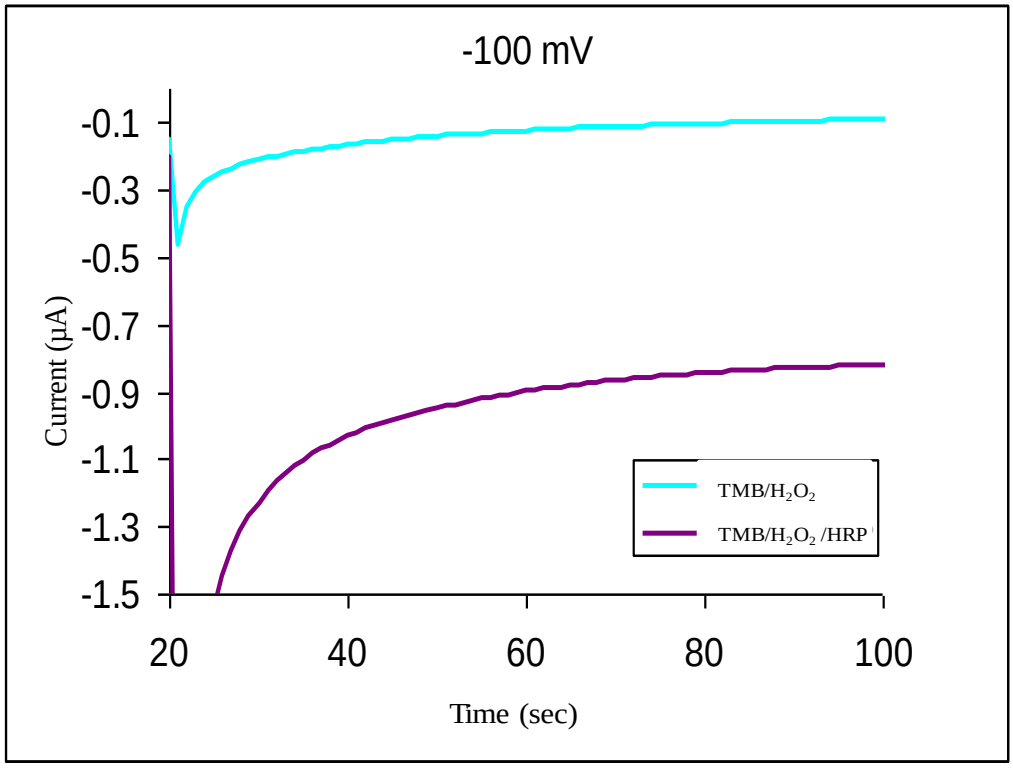


Figure 3.9: Current response of chronoamperometric studies of H_2O_2 and TMB with the addition of HRP (1 µg mL^{-1}) on bare SPGE at constant potentials -100 mV.

The electroactive species diffuse from the bulk solution to the electrode surface in order to react. The electrolytic process can be clearly observed as the current result of the electroactive species being transformed at the electrode surface. Using a redox enzyme such as HRP as the label in the immunoassay setup will result in a direct signal response with the enzyme concentration, which, in turn, is dependent on the antibody concentration bound to immobilised AFB_1-BSA. This format allows for non-competitive and competitive detection of analyte. Based on the type of result achieved in Figure 3.9 further optimisations were conducted using the gold sensor.

3.3.5 Potential determination

In order to obtain the optimum applied potential, the use of chronoamperometry detection for enzymatic activity using TMB/H_2O_2 substrate was investigated. The signal response of the enzymatic reaction using a TMB/H_2O_2 substrate based on the indirect non competitive format were conducted using AFB_1-BSA (1 µg mL^{-1}), blocked with 1 % PVA followed by $MAbAFB_1$ (10 µg mL^{-1}) and continued with anti-IgG-HRP (1 µg mL^{-1}). These were analysed by amperometry at different potentials from -600 to 600 mV to determine both the signal (S) and background (B) responses at each one, as shown in Table 3.1.

The S/B ratios were calculated. Data from Table 3.1 indicate that at -100 mV the S/B ratio response was the best for the assay. The high value of S/B was determined in the range from -200 to -100 mV and this range was the most suitable potential for amperometry measurement. The maximum value of S/B ratios was determined at -100 mV. Thus the potential of -100 mV was chosen as a suitable potential for the chronoamperometry detection of TMB/H_2O_2 whilst the background signal was still acceptably low.

Table 3.1: Current response of enzymatic reaction using TMB/H_2O_2 substrate based on the indirect non competitive format at different potentials. Experiments were conducted using AFB_1-BSA (1 µg mL^{-1}), blocked with 1 % PVA followed by $MAbAFB_1$ (10 µg mL^{-1}) and continued with anti-IgG-HRP (1 µg mL^{-1}). Backgrounds were conducted without $MAbAFB_1$ onto the gold surface of the electrode.

	Ercon			DuPont		
Potential, (E/mV)	B (µA)	S (µA)	S/B	B (µA)	S (µA)	S/B
600	2.24	3.09	1.38	1.15	4.52	3.93
400	0.57	0.96	1.68	0.82	1.36	1.66
200	0.13	0.31	2.35	0.72	0.72	0.99
150	0.24	0.15	0.65	0.70	0.39	0.55
100	0.28	0.08	0.29	0.60	0.17	0.28
50	0.03	0.13	0.29	0.71	0.18	0.26
0	-0.01	0.01	-0.88	-0.01	-0.007	0.82
-50	-0.05	-0.1	2.17	-0.07	-0.17	2.54
-100	-0.05	-0.66	13.49	-0.18	-1.84	9.93
-150	-0.1	-0.72	6.87	-0.39	-2.53	6.43
-200	-0.91	-5.77	6.29	-0.82	-3.86	4.66
-400	-3.17	-4.20	1.32	-6.19	-2.37	0.38
-600	-22.27	-2.67	0.12	-45.43	-12.61	0.28

Note: B = background; S = signal; S/B = Signal/Background

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3.3.6 Optimisation of TMB and hydrogen peroxide concentrations

Our substrate for the signal transduction of immune-conjugated HRP is an in house prepared TMB/H_2O_2 solution. Therefore, the most suitable TMB and H_2O_2 concentrations were examined for the optimal current signal by amperometric study. The substrate was applied based on the indirect non competitive immunoassay on a bare SPGE DuPont. The different concentrations of TMB from 0.1 to 10 mM were determined as well as 0.0015 to 0.15% concentrations of H_2O_2 (from dilution 30% of H_2O_2 solution).

As seen in Figures 3.10 and 3.11, the signal and background increased with the increasing of TMB and H_2O_2 concentrations. At the point where the maximum signal response started to plateau, the background response began to increase. Therefore, the most suitable concentrations for both chemicals are based on S/B values. The high value of S/B was obtained at 5 mM TMB and 0.075% H_2O_2 concentrations with the S/B ratio of 8.2 and 4.9. Therefore, the optimal concentration of TMB and H_2O_2 was chosen at the maximum value of 5 mM and 0.075% concentrations, respectively.

Optimisation of the mediated transduction system determined an effective concentration of 5 mM TMB in a combination of 0.075% H_2O_2 to give a maximum response, with high stability/reproducibility and without causing peroxide substrate inhibition. Volpe *et al*. (1998) found that the use of TMB and H_2O_2 as a substrate for electrochemical detection of low level HRP was successful and compared to hydroquinone (for HRP) and p-aminophenyl phosphate (PAPP) (for AP).

3.3.7 Optimisation of reagents concentrations

Development of the immunosensor using an immunoassay format with transducer immobilised antibodies has been traditionally used. However, the stability of the sensor relies on the quality of the immobilisation technique. Thus, both of competitive formats (direct and indirect) with stable antibodies and antigen bound to the sensor surface have been proven to produce more stable and reproducible sensors.

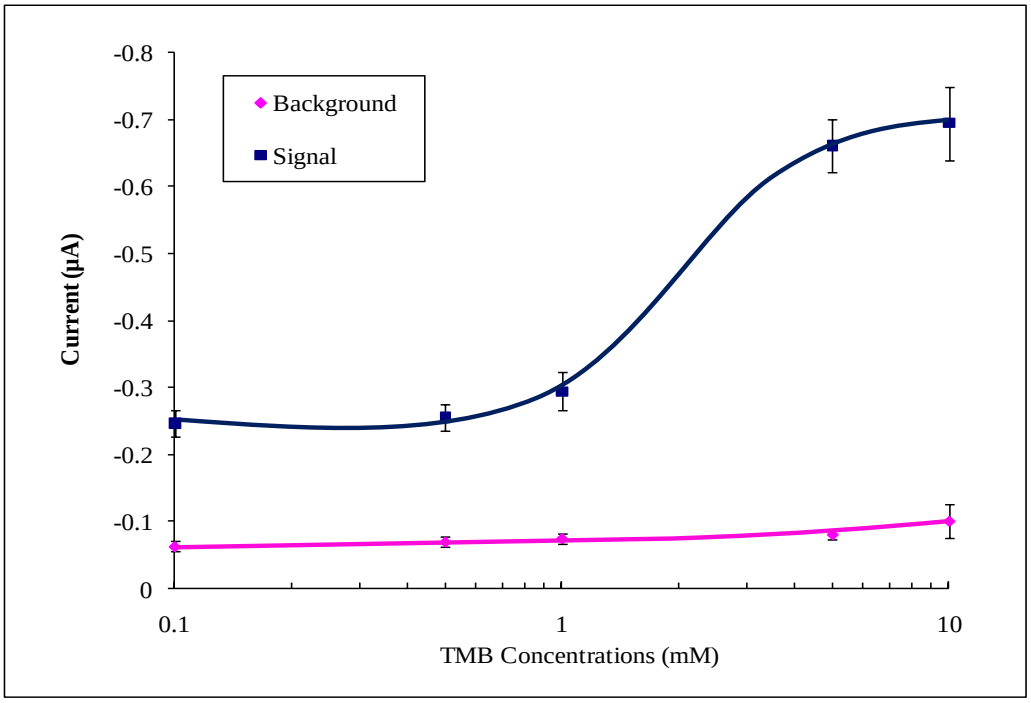


Figure 3.10: Mediated HRP response to different TMB concentrations (0.1 to 10 mM) with 0.075% fixed H_2O_2 concentration by constant potential -100mV. Screen-printed gold electrode used with immobilised AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (10 µg mL^{-1}) and continued with (Signal) and without (Background) 1 µg mL^{-1} anti-IgG-HRP. Error bars = SD, n = 3.

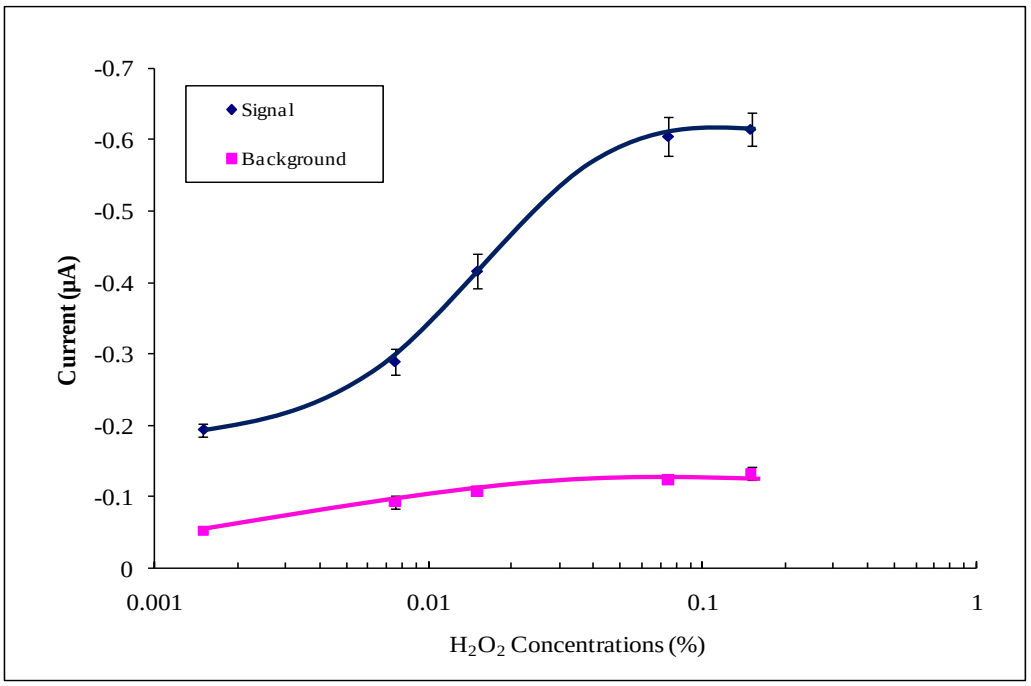


Figure 3.11: Mediated HRP response to different H_2O_2 concentrations (0.0015 to 0.15%) with 5 mM fixed TMB concentration by constant potential -100mV. Screen-printed gold electrode used with immobilised AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (10 µg mL^{-1}) and continued with (Signal) and without (Background) 1 µg mL^{-1} anti-IgG-HRP. Error bars = SD, n = 3.

3.3.7.1 Optimisation of Monoclonal antibody against AFB_1

The immobilization of capture antibody, monoclonal antibody (anti-aflatoxin B_1 antibody) on a bare gold working electrode is essential to produce a sensitive signal. The optimal amount found of monoclonal antibody concentration on the ELISA test for the indirect and direct format is 10 µg mL^{-1} and 20 µg mL^{-1}, respectively. In this case re-optimisation of the antibody concentration on the SPGE was applied by a different range of 1 to 50 µg mL^{-1}.

The specific binding for the indirect format was employed by immobilizing a fixed amount of AFB_1-BSA (1 µg mL^{-1}) then with $MAbAFB_1$ (1-50 µg mL^{-1}) before binding to the anti-IgG-HRP at 1 µg mL^{-1} concentration. In the case of the direct method, the specific binding was employed by immobilizing a fixed amount of anti-IgG unconjugated (10 µg mL^{-1}) as well as binding to $MAbAFB_1$ (1-50 µg mL^{-1}) and AFB_1-HRP (from Ridascreen kit) at (1:10 dilution) concentration.

The plot from Figure 3.12 shows a change in the current reading was present from -0.1 to -0.7 µA and -0.21 to -0.52 µA on the indirect and direct formats, respectively, depicting the dynamic concentration range of binding $MAbAFB_1$. This is directly proportional to the decrease in current value (increase in current response) observed by increasing the HRP catalysis. This suggests that the increase of signal response was due to the increase in the amount of monoclonal antibody bound, as expected.

The indirect immunoassay format (curve from Figure 3.12), shows that the current was dramatically increased from 5 to 10 µg mL^{-1} . The optimal concentration was based on the optimal signal at 10 µg mL^{-1}. This indicates that the optimal amount of specific binding of $MAbAFB_1$ was 10 µg mL^{-1}. Therefore, this concentration was used and it is similar to the one used for the ELISA method. This was selected as suitable for the coating and binding study.

For the direct assay method, the current was dramatically increased from 2 to 20 µg mL^{-1} and saturated after that. Therefore, the optima chosen of $MAbAFB_1$ concentration was about 20 µg mL^{-1}.

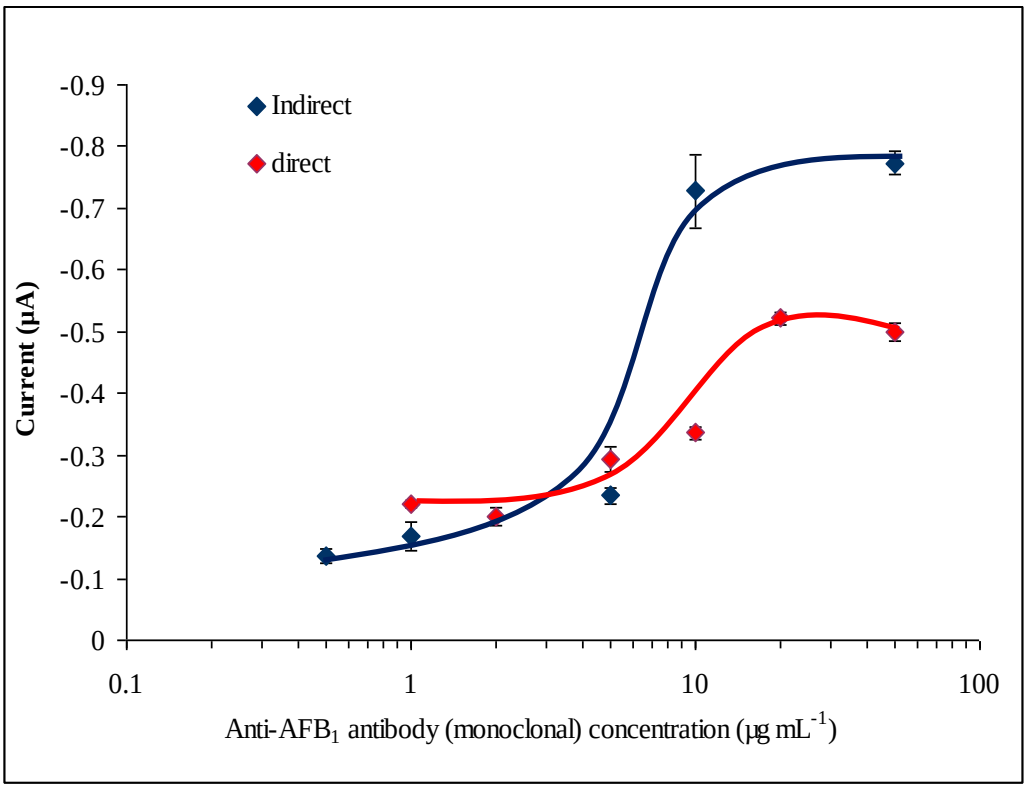


Figure 3.12: The signal of indirect and direct non competitive immunoassay for monoclonal antibody concentrations using SPGE DuPont by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). *Indirect format*: screen-printed gold electrode was coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (0 to 50 µg mL^{-1}) and continued with anti-IgG-HRP (1 µg mL^{-1}). *Direct format*: screen-printed gold electrodes were precoated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by $MAbAFB_1$ (0 to 50 µg mL^{-1}) and continued with AFB_1-HRP (1:10). Error bar = SD, n = 3.

3.3.7.2 Optimisation of HRP conjugate

The sensitivity of secondary anti-IgG-HRP (indirect) and AFB_1-HRP (direct), crucial for revealing the extent of reaction of the $MAbAFB_1$, was analysed. The amperometric response of the immunosensor depends on the amount of immobilised HRP conjugate (conjugate bound to the surface). In order to optimise the concentration of HRP conjugate, the solution containing different concentrations of anti-IgG-HRP (0 to 10 µg mL^{-1}) and AFB_1-HRP (1:100 to 1:1) were conducted. The variable of HRP conjugate was tested for obtaining a maximum response in order to obtain higher sensitivity of the immunosensor.

As shown in Figure 3.13, the current response rose dramatically up to 1 µg mL^{-1} of anti-IgG-HRP and then tended to saturate. The higher response was achieved at 1 µg $mL^{-1,}$ which is the optimal amount of anti-IgG-HRP bound to the optimal amount of $MAbAFB_1$ immobilised on the surface. Consequently, 10µl of 1.0 µg mL^{-1} of anti-IgG-HRP solution was used for further optimisation.

In the case of direct immunoassay for the variable concentration of AFB_1-HRP test, Figure 3.14 shows the current was increased up to 1:10 dilution to achieve maximum response. Therefore, this concentration was chosen for the direct immunoassay test routinely in order to obtain maximum sensor sensitivity. The response was slightly decreased when the concentration of more than 1:10 dilution was applied. The reason for this phenomenon has already been discussed in the ELISA chapter (Chapter 2).

Both of the optimal results were similar to that achieved with the ELISA. In terms of current detection, a higher current response was present on the indirect method than on the direct method, which indicates that the indirect method may produce better sensitivity of the assay.

3.3.8 Development of competitive assay on SPGE (ERCON)

3.3.8.1 Passive adsorption immobilisation

The optimal concentration of indirect and direct immunoassay reagents was subsequently transferred to an immunosensor format on the SPGE Ercon. Then, the competitive immunoassays were measured using chronoamperometry. The competition assay for the calibration curve were prepared prior to each assay by using 10 fold serial dilution of AFB_1 standard solution in buffer in the range of 0–100 µg L^{-1}. A change in current response was observed at a potential (-100 mV) by adding a mixture of 5 mM TMB and 0.075% H_2O_2 to the surface of the gold electrode.

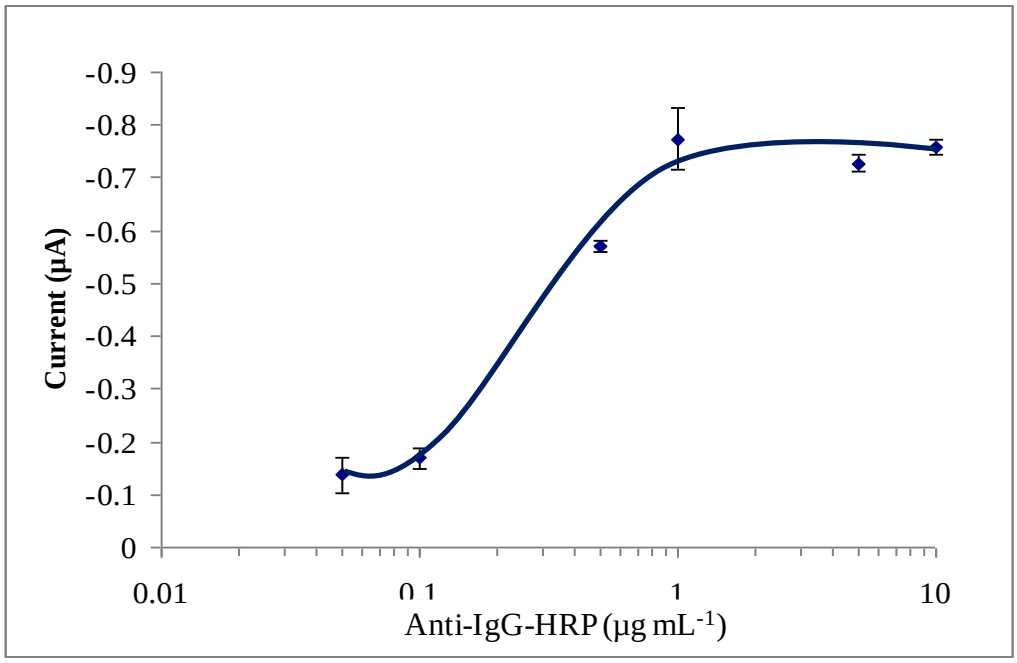


Figure 3.13: The signal of indirect non competitive immunoassay for anti-antibody-HRP concentrations using SPGE DuPont by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). Screen-printed gold electrodes were coated with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (10 µg mL^{-1}) and continued with anti-IgG-HRP (0 to 10 µg mL^{-1}). Error bar=SD, n=3

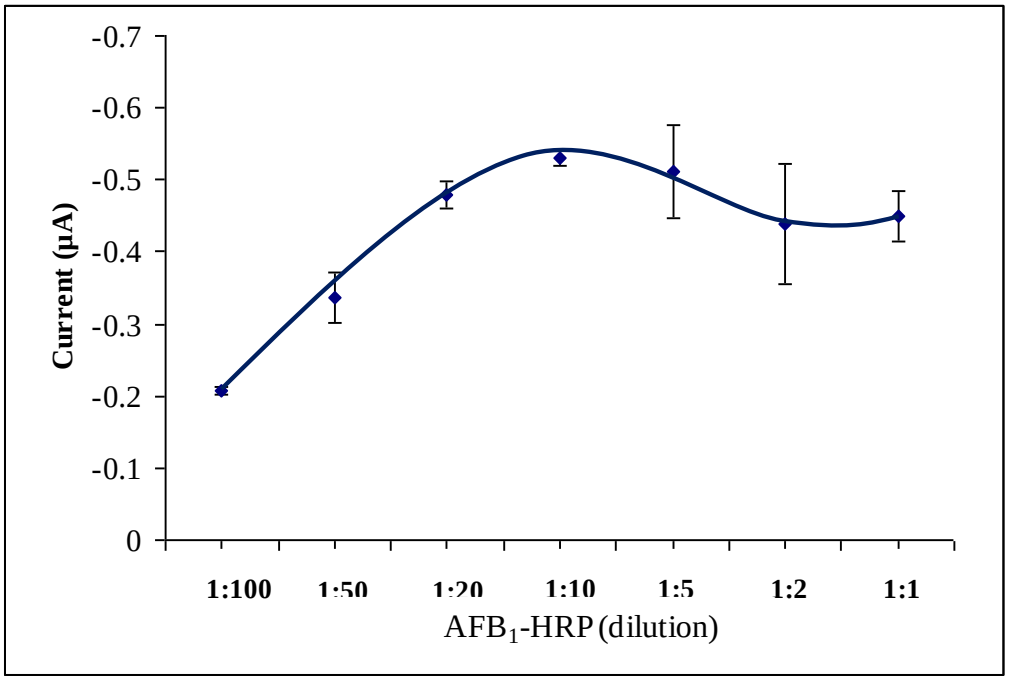


Figure 3.14: The signal of direct non competitive immunoassay for AFB_1-HRP concentrations using SPGE DuPont by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). Screen-printed gold electrodes were precoated with anti-IgG (10 µg mL^{-1}), followed by $MAbAFB_1$ (20 µg mL^{-1}) and continued with AFB_1-HRP (1:100 to 1:1). Error bar = SD, n = 3.

The plot of the calibration curves were presented in Figure 3.15. For both curves, the result shows that the signals were decreased with the increasing of the AFB_1 concentration (inversely proportional). For the indirect competitive method, the decrease in current means less capture antibody ($MAbAFB_1$) on the surface (less anti-IgG-HRP binding to the $MAbAFB_1$). The more AFB_1 competitor, the less antibody binds to the AFB_1-BSA on SPGE. Therefore, the less antibody binds, the less secondary antibody labelled HRP bound to the SPGE, which results in a less response. This is most of the $MAbAFB_1$ was bound to the free AFB_1 and washed out. In the case of the direct competitive method, the more AFB_1 competitor, the less AFB_1-HRP to be bound to the $MAbAFB_1$ on the surface, which most of the $MAbAFB_1$ was bound to the free AFB_1.

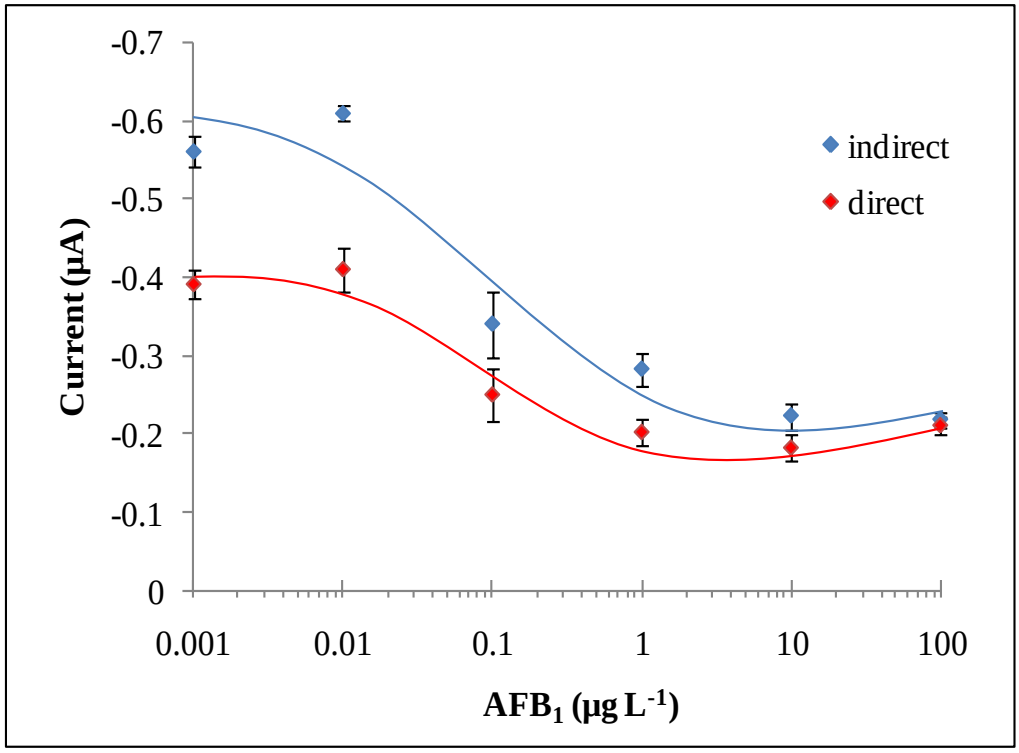


Figure 3.15: The competitive response curve for AFB_1 detection by passive adsorption on gold working electrode (SPGE Ercon). Current measurement was by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Indirect: screen-printed gold electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MabAFB_1$) (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (1 µg mL^{-1}). Direct: screen-printed gold electrodes were precoated with anti-IgG (10 µg mL^{-1}), followed by $MAbAFB_1$ (20 µg mL^{-1}) and continued with AFB_1-HRP (1:10 dilution). Error bar = SD, n = 3.

The working range for both curves was slightly similar by 0.01 to 1 µg L^{-1} (linearity range). This suggests that the use of the screen-printed gold electrode (Ercon) as an electrochemical immunosensor can potentially obtain a low detection limit in the assay [lower than the maximum level 1 to 20 µg L^{-1} of aflatoxin B_1 (the level required officially by the European Union)]. For further experiments, the indirect immunoassay format was chosen because of its present higher current response, with a wide current range and still producing a low background reading**.**

The calibration curves were conducted on the gold surface for AFB_1 detection via physical adsorption immobilisation. It was simplest and rapid, just requiring incubation of the reagents to be immobilised on the support. However, the orientation of the specific binding is dependent on the strength of interaction between the gold surface and the molecules immobilised. The random antibody orientation can lead to the obstruction of binding sites and may cause a loss of affinity or activity. Thus, covalent immobilisation on a chemical modified gold surface needs to be developed as it allows a more stable layer and builds the correct orientation of the binding sites (Bilitewski, 2006). In order to cover all the points above, attachment of chemical compounds for covalent immobilisation on the gold working surface was investigated.

However, the multi-stage screen printing DEK model 248 machine in Cranfield University broke down; therefore the work of using these sensors had to stop. To continue with covalent binding immobilisation, further experiments were continuously run using the DuPont sensors.

3.3.9 Competitive assay on SPGE DuPont

3.3.9.1 Characterisation of the formation of thiols monolayer

To enhance the sensitivity of the immunosensor system, covalent antibody immobilisation using a thiol groups chemical by the formation of a thiolate bond onto the gold surface was applied. Attractive van der Waals forces between the alkyl thiol chains enhance the stability and orders of the self assembled monolayer (SAM). The

purpose of the study is to increase the orientation of specific binding antibodies/antigens and enhance the stability of the immobilised reagents.

To choose which thiol groups are suitable for electrochemical immunosensor detection, three types of thiol compounds were investigated. These were: long-chain alkanethiol (11-Mercaptoundecanoic acid) and shorter chains (Lipoic Acid and Dithiodipropionic Acid). The characterisation of the thiol groups was constructed by cyclic voltammetric analysis using 5 mM potassium ferrocyanide solution in 0.1 M KCl to investigate the properties of the formation on the gold surface. Generally this redox behaviour of a reversible couple was applied especially for measuring the density and sensitivity of the monolayer.

As shown in Figure 3.16, cyclic voltammograms of redox peaks were reduced at the different lengths of thiols that deposited on the surface compared to bare gold. These indicated that the modified gold surface was covered with the thiols monolayer. The performance of coverage was indicated by the level of reducing of redox peaks. Result show, the long chain of thiols (11-mercaptoundecanoic acid) present smaller redox peaks than those with shorter chains (Lipoic acid and Dithiodipropionic acid), indicates more ordered alkanethiol covered on surface. Therefore, the long chain with the –COOH thiol terminal on the surface will provide more antibody immobilisation in order to increase the orientated antibody binding.

In many cases in immunosensor development, 11-mercaptoundecanoic acid has been used to generate carboxylic groups on the gold surface by self assembling of the thiol compound due to increased protein immobilisation and reduced nonspecific adsorption (Lee *et al*., 2005; Bilitewski, 2006; Silva *et al*., 2008). However, it can be deduced that the current was much lower than that of the short chain thiols modified where the current could not penetrate to the surface (Brito *et al*., 2003). In this case, the antibody binding could be increased with the increasing of the thiols surface, but the penetration of electrons from the solution to the electrode was blocked, hence the reduction in current.

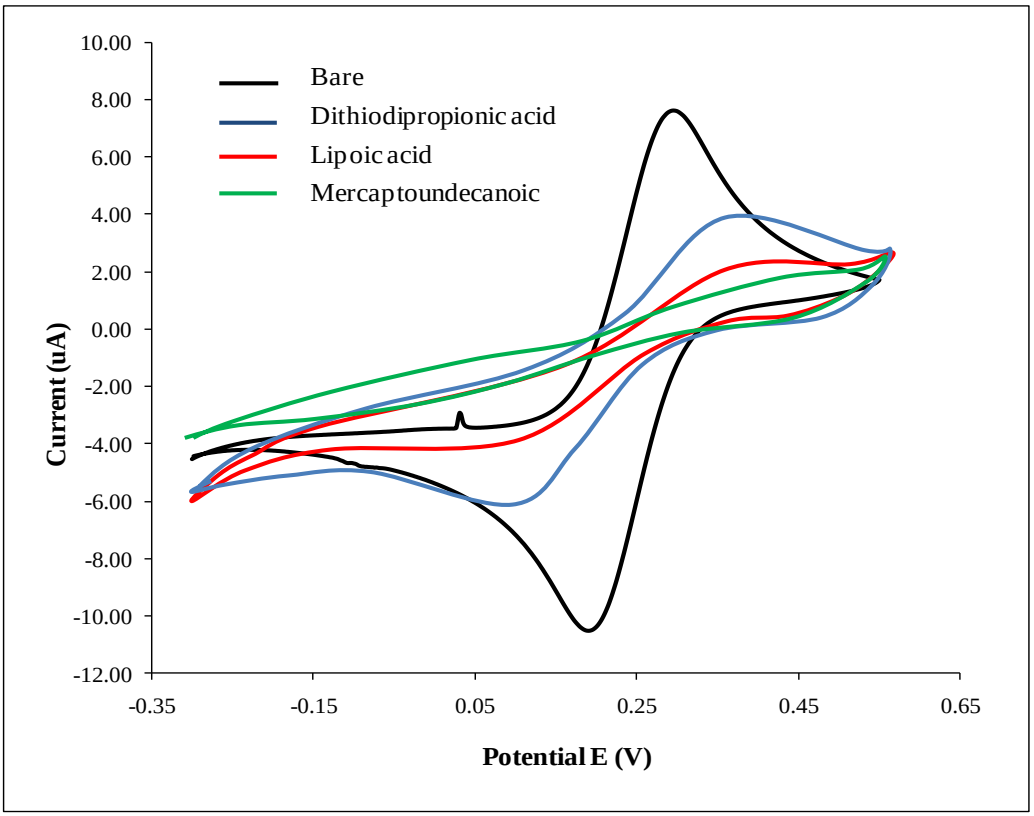


Figure 3.16: Cyclic voltammograms recorded in a 5 mM ferrocyanide solution in 0.1M KCl, versus Ag/AgCl reference electrode after deposition with different thiols compound in comparison with bare gold electrode. The scan rate was set at 20 mVs^{-1} and set potential was set between -0.4 to 0.5V

In the case of the electrochemical detection, it was measured using the amperometry technique which needed to measure the reduction of current on the electrode surface. Figure 3.16 shows that the 3,3-dithiopropionic acid (DTDPA) allowed the current to penetrate through the monolayer and undergo electron transfer on the surface, which is better than lipoic acid modified. This could be observed from the cyclic voltammograms which showed a higher redox peak than 11-mercaptoundecanoic acid and lipoic acid. Based on this, the short chain of (3,3-dithiodipropionic acid) still covered the surface and gave a good current transfer; it was chosen for the study of antibody immobilisation in order to increase the sensitivity of the immunosensor.

The illustration of the covalent antibody immobilisation through amine coupling on the SAM thiol sulphides modified surface is presented in Figure 3.17. The SAMs was formed by alkanethiols through the formation of a strong gold metal-thiolate bond on

the gold surface and the provision of a free carboxylate group for linkage with the protein. Because of the stability, orientation and ability to functionalise the terminal groups on the molecules they can offer a very convenient method for immobilisation of biomolecules to gold surfaces especially for this immunosensor development (Vaughan *et al*., 1999). The SAMs thiol modification using 3,3-dithiodipropionic acid method is most frequently used in optical immunosensors (Vaughan *et al*., 1999; Wong *et al*., 2002; Park *et al*., 2004; Mazumdar *et al*., 2007) but not in electrochemical immunosensors. The covalent immobilisation through this method for calibration curve of AFB_1 detection using electrochemical detection was investigated.

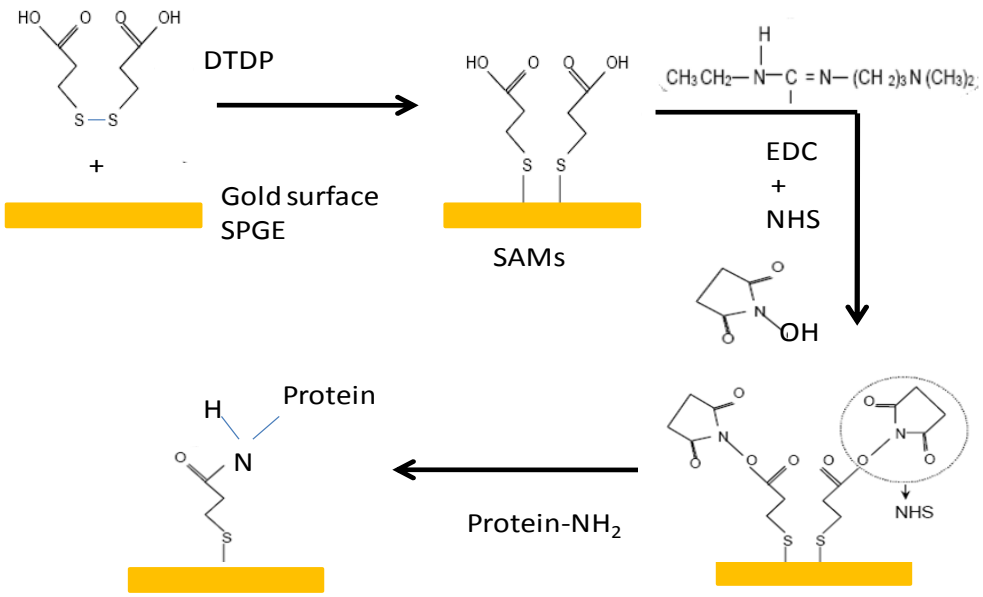


Figure 3.17: Immobilisation of a protein (AFB_1-BSA) to the gold surface through an activated SAMs thiol modified surface. DTDP = 3,3-dithipropionic acid, EDC=1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride, NHS = N-hydroxysuccinimide.

3.3.9.2 Passive adsorption and covalent immobilisation calibration curve

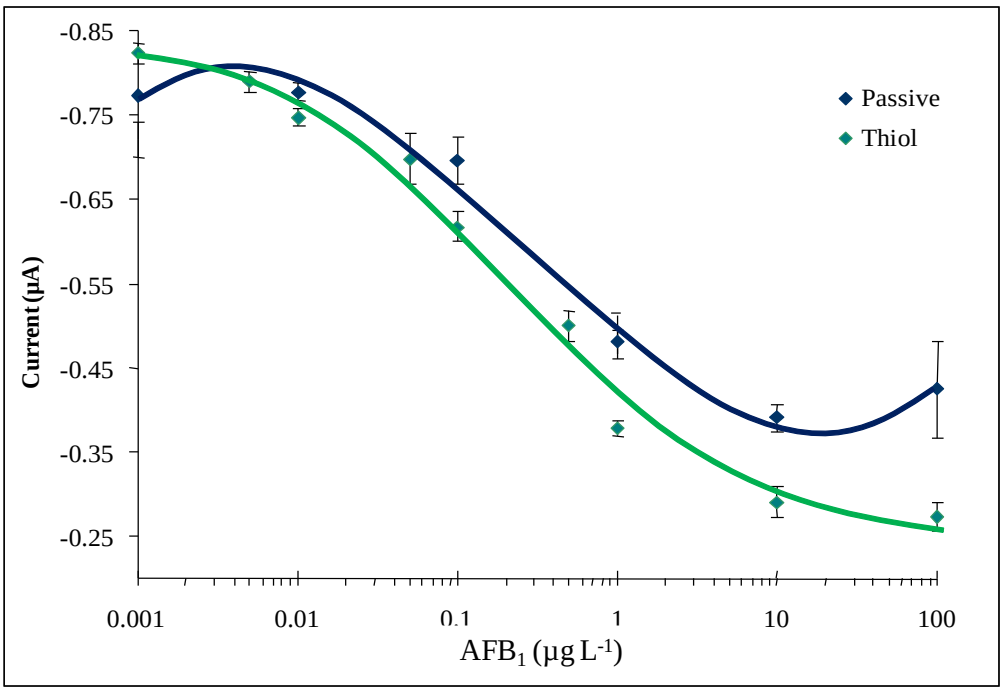
A repeat of the indirect competitive assay experiment for a calibration standard curve construction using gold ink electrodes was carried out in order to compare the

sensitivity of the assay. With the same concentration of reagents and conditions, it was subsequently transferred to the SPGE DuPont. In this study, passive and covalent immobilisation were employed. About 5 mM of DTDPA in ethanol was coated on the surface before it was immobilised with antibodies and antigen. The calibration curve were prepared prior to each assay by using 10 serial dilution of AFB_1 standard solution with concentrations in the range 0-100 µg L^{-1}.

Figure 3.18 presents different plots of the calibration curve at different surface immobilisations (passive and covalent). From the Figure, with the increasing of AFB_1 concentration, a decreased change in current was obtained. Therefore, a decrease in current, with the high amount of free AFB_1 bound to the $MAbAFB_1$ was observed and that provided fewer antibodies bound to immobilise the AFB_1 conjugate on the surface.

The data of the plots was then illustrated in Table 3.2 by non-linear regression and linear regression as well as LOD calculated where the sensitivity of the immunosensor could be observed. Based on this, the treated and untreated surface was found similar in linear range of detection and coefficient of determination (R^2) with a small value of coefficient of variation (CV%). A low detection limit was also obtained, which was below that of using the Ercon immunosensor. According to Seurynck-Servoss *et al*, in various studies on immobilisation methods either covalent or physical adsorption has been found to have similar binding depending on the types of surfaces (Seurynck-Servoss *et al*., 2007).

However, in this case, the competitive immunoassay on the thiol modified surface presented a low background reading and showed high sensitivity of AFB_1 detection in buffer. A low background reading (nonspecific binding) with a higher specific binding antibody on the modified surface is an important factor for enhancing the LOD. According to Peluso *et al*, the increasing of orientated antibody attachment through covalent binding also can improve the binding activity (Peluso *et al*., 2003).



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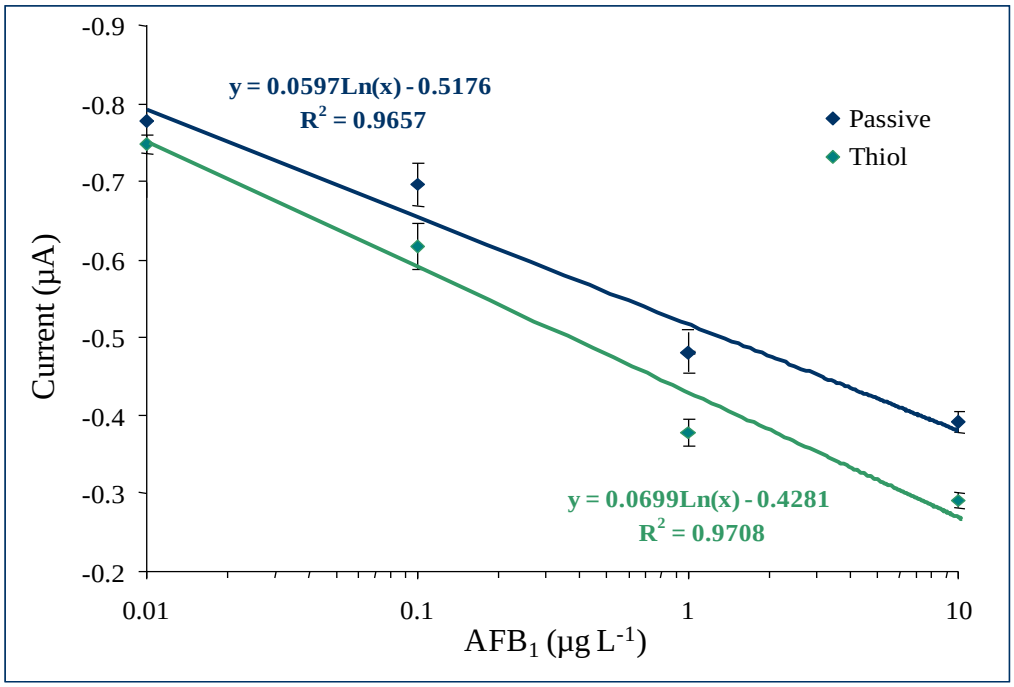


b

Figure 3.18: (**a**) The competitive response curve for AFB_1 detection by passive adsorption and covalent binding (Thiol modified) on gold working electrode (SPGE Ercon). (**b**) Linearity graph for AFB_1 detection. Current measurement was by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Screen-printed gold electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MabAFB_1$) (10 µg mL^{-1}) and free AFB_1 (0 to 100 µg L^{-1}) before continuing with anti-IgG-HRP (1 µg mL^{-1}). Error bars = SD, n = 3.

Table 3.2: Comparison data within covalent and passive adsorption immobilisation in indirect competitive assay on bare and thiol-modified SPGE.

Immobilisation surface	working range (µg L^{1})	LOD (µg L^{1})	R^2	CV%
Passive	0.01 to 10	0.006	0.97	4.5
Thiol-Modified	0.01 to 10	0.005	0.97	3.2

n=3

In this experiment the potential of using SAMs of short chain thiol (3,3-dithiopropionic acid) as a precoating for covalent antibody immobilisation has been demonstrated. The most important factor here is that the surface coated can still allow the diffusion of electrons to the surface.

There is no report on the previous study on electrochemical detection for AFB_1 detection on the DTDPA thiol monolayer application on a gold surface. However, the sensitivity of the detection compared with other electrochemical immunosensor detection is listed in Chapter 6. Therefore, this electrochemical immunosensor based on thiol modified on gold screen-printedelectrode was successful and the limits of detection are still lower than ELISA and cover the permitted level.

According to these results, both of the immobilisation strategies will be used in the next step for the improvement of the immunosensor performance with gold nano-particles.

3.3.10 Application of gold nano-particle to enhance the sensor response.

3.3.10.1 Formation of immuno gold nano-particle for signal amplification

The use of functionalized gold nano-particles to achieve better sensitivity compared to conventional electrochemical immunosensors was investigated. These gold nano-particles were targeted as a smart system for interfacing biomolecule recognition in the electrochemical of nano-immunosensors. Due to their large surface area and high surface energy (Luo *et al*., 2006), these gold nano-particles can absorb protein strongly. In this study the HRP and antibody were immobilised on the gold-nano-particle (Figure 3.18), and then attached to a bare and modified thiol monolayer gold surface. The purpose of the study is to improve the immunosensor signal using this gold screen-printed electrode, in order to improve the immobilisition steps (fewer steps) and sensitivity of the sensor. Currently, in immunosensor development, gold nano-particles have also been employed to enhance the immunosensor signal in Surface Plasmon Resonance (SPR) or Quartz Crystal Microbalance (QCM) responses (Uludag & Tothill, 2010).

Figure 3.19 illustrates the application of gold nano-particles in amplification immunosensor signals using the ELISA assay format. A theoretically amplified immunosensor signal is based on the increasing of enzymatic activity on the surface by the increase of enzyme label binding to the surface. Therefore, the increased amounts of HRP molecules on gold nano-particles will increase the enzyme-substrate reaction and will substitute a limited HRP molecule to one antibody molecule in conventional system.

In this procedure, the application of anti-IgG-HRP conjugate was replaced with gold nano-particle conjugate with anti-AFB_1 antibody ($MAbAFB_1$) and HRP. The gold-nano particle conjugate is prepared based on the adsorption of the HRP and antibody onto the gold nano-particles through a combination of ionic and hydrophobic interactions. Since most of the nano-particles carry charges, they can electrostatically adsorb biomolecules with different charges.

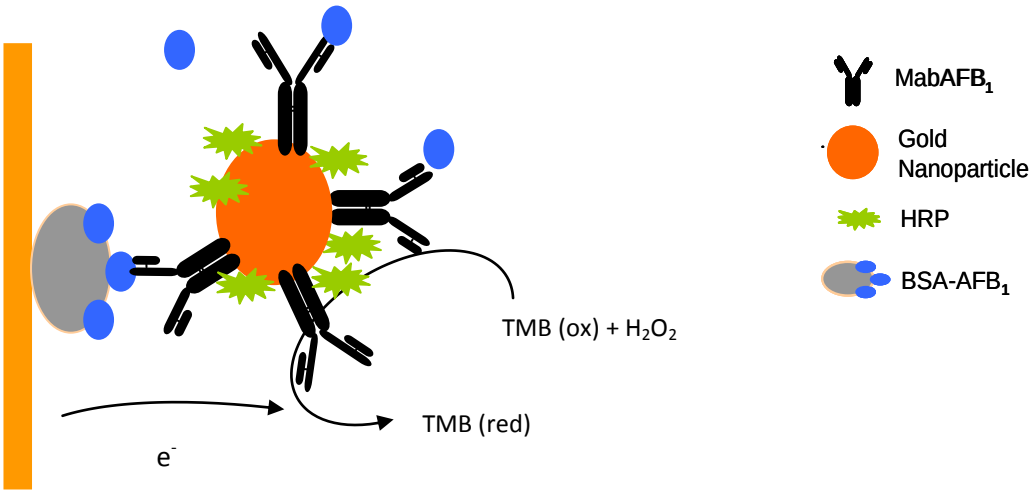


Figure 3.19: Schematic diagram of the assay formation of AFB_1-BSA immobilised and coated immuno-gold colloid conjugated HRP on working surface of SPGE for amplification of immunosensor signal.

The complete immunoassay format using immuno gold nano-particles for competitive detection was present as well in Figure 3.19. The bare and modified gold surface was coated first with AFB_1-BSA before adding the blocking reagent (1%PVA) then the competition within free AFB_1 and immuno gold nano-particles. Using this format for the development of the electrochemical immunosensor provided a new technique with fewer immobilisation steps (simple test) compared to the indirect immunoassay method.

3.3.10.2 Electrochemical characterisation of the electrode with nano-particles

Cyclic voltammetry

The electrochemical characteristic of immobilised protein and coated immuno gold nano-particles on the support electrodes was studied using CV in the presence of $Fe(CN)_6$. These experiments were run to obtain information about reagent immobilization and electrons transferred activity. Figure 3.20 shows the cyclic voltammograms of bare electrodes (SPGE), electrodes with immobilised BSA-AFB_1,

BSA-AFB_1 and gold nano-particles (NP) (SPGE/BSA-AFB_1/NP), SPGE/ BSA-AFB_1/immuno gold nano-particles (NP-Ab) and SPGE/ BSA-AFB_1/immuno gold nano-particles conjugated HRP (NP-Ab-HRP). All the electrodes represent a reversible cyclic voltammogram at different levels of the peak currents.

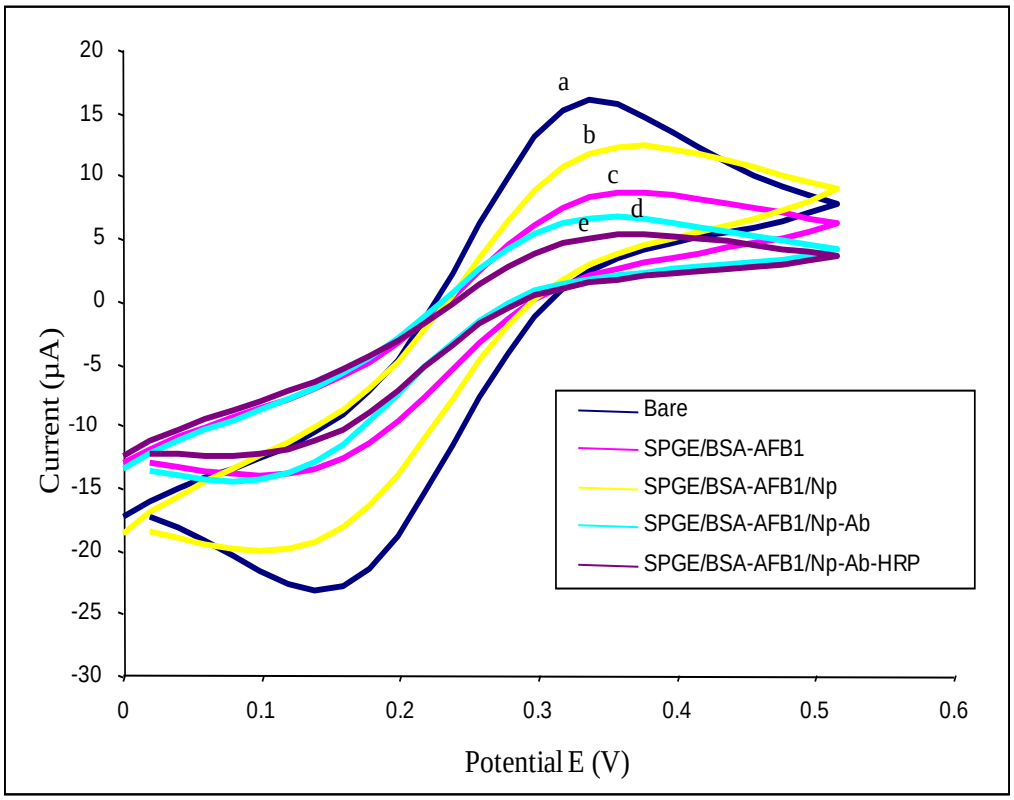


Figure 3.20: Cyclic voltammograms of the different electrodes in the present of 5 mM $Fe(CN)_6$ in 0.1M KCl on (a) bare gold electrode (SPGE), (b) SPGE/BSA-AFB_1, (c) SPGE/BSA-AFB_1/NP (d) SPGE/BSA-AFB_1/NP-Ab (e) SPGE/BSA-AFB_1/NP-Ab-HRP. The CVs was measured at scan rate 40 mVs^{-1}.

A high peak current of CV (curve a) was observed on the bare electrode, which suggested that the electrons transferred activity was taking place on a clean surface area. The CV (curve c) was then reduced after the protein was immobilised on the electrode system. This indicates that the protein was covered on the support area in a sort of way and thus blocked some redox reaction. Whenever the gold nano-particles (NP) were deposited on the surface, the CV current was increased (curve b). According to Luo *et al*, (2006) this phenomenon suggests that nano-gold is an important material for enhancing the electron transfer between redox proteins and electrode surfaces similarly to electrical wires.

After the immuno gold nano-particles were coated on the working electrode, the redox peak CV decreased (curve d), shows that the antibody had been immobilised on the NP and covered the electrode surface. In the case of the immuno gold nano-particles conjugated HRP on the surface, the CV also showed a decrease in current than those CVs (curve e). This indicates the electrode surface was covered after the HRP was immobilised on the immuno gold particle. However, the redox reaction in curve (e) showed that electrons can penetrate on the surface. The electrons transfer may be in between the redox proteins on the gold particle surface and the electrode surface.

This experiment shows that the protein molecules (antibody and enzyme) had been adsorbed on the gold nano-particle.

Chronoamperometric

The test using chronoamperometry, as seen in Figure 3.21, shows that different current responses were achieved through the use of TMB/H_2O_2 as the enzyme substrate system. The current produced was amplified using an immuno-gold nano-particle-HRP conjugate at -100 mV vs. onboard Ag-AgCl pseudo-reference electrode. The current response increased when HRP was present on the electrode, which was in step 5 and step 6. The phenomena of electroactive reaction has been discussed in section 3.3.4. According to Fu (2008), this phenomenon suggests that HRP could keep electrocatalytic activity and catalyze the reduction current on the surface.

The Figure shows the increase of current response with the application of the gold nano-particles HRP and the antibody (step 5) compared to conventional immunoassay (step 6). The increasing of the amount of HRP immobilised on the conjugated gold nano-particles may raise the enzymatic reaction on the surface. This is because the large surface area of nano-particles can normally increase the amount of HRP adsorbed on the nano gold surface as an enzyme label (Luo *et al*., 2005). According to Luo *et al*, the conductivity properties of nano-particles make them suitable for enhancing the electron transfer between the active centre of enzymes and electrodes acting as electron transfer mediators (Luo *et al.*, 2005). The increasing of the current response may improve the sensitivity of the assay.

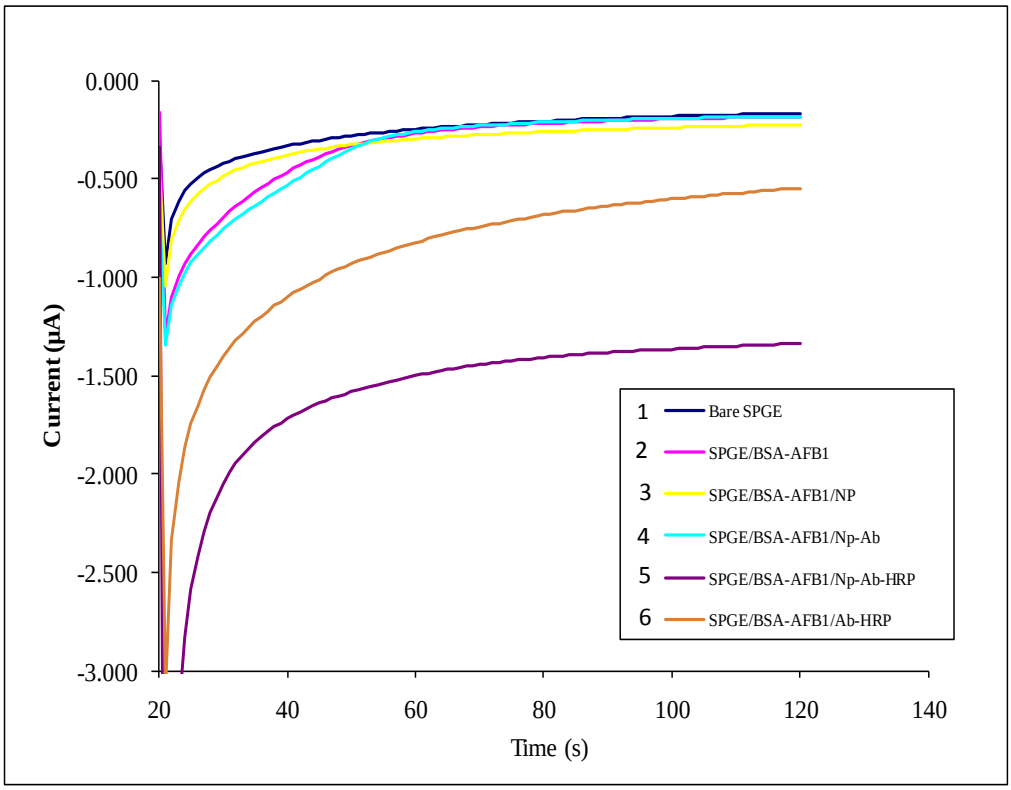


Figure 3.21: Chronoamperogram of the different electrodes present in 5mM TMB and 0.075% H_2O_2 in 0.05M phosphate citrate buffer prepared in 0.1M KCl on (1) bare gold electrode (SPGE), (2) SPGE/BSA-AFB_1, (3) SPGE/BSA-AFB_1/NP (4) SPGE/BSA-AFB_1/NP-Ab, (5) SPGE/BSA-AFB_1/NP-Ab-HRP and (6) SPGE/BSA-AFB_1/Ab-HRP. The chrono-amperometry measurement was recorded at -100mV constant potential.

3.3.10.3 Optimisation of immuno gold nano-particle sensor

In order to maximise the current response of the assay, the immuno gold nano-particles labelled with HRP was optimised. In this case, the nano-particle conjugated proteins were prepared first using a fixed concentration of 10 µg mL^{-1} anti-aflatoxin B_1 antibody ($MAbAFB_1$) and then the range of 1 to 1000 µg mL^{-1} of HRP onto the different dilutions of gold colloids containing nano-particles size 20 nm in diameter. The non-competitive assay was constructed based on the indirect immunoassay format on the gold surface SPGE.

The different ranges of HRP were optimised first, based on the maximum concentration of gold colloid solution at a fixed concentration of captured $MAbAFB_1$.

After the incubation and centrifugation, the HRP bound to the antibody gold particles was assayed with TMB-H_2O_2 substrate system in order to measure the HRP activity which was correlated with the HRP bound to the antibody-gold particles. From Figure 3.22, it can be seen that the current response was dramatically increased when the HRP concentration was increased from 1 until 100 µg mL^{-1} showing a maximum HRP binding to the antibody-gold particles. This indicates that the maximum HRP concentration bound to the immuno gold nano-particles to achieve a higher current response was 100 µg mL^{-1}.

The optimal concentration of gold colloid solution was also investigated, based on a fixed amount of antibody (monoclonal anti-aflatoxin B_1 antibody) and immobilised enzyme (HRP). Figure 3.23 shows that the current response decreased with the decreasing of gold colloid concentration. The decreased concentration of gold colloid reduced the amount of nano-particles, which may reduce the protein immobilisation.

This undiluted gold colloid produced the maximum amount of nano-particles that can provide a large surface area for the orientation of $MAbAFB_1$ and HRP absorbed compared to diluted gold colloid. Therefore, the higher current response for 10 µg mL^{-1} of anti-aflatoxin B_1 antibody and 100 µg mL^{-1} of HRP adsorbed to gold-particles was undiluted gold colloid concentration. The high amounts of HRP bound on the nano-particle surface may influence the redox-enzymes activity which provides a high current response of the sensor (in Section 3.3.10.2). Based on the results achieved, a 100 µg mL^{-1} of HRP conjugated to the undiluted gold colloid coated with a 10 µg mL^{-1} of anti-aflatoxin B_1 antibody was used to prepare the immuno gold nano-particle conjugate for immunosensor construction.

3.3.10.4 Competitive assay using immuno gold nano-particles

The immuno gold nano-particle conjugate HRP concentrations were used in immunosensor construction with the indirect ELISA assay format and were applied for both type of immobilisation (physical adsorption and covalent immobilisation by thiol monolayer) to construct the AFB_1 calibration curve.

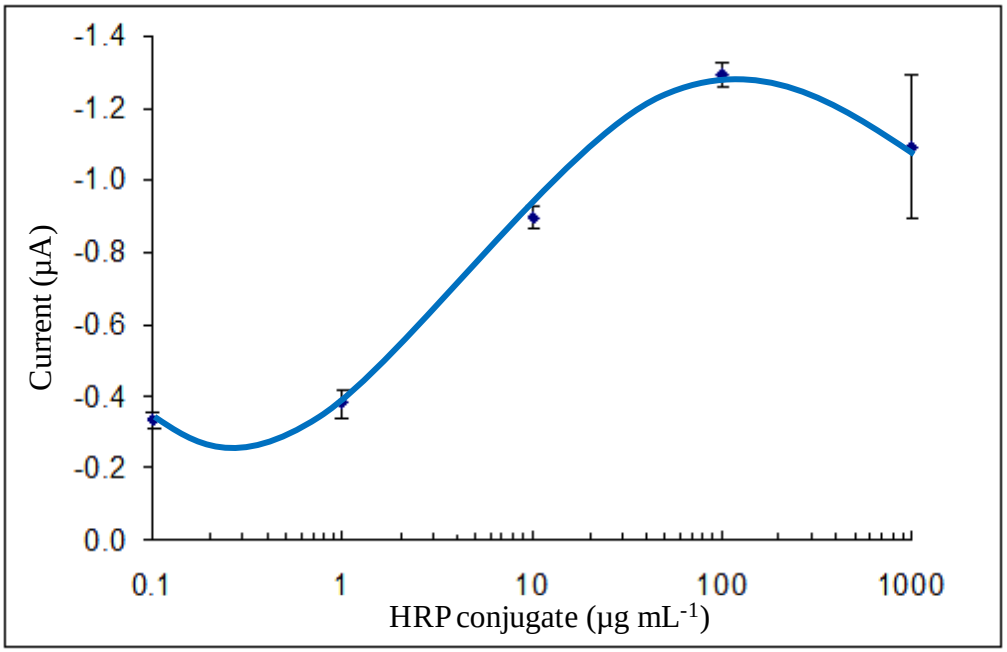


Figure 3.22: The current response of the different HRP concentrations conjugated to immuno gold nano-particles in an indirect non competitive assay. The gold working electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by immuno gold nano-particle conjugated HRP. The detection was by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). Error bar=SD, n=3.

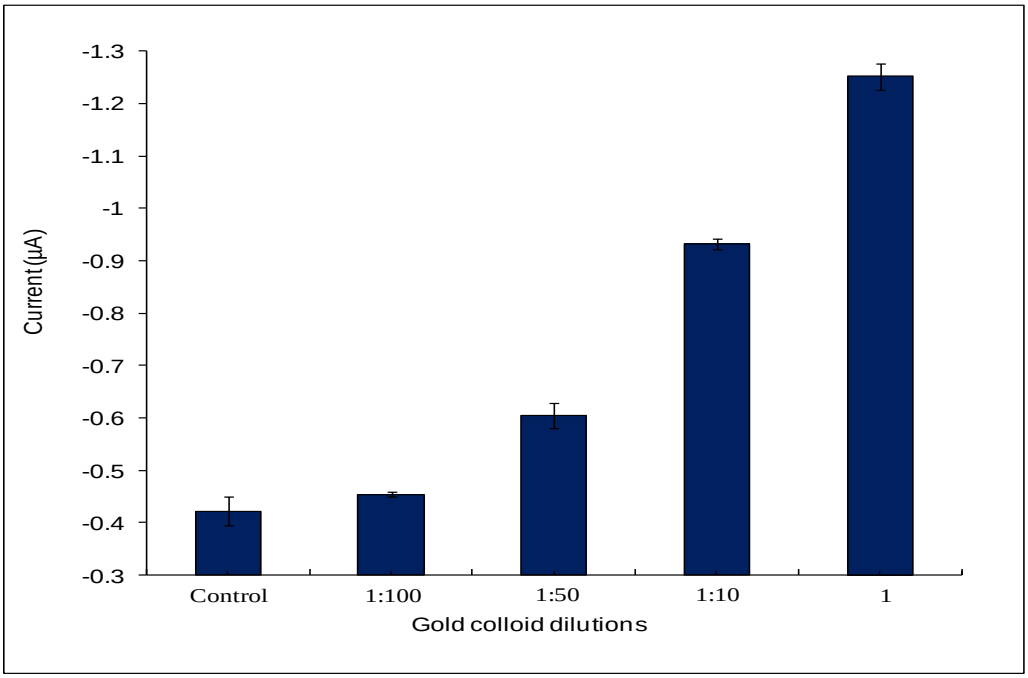


Figure 3.23: The current response of the different gold colloid concentrations for antibody and HRP immobilised on nano-particles, in an indirect non competitive assay. The gold working electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by immuno gold nano-particle conjugated HRP. The detection was by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). Error bar=SD, n=3.

The competition system was employed within free AFB_1 concentrations at a range of (0 to 100 µg L^{-1}) and immuno gold nano-particle-HRP through optimal conditions and concentrations of reagents as described previously.

Two different protocols were investigated: first, free AFB_1 and immuno gold nano-particle-HRP were mixed together and directly onto the surface (for rapid method); second, pre-incubation was carried out first for 30 minutes, then adding onto the surface. Figure 3.24 shows the signal response was increased when the pre-incubation step for 30 min within free AFB_1 and immuno-gold nano-particle conjugated HRP was applied to the sensor surface. A one-fold increase of signal/background ratio resulted from this study compared to without a pre-incubation step in the competitive assay. In this case, the sensitivity of the analysis may increase with the increasing of the specific binding of the AFB_1 with the immuno gold nano-particle sites. This technique has been used especially for promoting the binding of the free AFB_1 with the antibody before the competition was applied (Micheli *et al*, 2005).

A typical standard curve of AFB_1 using the application of nano-particles based on the indirect competitive format assay was obtained in Figure 3.25. In this Figure, the sensitivity of the assay was compared using physical AFB_1-BSA adsorped and immobilised using SAM thiol monolayer on the SPGE. As can be seen from the Figure, with increasing AFB_1 concentration the decrease change in current was obtained. For the physical absorption, the current increase was inconsistent with decreasing AFB_1 concentration (R_2=0.87) compared to covalent immobilisation on the surface (R_2=0.94). This is due to the unstable immuno gold nano-particle conjugate HRP immobilisation and unorientated antibody bound to analytes on the gold surface. With the covalent immobilisation, the specific binding antibody is more stable and more properly orientation binding. This is to maximise the ability of specific binding at the lower concentration of analyte. According to Templin *et al*., the advantages of using a modified surface of covalent binding over passive adsorption is to anchor the proteins to a substrate surface which provides a high random orientation of antibodies binding (higher binding capacities) (Templin *et al*., 2002).

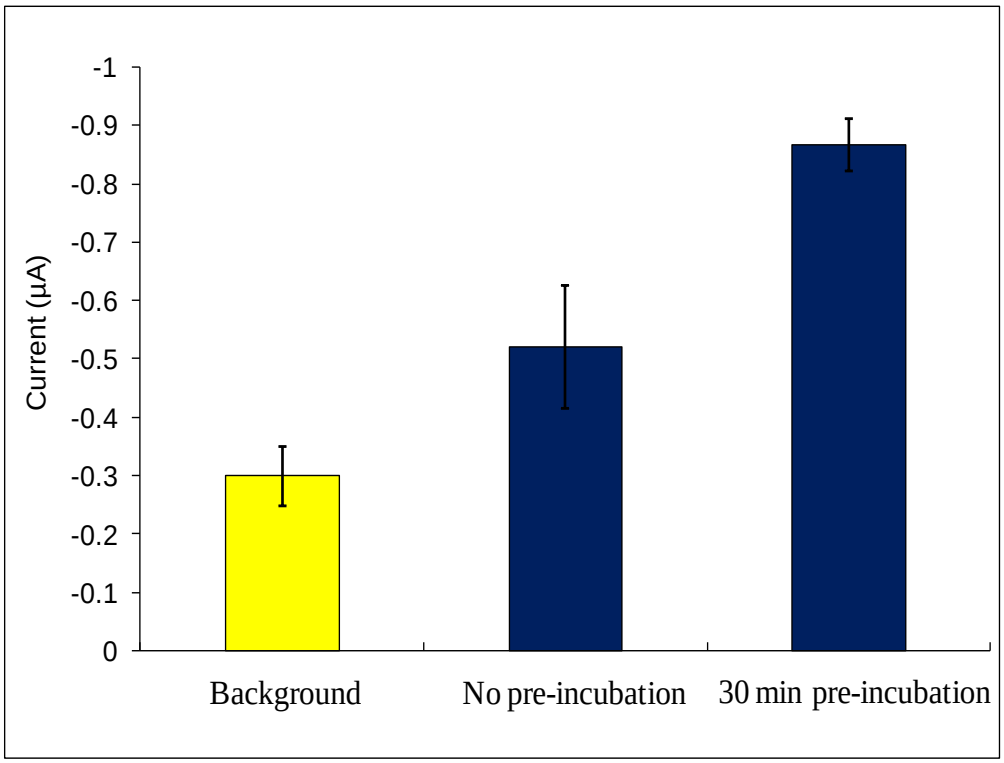
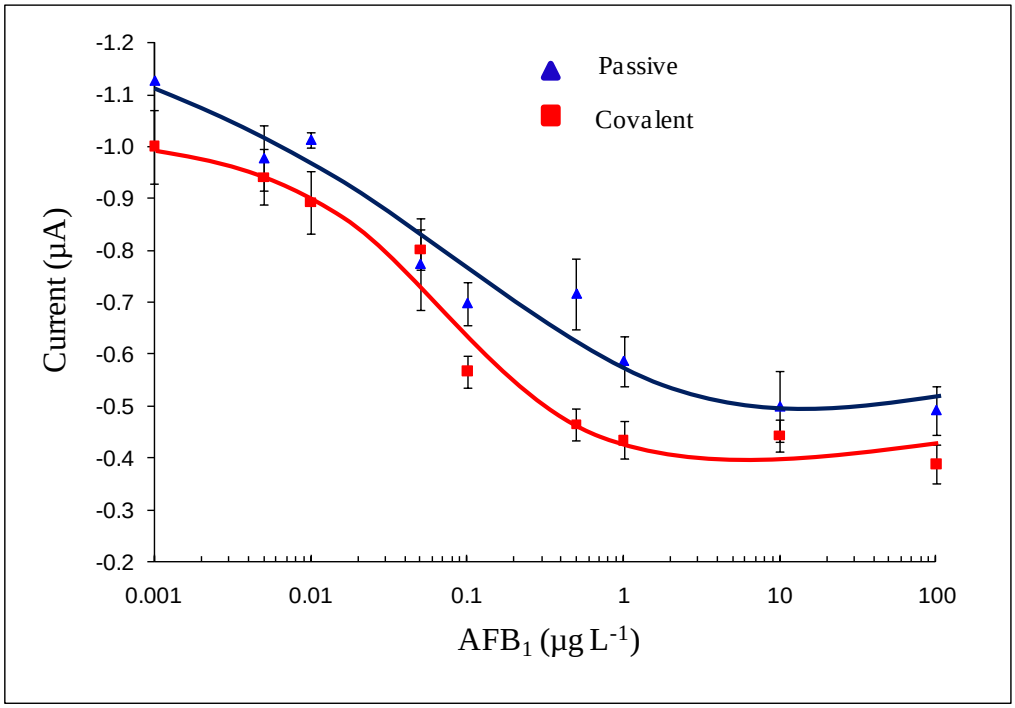


Figure 3.24: Current response of indirect competitive assay; with no pre-incubation and 30 minute pre-incubation with the sample. The gold working electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA and followed by the competition of 0.01µg L^{-1} free AFB_1 and immuno gold nano-particle conjugated HRP. The detection was by chronoamperometry (-100 mV) and using TMB (5 mM) and H_2O_2 (0.075%). Error bar=SD, n=3.

However, the results show that a slightly lower signal response was present by the SAM thiol monolayer compared to physical adsorption. This might be due to random self-assembly of the thiol and protein immobilised causing it to form an electrically blocking structure (Ivanova *et al*., 2006). With the covalent immobilisation method, the background current was lower and the signal achieved still higher. Thus, a high range of signal/background was obtained when the competitive reagents were deposited on gold coated by the SAM thiol monolayer (~ 3 unit S/B) compared to physical adsorption (~ 2.3 unit S/B).



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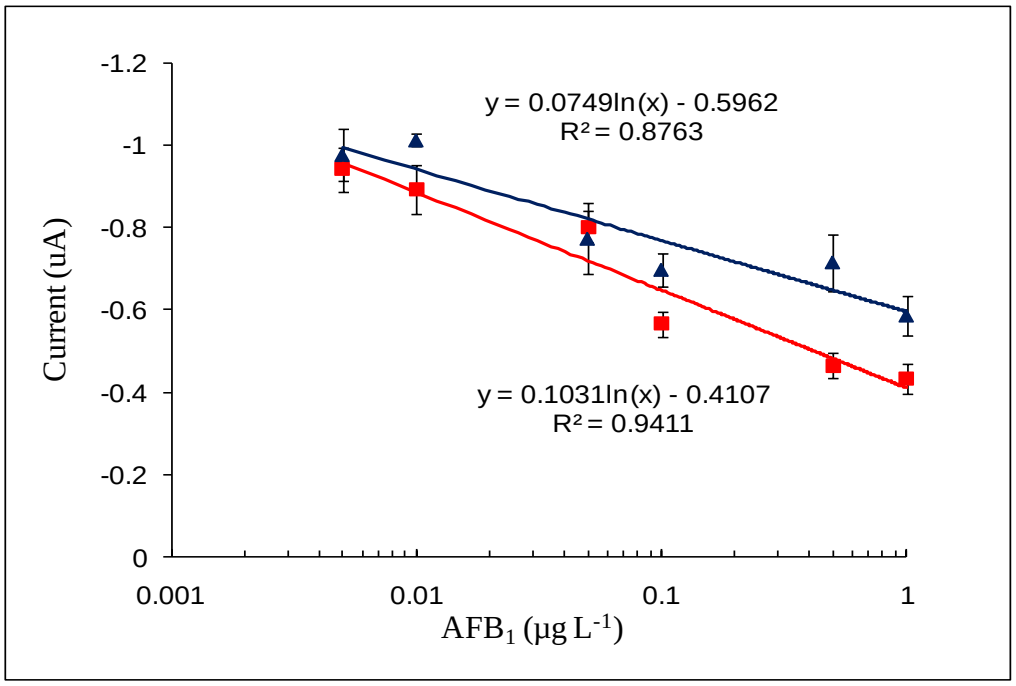


b

Figure 3.25: (a) The competitive response curve for AFB_1 detection using immune gold nano-particles through passive adsorption and covalent binding (Thiol modified) on gold working electrodes (SPGE Dupont). (b) Linearity graph for AFB_1 detection. Current measurement was by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Screen-printed gold electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by immune gold nano-particles and free AFB_1 (0 to 100 µg L^{-1}). Error bar=SD, n=3.

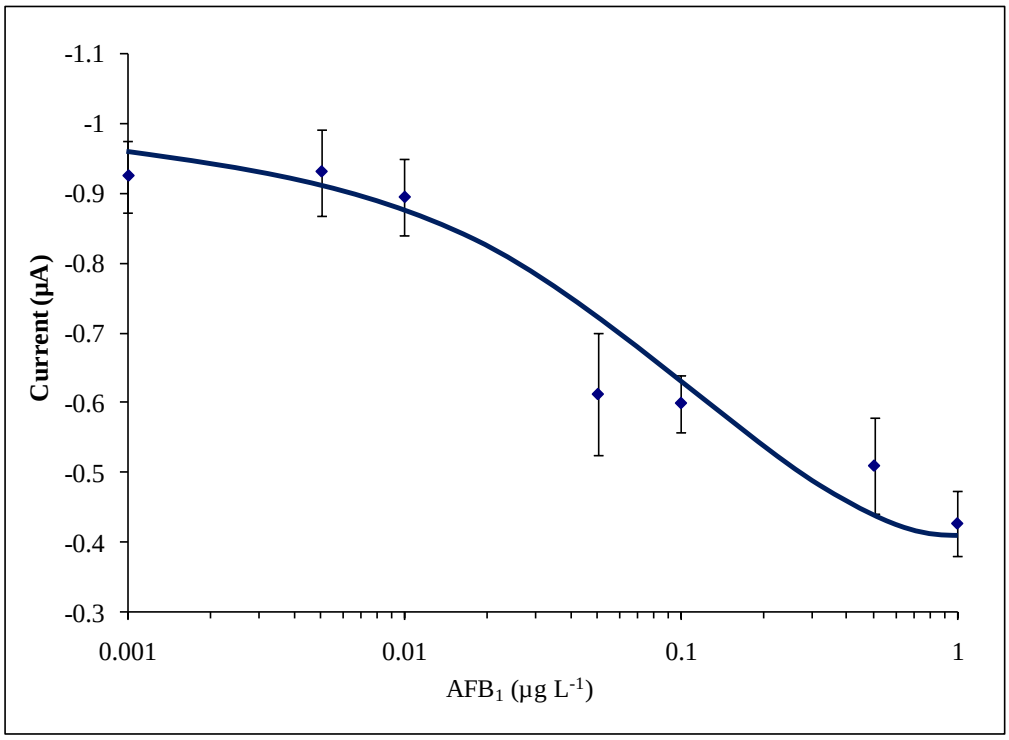
Due to the stability and higher signal obtained from the application of the immuno gold nano-particles, the immuno gold nano particle sensor through covalent binding presented a R^2 value of 0.94 and %CV of 6.8 with LOD of 0.001 µg L^{-1}. This can be compared to passive adsorption with R^2 value 0.88 and %CV of 8.6 and LOD was 0.002 µg L^{-1}. Both sensors have a working range of 0.005 to 1 µg L^{-1}. The calculation was fitted using a non-linear four-parameter logistic calibration curve as described previously. The sensitivity of the immunosensor detection using different approaches of nano-particle application for AFB_1 detection has been previous reported (Liu *et al.*, 2006; Owino *et al.*, 2008; Sun *et al.*, 2008). In this case, the different approach of nano-particle immobilisation (different format), the reduced of immobilisation step (more simple) with increased the sensitivity assay was achieved.

3.3.11 AFB_1 detection in peanuts

3.3.11.1 Competitive method of detection used with sample matrix

The performance of the immuno gold nano-particle sensor for the determination of AFB_1 content in peanut sample was then conducted. In this study, we chose peanut as a model system for examining the feasibility of applying the proposed immunosensor in real samples since peanut is one of the most contaminated commodity with aflatoxins producing fungus. Normally, the extract of food sample especially in solid based samples contains several matrix components, which can interfere with the immunoassay results, causing false positive results (Acharya and Dhar, 2008). Therefore, studies were carried out to assess the possible matrix interference in the presence of AFB_1 (spiked) in non-infected peanut extract. The non-infected peanut sample was then extracted using 70% of methanol and diluted by 1:1 in PBS for preparing a standard solution. The calibration curve of AFB_1 in peanut extract was then developed using these peanut samples.

The calibration curve results presented in Figure 3.26 show a limit of detection of ~ 0.001 µg L^{-1}, R^2 value was 0.94 and %CV ~ 9 with the linear range of 0.005 to 1 µg L^{-1}. The resulting data was found from the non linear and linear regression of calibration curve obtained. As compared to the immuno gold nano-particle sensor in



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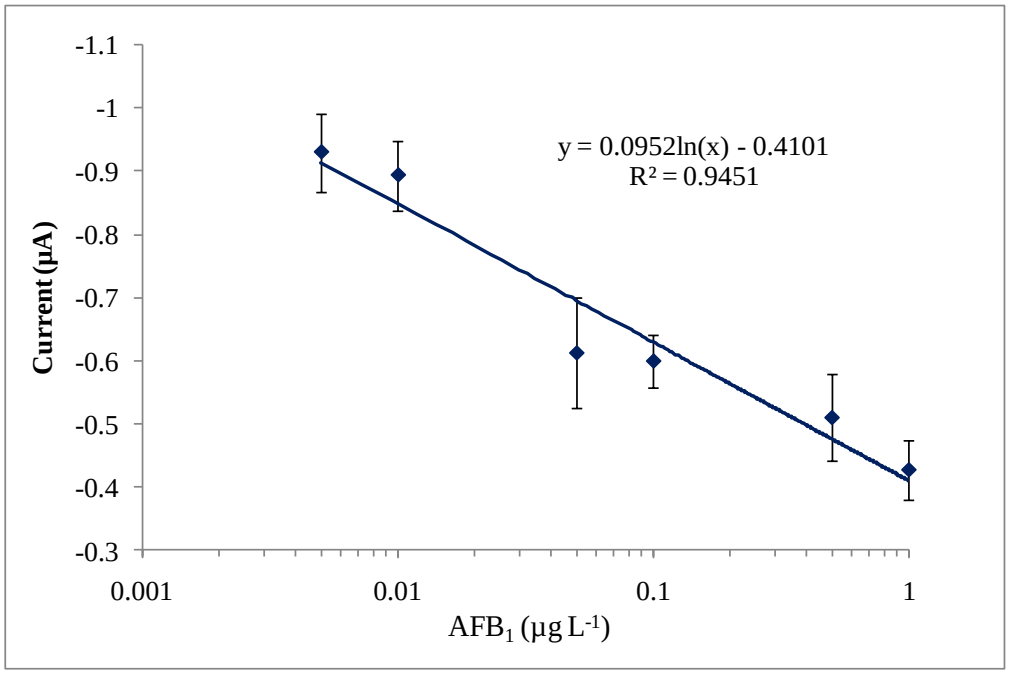


b

Figure 3.26: (**a**) The competitive response curve for AFB_1 detection in non infected peanut extract using immuno gold nano-particles through covalent binding (Thiol modified) on gold working electrodes (SPGE Dupont). (**b**) Linearity graph for AFB_1 detection. Current measurement was by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Screen-printed gold electrodes were immobilised with AFB_1-BSA (1 µg mL^{-1}), blocked with 1% PVA followed by immuno gold nano-particles and free AFB_1 (0 to 1µg L^{-1}). Error bar=SD, n=3.

buffer, a result was similarly found, which showed no or low matrix interference in the present assay. There is therefore no significant difference in current intensities between the sample extract and the assay buffer.

The dilutions of peanut extract with 1:1 of assay buffer also reduce the matrix interference. Mainly, if there is any low matrix interference on the assay, it is from sample co-extractants interfering with the enzyme reaction on the enzyme conjugate (Acharya & Dhar, 2008).

3.3.11.2 Sample analysis

In this section, the proposed electrochemical immunoassay and HPLC method for the determination of AFB_1 in peanut samples were carried out. HPLC analysis is an important method for confirmatory tests of any proposed method developed. For this, uninfected peanut extracts were spiked with AFB_1 ranging from 0 to 250 µg kg^{-1}, extracted, diluted with buffer and assayed, in order to monitor the performance of the sensor and analytical recovery. At the same time, unknown AFB_1 content in real samples were also extracted and analysed in order to further investigate the possibility of the newly developed method.

In our case, the assay was carried out with and without clean-up processes after extraction steps with the purpose of providing a rapid and simple procedure similar to the AFB_1 ELISA kit. Then, analyses of samples were carried out using normal calibration curves of peanut extract of HPLC and immunosensor. The AFB_1 content was calculated on the basis of the peanut extracted regression equation. For each concentration, three replicates were applied for the detection.

The calculated recoveries for with and without clean-up using the gold nano-particle immunosensor are shown in Table 3.3. This gives varied results from 93.2 to 118 % and from 76.9 to 87.8 % with overall %CV of 8.7 and 8.5% respectively, for with and

Table 3.3: The average value and recovery of AFB_1 content from peanuts and peanuts spiked using the indirect gold nano-particle imunosensor and HPLC. Two different extraction methods (with and without clean-up) were analysed using the indirect immuno gold nano-particle sensor and compared with the HPLC method.

Samples	AFB_1 added ($\mu g\ kg^{-1}$)	Measured value ($\mu g\ kg^{-1}$)								
		HPLC			Electrochemical Immuno gold nano-particle sensor					
		Clean-up			without Clean-up			Clean-up		
		Found	%CV	%Recovery	Found	%CV	%Recovery	Found	%CV	%Recovery
Non-infected peanut	0	nd			less than 0.001			less than 0.001		
	4	5.25± 0.3	5.7	131	3.21 ± 0.25	7.8	80.3	4.2 ± 0.42	10	105
	40	40.3 ± 1.7	4.3	100.7	34.36 ± 1.73	5.0	85.9	37.3 ± 1.98	5.3	93.2
	100	106.3 ± 7.3	6.9	106.3	76.90 ± 7.54	9.8	76.9	118 ± 9.23	7.8	118
	250	255 ± 24.7	9.8	102	219.6 ± 24.54	11.2	87.8	240 ± 27.72	11.5	96
Average			6.7	110		8.5	82.7		8.7	103.1
PSM	unknown	8.26 ± 0.82	9.9		4.1 ± 0.9	21.9		7.6 ± 0.45	5.9	
POMM	unknown	250.3 ± 9.65	3.7		233.6 ± 20.12	8.6		240 ± 21.35	8.9	
PTUK	unknown	nd			0.07 ± 0.008	11.4		0.06 ± 0.008	13.3	
POMUK	unknown	nd			0.05 ± 0.007	14		0.03 ± 0.003	10	

PSSM = Peanut Supermarket Malaysia; POSM = Peanut Open Market Malaysia; PTUK = Peanut Tesco UK; POMUK = Peanut Open Market UK. nd = non detected. Average value of three measurements (mean ± SD).

without clean-up samples. Thus, the data demonstrated that the accuracy of the present method was excellent, being in the range of 60 to 120% (classed as valid) (Muscarella *et al*., 2008) and presenting CV less than 10%. The recovery after the clean-up processes was shown to be higher than without clean-up. This is because the foreign substances that cause the interference in peanut extract were removed using the clean up procedure. These foreign substances such as lipids and proteins can usually inhibit the signal response. These materials can have lesser or greater retention than the analytes of interest.

However, the recovery result of without clean-up (simple extraction) method for AFB_1 detection was still acceptable, which indicates the strong specific binding of antibody ($MAbAFB_1$) to the analytes of interest in the content of interference in the peanut matrix.

The analytical performance of the nano-particle immunosensor was then compared to HPLC due to confirmatory tests and validity results for the reliability of the device. In this case, the calculated recoveries for HPLC were in the range of 100.7 to 131% with an average value of 110%. From the average value result of recovery, this indicates that the nano-particle immunosensor had a satisfactory agreement with HPLC. This was confirmed with the application of both methods on the detection of AFB_1 in peanut samples. These data show that there is no significant difference between the results given by two different methods such as samples PSM and POMM. However, the non-detected value of AFB_1 in samples PTUK and POMUK show the value is lower than the limit of detection of HPLC (1-5 µg kg^{-1}) (Shuster *et al*., 1993).

In the case of the immunosensor detection, the non-detected samples from the HPLC can still be detected through the sensor because the values are higher than 0.001 µg Kg^{-1} (limit of detection for nano-particle immunosensor). The present method here was shown a very sensitive sensor device and acceptable for AFB_1 detection.

3.4 Conclusions

This work describes an electrochemical immunosensor of SPGE and the application of gold nano-particles for the detection of AFB_1 in peanut samples. Assays were subsequently transferred from optima concentration and conditions on the ELISA method to the in-house screen-printed gold electrodes. A preliminary study on electrochemical immunosensors by cyclic voltammetry and chronoamperometry measurement has been developed. The use of TMB and H_2O_2 as an HRP substrate for immunoassay with electrochemical detection has been also investigated. The optimal concentrations of TMB and H_2O_2 for enzyme activity were 5 mM and 0.075%, respectively. A suitable constant potential for immunosensor measurement on both electrodes were -100 mV versus Ag/AgCl.

The optimum data for indirect assay were 1 µg mL^{-1} of BSA-AFB_1, 10 µg mL^{-1} of monoclonal antibody and 1 µg mL^{-1} of anti-antibody labelled HRP. For the direct immunoassay the data were 20 µg mL^{-1} of monoclonal antibody, 10 µg mL^{-1} of anti-IgG and 1: 10 dilution of AFB_1 labelled HRP concentration. The indirect competitive method based on passive absorption using SPGE Ercon presented better in current response, wide current range and low background reading compared to the direct method. Furthermore, transferring by the indirect competitive on covalent binding of SAMs DTDPA thiol surface of SPGE DuPont presented a better random orientated antibody compare to passive adsorption. The immunosensors exhibited working ranges of 0.01-10 µg L^{-1} and the LOD was 0.005 µg L^{-1}.

Based on the covalent indirect competitive method using gold nano-particles immobilised with antibody and HRP (immuno gold nano-particle labelled HRP) on the SPGE, the sensitivity of the assay was enhanced, and also fewer steps of incubations were proposed. To construct the competitive immunoassay method, the bio-sensing components of HRP conjugated immuno gold nano-particles were deposited on the immobilised AFB_1-BSA on the thiol surface. The sensitivity of the assay was increased 5 times (LOD = 0.001 µg L^{-1}) when immuno gold nano-particle sensors were used compared to conventional indirect immunosensors. The improvements of the orientation binding and lower non specific binding was seen by employing a SAMs thiol monolayer on the gold working surface rather than passive

adsorption immobilised. The calibration curve displaying a dynamic range, covering a lower concentration of interest and also covering a detection of about 1-20 µg kg^{-1} of AFB_1 (level permitted). The high value of recovery of AFB_1 in peanut spiked showed a better performance of the proposed immunosensor which was around 93.2 to 118 % and 76.9 to 87.8 % for clean-up extraction and direct extraction (rapid), respectively. The reliability of both immunosensor formats for the analysis of AFB_1 in spiked or real samples was verified by comparison of the data with the fully validated confirmatory HPLC results.

We have demonstrated that the electrochemical immunosensor using gold working electrodes and with the application of gold nano-particles for AFB_1 detection shows a working range and a detection limit that were acceptable for detecting levels below the maximum level of regulatory relevance.

CHAPTER 4

DEVELOPMENT OF ELECTROCHEMICAL IMMUNOSENSOR FOR FUMONISINS ANALYSIS

4.1 Introduction

Other than aflatoxin, fumonisin (Fms) is a mycotoxin that is difficult to eliminate from the food chain. Normally, a program of monitoring the surveillance of fumonisin level is therefore necessary to ensure safe exposure, especially in the food chain. Analytical techniques for fumonisin detection are typically by chromatographic methods. This includes thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and post-hydrolysis gas chromatography, and these usually involve extraction procedures and clean-up processes (Sydenham & Shephard, 1996; Shephard, 1998). However, analyses by these methods are a difficult task and require a lengthy sample clean up especially when there are only trace amounts of fumonisins in matrix samples. Working with some chromatography methods also needs a form of chemical derivatization before detection is possible, with the exception of mass spectrometry.

The development of rapid and reliable methods must become a priority to comply with the level requirement. Because of this necessity, a number of fumonisin antibodies have been developed to allow for a faster analysis by immunoassay techniques. Immunoassay methods have been developed using polyclonal and monoclonal antibodies for fumonisin detection because of their adaptability, simplicity and selectivity (Wang *et al*., 2006). In the case of fumonisin detection, many immunosensor have been reported using optical sensing such as surface plasmon resonance (Mullet *et al*., 1998) and fiber optic sensor(Gaag *et a*l., 2003; Daly et al., 2000; Thompson and Maragos, 1999). However, no reports on electrochemical immunosensor methods have been used. This method has been proven to be a sensitive analytical tool, obtaining low detection limits and offering reduced instrumentation costs compared to its optical counterpart.

In this study, the development of electrochemical immunosensors using screen-printed gold electrodes for fumonisin detection was investigated. This is an attractive alternative to enhance the sensitivity compared to the common carbon electrode. The study will describe the immobilization of biological molecules (antibody receptors) on the surface by optimisation of ELISA before transferring to the electrode surface. The principle of immunoassays combined with electrochemicals using screen-printed

electrode (electrochemical immunosensors) has been previously reviewed in Chapter 1 (1.6).

The assay format is a direct system, using first immobilisation by precoating of anti-IgG before coating with anti-fumonisin antibody (monoclonal antibody) followed by competition of free fumonisins and HRP conjugated fumonisins. Normally before all the reagents were immobilised on the electrodes, optimisation by ELISA test through checkerboard method on microwell plate was first investigated. The optimum conditions and concentration of reagents were subsequently transferred to the electrode surface. The electrochemical detection involves chronoamperometry and uses a TMB/H_2O_2 substrate catalysed by horseradish peroxidase that has been widely used for screen-printed immunosensors (Butler *et al*., 2006; Crew *et al.,* 2007).

4.2 Materials and Methods

4.2.1 Chemicals and reagents

Mouse monoclonal antibody against fumonisin (MAbFms) was purchased from Abcam (Cambridge, UK). Standard solution of fumonisins, fumonisin labelled with HRP and chromogen/substrate was supplied from Veratox kit, Neogen Corporation. Affinity purified anti-antibody unconjugated, anti-mouse IgG (H+L) from goat for the direct format was obtained from Pierce (U.K) Ltd. Fumonisins standard solution (Fumonisin B_1 and Fumonisin B_2) were purchased from Sigma-Aldrich Co. Ltd (Gillingham, United Kingdom (UK).

Other reagents – polyvinyl alcohol (PVA), 30% hydrogen peroxide (H_2O_2), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (powder), and potassium chloride – were purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). TMB solution was purchased from Insight Biotechnology (UK).

4.2.2 Buffer Solutions

A 0.1 M carbonate buffer, pH 9.6 was prepared similarly to the previous method in chapter 2. This buffer was for the immobilization of anti-IgG unconjugated (precoating) on both microplates and electrodes. A 0.01 M phosphate buffered saline (PBS), pH 7.4, used for preparation of MAbFms standard, blocking solution, dilution of antibodies and washing solution was purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). The washing solution was prepared by adding 0.05% Tween-20 (v/v) to the PBS (PBS-T). The fumonisins standard solution was prepared by diluting the stock solution (in methanol) with PBS. A 0.05 M Phosphate citrate buffer, pH 5.0 used in preparation of the TMB solution, was also obtained from Sigma-Aldrich.

4.2.3 Apparatus

The micro well polystyrene plates, MaxiSorp (Nunc Immuno) was purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). The spectrophotometric analysis for colour ELISA development was performed by a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, Fisher Scientific (Loughborough, UK).). The SPGE was fabricated by DuPont with printing pastes used are 107255-135E carbon, BQ331 gold, 5874 Ag/AgCl and 5036 blue encapsulant. This electrode was designed following collaborative work with DuPont to improve on the in-house electrodes. Incubations for every step of the reactions were carried out by Labsystem iEMS incubator/shaker HT. Electrochemical measurements were performed using AUTOLAB model PGSTAT with multichannel GPES software from Eco Chemie (Utrecht, Netherlands). For a comparative study for fumonisins analysis, Agilent 1200 HPLC was used, from Agilent (Berks, UK).

4.2.4 Preparation of stock solutions

Stock solutions of reagents for the immunoassay study of fumonisins analysis were prepared as listed in Table 4.1.

Table 4.1: Preparation of stock solutions of reagents for immunoassay study of fumonisins analysis.

Reagents	**Stock solution Concentration**	**Preparation**
Goat anti-mouse IgG (H+L)	100 µg mL^{-1}	25 µl in 475 µl of 0.1 M carbonate buffer pH 9.6
Anti-fumonisin antibody (MAbFms)	1:5 dilution	100 µl of in 400 µl of 0.01 M PBS pH 7.4
Free Fumonisins	6 µg mL^{-1} (ppm). PBS pH 7.4.	Standard solution was diluted in 0.01 M
Fumonisin-HRP conjugated	1:2.5 dilution	50 µl of in 75 µl of 0.01 M PBS pH 7.4

4.2.5 Preparation of standard solution

The Fms standard solution from Sigma was dissolved in methanol (HPLC grade) and stored at -18ºC in a tightly capped and dark bottle. A 6 µg mL^{-1} of stock solution of Fms was diluted to the concentration range of 0-3000 µg L^{-1} in 0.01 M PBS, pH 7.4 for a calibration standard of ELISA and immunosensor test.

4.2.6 Spectrophotometric ELISA for fumonisins analysis

The immunoassay for fumonisins detection was developed and optimised by the direct competitive method in the 96 microwell plates. It is based on enzyme reaction to produce colour and then measured by the spectrophotometric technique.

4.2.6.1 Optimisation by ELISA Procedure (checkerboard method)

ELISA procedures were carried out first to evaluate and optimise the MAbFms coating range (1:25 to 1:5000 dilutions) and commercial Fms-HRP (1 to 1:50 dilutions) using the checkerboard method. The tests were performed in a 96-

microwell plate in accordance with a direct ELISA format based on the method described by Anna *et al*., (2005) (Figure 4.1). The first antibody coating and binding sites reagents were incubated for 2 hours at 37 ºC.

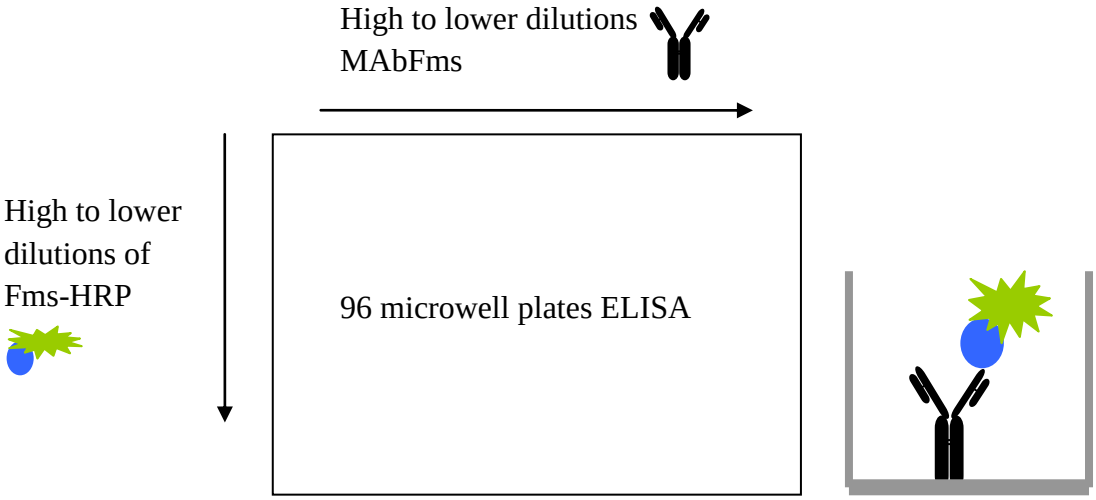


Figure 4.1: Example schematic diagram of checkerboard design for optimisation of various dilutions of MAbFms and Fms-HRP in microwell plate.

The optimisation of precoating immobilization using goat anti mouse IgG (H+L) (IgG) was also investigated (Figure 4.2). Various concentrations of reagent from 0 to 50 µg mL^{-1} were immobilised for 18 hours at 4ºC in the microwell plate before coating with optimal MAbFms concentration (2 hours at 37ºC) and labelling by optimal Fms-HRP concentration.

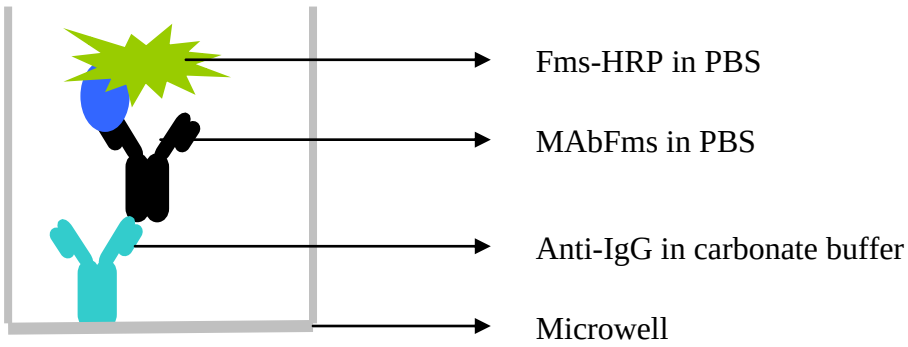


Figure 4.2: Precoating and coating step of capture antibody in direct immunoassay format using goat anti mouse IgG (H+L) and MAbFms, respectively.

4.2.6.2 Calibration curve of fumonisin developed by ELISA

Experiments were based on a competition between Fms-HRP and free Fms in a sample or standard for the binding sites of antibodies immobilised on the pre-coated well. The optimal concentration of reagents and conditions were used in producing a standard curve of Fms ranging from 0 to 3000 µg L^{-1}. The assay was done by following the procedure in Figure 4.3.

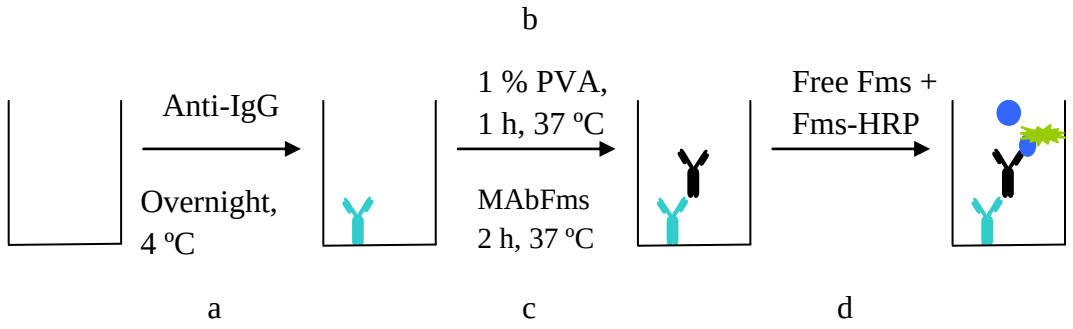


Figure 4.3: Schematic diagram of ELISA system for calibration curve of fumonisins detection until step (d).

The competitive assay was carried out using the following procedure; the microtiter Plate was pre-coated with anti-mouse IgG antibody (20 µg mL^{-1}, 50 µL/well) in 0.1 M carbonate buffer pH 9.6, for 18 hours (overnight) at 4 °C, followed by washing twice with 150µl/well phosphate buffered saline containing Tween 20 (PBS-T) and once with PBS alone. The plate was then blocked with 1% PVA (50 µL/well) and incubated for 1 hour at 37 °C. After washing as above, the plate was coated with anti-Fms monoclonal antibody (1:50 dilution, 50 µL/well) in PBS for 2 hours incubation at 37 °C, followed by washing. The competition solution was prepared by mixing (50 µL/well) of free fumonisin B_1 (0–3000 µg L^{-1}) in PBS with fixed dilution of a solution of fumonisin-HRP conjugate (1:5 dilution) in PBS. The competition reaction was allowed to proceed for 30 minutes at 37 °C and then rinsed with PBS-T (twice), followed by PBS (once). Finally, the absorbance was measured by the addition of TMB substrate solution (50 µL/well) to each well and incubated at room temperature for 15 minutes before added a 25 µL of stop reagent (H_2SO_4) and then measuring at 650 nm using the plate reader.

4.2.7 Electrochemical immunosensor for fumonisin analysis

The optimal concentration and condition from the ELISA method were subsequently transferred to the gold working electrodes of SPGE for the immunosensor test. The measurement of the current for the direct method immunoassay on SPGE was performed by chronoamperometry at a constant potential -100 mV.

4.2.7.1 Immobilisation by physical adsorption

The general protocol of the adsorption procedure in the initial study is described below. About 10 µl of 10 µg mL^{-1} goat anti-mouse IgG (H+L) (anti-IgG) primary layer (capture species) in a carbonate buffer were immobilised on the surface of the gold electrodes then incubated overnight at 4ºC. The electrodes were then washed using PBS-T (twice) followed by PBS (once). Blocking reagent was added on the electrode surface with 1 % PVA in PBS before being incubated for 1 hour at 37ºC. About 1:50 MAbFms antibody capture was coated on the gold working electrodes then incubated for 2 hours at 37ºC. For the assay a 10 µl mixture of 1:2.5 of antigen labelled HRP (Fms-HRP) and free Fms (0 to 6000 µg L^{-1}) concentration was added onto the gold surface. The electrode was then incubated for 1 hour at 37ºC. During each step of the assay, washing procedures were carried out between immobilization (precoating/coating), blocking, competition and detecting, by rinsing each electrode twice with PBS-T and once with PBS. The signal generated was performed by the addition of TMB/H_2O_2 solution onto the electrode surface.

All incubations were performed at 37ºC in a humidity chamber to avoid evaporation. The assay format used is illustrated in Figure 4.4.

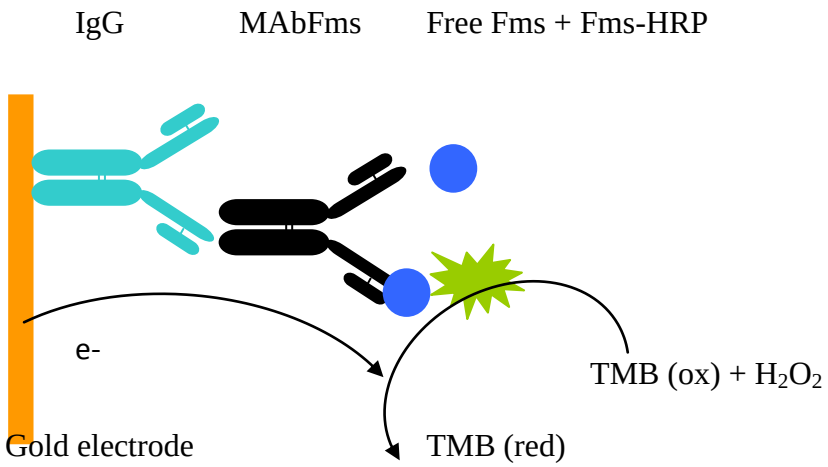


Figure 4.4: Schematic diagram of electrochemical direct competitive immunoassay with passive adsorption based on on gold surface electrode for fumonisins detection. The immunoassay was performed by precoating of anti-IgG (10 µg mL^{-1}) followed by coating with MAbFms (1:50) and competition within free Fms (0 – 3000 µg L^{-1}) and Fms-HRP (1:5).

4.2.7.2 Immobilisation on the gold modified surface

The assay was carried out following the procedure in paragraph 4.2.7.1, which meant that all the reagents were immobilised onto the gold modified surface of the working electrode. The gold surface was modified by immersing in 5 mM of 3, 3-dithiodipropionic acid (DTDPA) (selected thiol compound – the best modified thiol refer to AFB_1 assay) in absolute ethanol, and the sensors were left it in the dark for 24 hours at room temperature.

4.2.7.3. Different incubation techniques used in the competition assay

The assay was carried out following the procedure listed in section 4.2.7.1. on the modified gold surface. However, after the gold surface was immobilised with anti-IgG and MAbFms, the competition assay was carried out first by adding 5 µl of free Fms (0 to 6000 µg L^{-1}) onto the support for 10 minutes (pre-incubation times) before the

addition of 5 µl of Fms-HRP (1:2.5 dilution). Then the electrode was incubated for another 50 minutes at 37ºC. Second, 10 µl of mixed free and labelled Fms were directly added on the modified electrode surface for 60 minutes of incubation.

4.2.7.4 Effect of incubation time on the modified gold surface

Two different experiments were conducted for the incubation time study. First, the optima of incubation time for specific binding on the surface was investigated. The assay was carried out by immobilisation of 10 µg mL^{-1} goat anti-mouse IgG (H+L) and 1:50 dilution of MAbFms, following the procedure as in section 4.2.7.1. on the modified gold surface. Then, to the modified gold surface was added 1:5 Fms-HRP for 1, 10, 20, 30, 40, 50 and 60 minutes before measuring the signal. The optimum result from this experiment was used for the second part of the experiment.

The second experiment was performed for optimizing pre-incubation time in the competition step of the direct immunoassay on the modified gold surface. The assay was carried out by immobilisation of 1º antibody (10 µg mL^{-1} goat anti-mouse IgG (H+L) and 2º antibody (1:50 MAbFms), following the procedure in paragraph 4.2.7.1 on the modified gold surface. Then, the competition assay was carried out by adding 5 µl of free Fms (0 to 6000 µg L^{-1}) onto the support for 1, 10, 30 and 60 minutes (pre-incubation times) before the addition of 5 µl of Fms-HRP (1:2.5 dilution). After that, the electrode was incubated for another 30 minutes at 37ºC before measuring the signal.

4.2.7.5 Cross reactivity study

The assay was carried out by following the procedure in section 4.2.7.1 on the modified gold surface, using three different standard solutions of fumonisins: fumonisin from the Neogen Kit, fumonisin B_1 (FmB_1) and B_2 (FmB_2).

4.2.8 Calibration curve for fumonisin detection

The calibration curve for fumonisins detection was investigated using the optimal concentration and condition (pre-incubation and incubation time) on the modified gold surface of SPGE. The assay was carried out by immobilisation, first with a pre-coated antibody (10 µg mL^{-1} goat anti-mouse IgG (H+L) before being coated with 1:50 MAbFms. Then, the assay was carried out by adding 5 µl of free Fms (0 to 6000 µg L^{-1}) onto the support for 30 minute (pre-incubation times) before the addition of 5 µl of Fms-HRP (1:2.5 dilution). The electrode was then incubated for another 30 minutes at 37ºC before the signal was measured.

4.2.9 Precision and stability of the sensor

For a precision study, the experiment was conducted by analyzing four concentration levels of fumonisin (10, 50, 100 and 500 µg L^{-1}) using 9 electrodes per run of each level on the SAM modified gold surface of the screen-printed electrode. The assay was based on the direct competitive method.

The storage stability of the sensor was investigated by analyzing 8 weeks of modified gold surface with antibody immobilised stored at 4ºC. For every week of analysis, the assay was conducted by adding free Fms (50 µg L^{-1}) and labelled Fms (1:5 dilution) on the modified gold surface immobilised before measuring the signal.

4.2.10 Corn (maize) samples analysis

Dried corn (maize) samples were collected from the United Kingdom and Malaysia. Samples were then ground using a food processor or blender at medium speed for 3-5 minute until all the corn was blended. Samples were then packed properly in plastic bags and stored in a fridge (0-4ºC) before use (to avoid contamination). For the spiked sample, a fumonisins solution (Neogen Kit) was spiked on the corn samples at concentrations of 50, 250, 500 and 2500 µg kg^{-1}. The mixture was manually shaken for 30 seconds and then kept overnight to obtain a homogeneous mixture.

4.2.10.1 Extraction without clean-up

Sample preparation and extraction were conducted following the procedure as described by the Veratox ELISA kit (Neogen Corporation, UK). The samples or spiked samples of five grams of ground corn was used, mixed with 25 mL of 70% methanol and 30% water and then shaken vigorously for 3 minutes. The extract was filtered through a Whatman #1 filter paper to remove the solid material and the filtrate was then collected and diluted with 1:10 of 0.01 M PBS pH 7.4 for analysis without further preparation.

4.2.10.2 Clean up using C-18 solid phase extraction (SPE)

For this procedure, a samples extraction was conducted similar to the procedure described above. Sample filtrates were collected and cleaned using C-18 SPE (Waters, Milford, MA) following the procedure accompanying the SPE columns. The C-18 SPE column was first conditioned by sequentially passing 5 ml methanol and 5 mL water through the column. A 4 mL of sample filtrate was then passed through the column, followed by 6.0 mL of deionised water. The fumonisin was then eluted from the SPE column by rinsing with 2.0 mL of methanol : water (70:30).

Immunosensor detection for calibration curve

A 1 ml of sample eluted was dried under nitrogen stream and re-dissolved with 1 ml of free fumonisins in 0.01 M PBS pH 7.4 to give a standard series of 0, 0.1, 1, 10, 100, 1000 and 2000 µg L^{-1} which were used for the preparation of the immunoassay calibration curve. Then, 5 µl of the sample was mixed with 5 µl of MAbFms before being transferred onto the gold working electrodes.

Pre-column derivatisation for HPLC detection

The sample eluted was dried under nitrogen stream, and re-dissolved with 2 mL of acetonitril /water (1:1). Pre column derivatisation was first carried out before samples were then injected into the HPLC column. The process of derivatisation was started by transferring about 25 µl of samples or fumonisins standard in the small test tubes before the addition of 225 µl OPA reagent (OPA = 40 mg OPA in ml methanol, and

diluting with 5 ml 0.1M disodium tetraborate solution (3.8g in 100 ml H_2O and 50 µl 2-mercaptoethanol). About 10 µl of the sample was then injected into the LC system within 1 min after the addition of the OPA reagent.

HPLC system

HPLC parameters for Fms measurement are described below using the isocratic system:

Mobile phase: Methanol/0.1M sodium dihydrogen phosphate solution (15.6 g in 1 L H_2O) (77+23) and adjust pH to pH 3.3 using phosphoric acid.
Column: C18 (Nucleosil 100 5 µm 15 x 0.46 cm)
Flow rate: 1.0 ml/min
Detector: Fluorescence detector (335 nm excitation and 440 nm emission)

Each experiment was carried out in triplicate and each value was the mean of three determinations.

4.3 Results and Discussion

4.3.1 Optimisation of reagent by ELISA method

A checkerboard titration is a single experiment in which the concentration of two components is varied in a way that will result in a pattern. As shown in Figure 4.5 the anti-fumonisins antibody (MAbFms) is serially diluted across the plate and the HRP-labelled fumonisin (Fms-HRP) is serially diluted down the plate. After adding the substrate, the colour was developed by the reaction of HRP. A high absorbance reading was obtained at range dilutions of 1:25 to 1:50 of MAbFms and dilutions of 1:1 to 1:10 of Fms-HRP, indicating high specificity of binding for the combination of both reagents. From this checkerboard matrix, the following curves are generated (Figure 4.6).

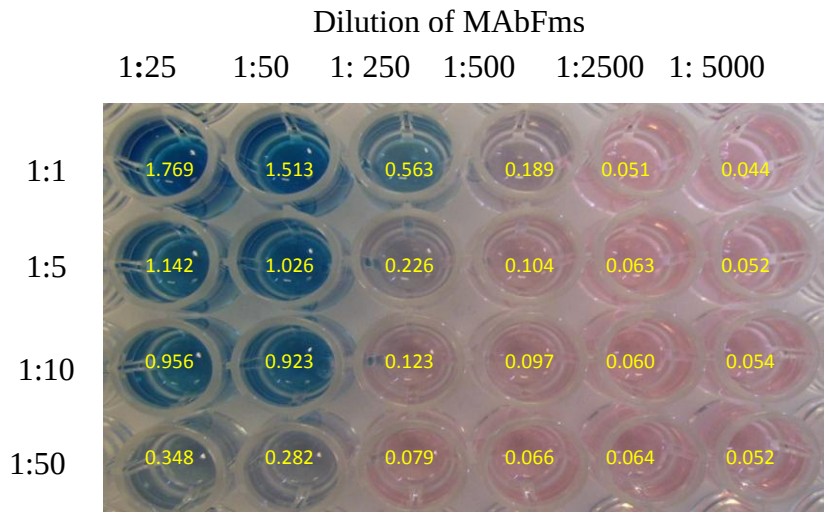


Figure 4.5: Checkerboard test for optimisation of two components: MAbFms and Fms-HRP. The yellow numbers represent the absorbance reading at 650 nm.

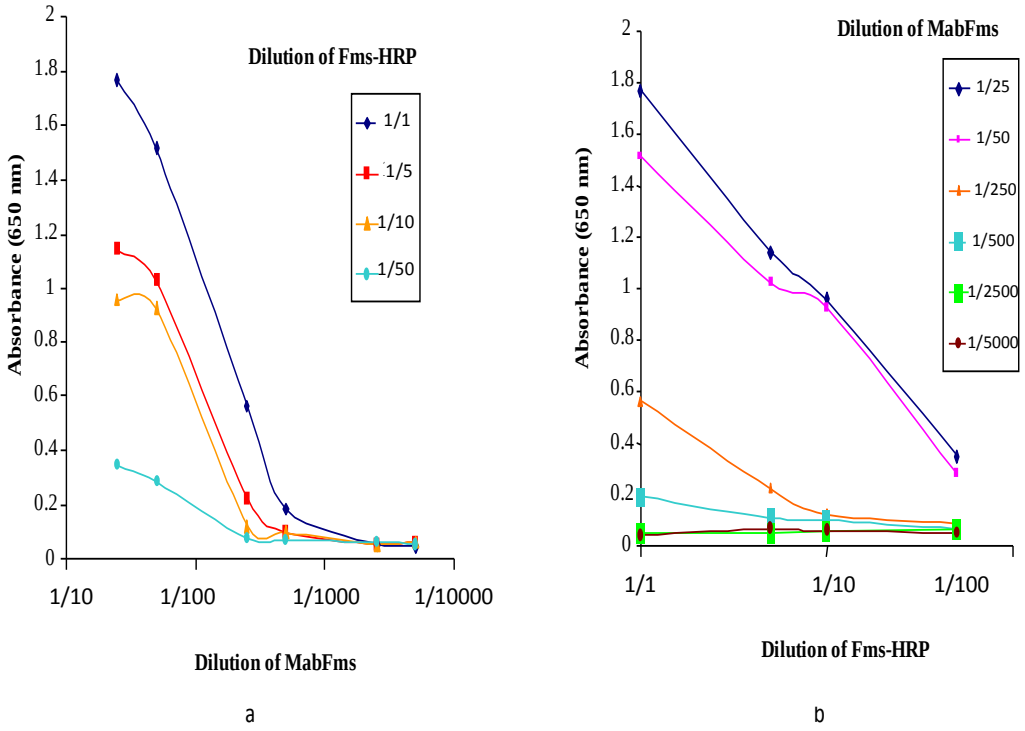


Figure 4.6: Optimisation of plate coating by a) MAbFms and b) Fms-HRP dilution in the direct non-competitive checkerboard ELISA. Plates were coated with variable dilutions of both reagents.

As shown from Figure 4.6, the increasing of the combination of the MAbFms and Fms-HRP concentration increased the absorbance reading (high specific binding). However, a high combination of concentration may also provide a high non specific binding that produces high background reading (less sensitivity) (Sadana & Chen, 1996). So, in this case, the absorbance reading was still obtained with a high specific binding at 1:5 of Fms-HRP and 1:50 of MAbFms and was chosen for the assay.

The use of anti-IgG to pre-coat solid face surfaces before immobilising the anti-capture antibody has been reported to increase the detection limit of aflatoxin M_1 (Micheli *et al*, 2005). Therefore, to maximize the ELISA signal, the use of pre-coated microtitre wells was investigated in this study. Then, the concentrations of reagents of an anti-mouse IgG antibody (IgG) were placed on the wells. The fixed amount of MAbFms (1:50 dilution) and Fms-HRP (1:5 dilution) were used for the optimizing of the pre-coating reagent. Figure 4.7 shows the absorbances reading of the precoated IgG treated on the well. The signal was increased with the increasing of the anti-mouse IgG antibody (IgG) coated on the well, while a dramatic increase was achieved at a range between 1 to 20 µg mL^{-1}. A range from 5 to 20 µg mL^{-1} concentrations is suitable for assay development, and therefore 20 µg mL^{-1} was chosen for further experiments.

The results achieved (Figure 4.8) show that the use of pre-coated wells produced a much greater signal than non-coated wells. The signal/background (S/B) was ~ 14 and 10 for pre-coated wells and non-coated wells respectively, which for the pre-coated wells is an increase of about 40% compared to the non-coated wells. This indicates that the use of pre-coating of the anti-IgG in this system improved the signal reading in the assay. The increase in the signal indicates a better binding orientation of the antibodies in the assay and therefore it will be applied in future tests and sensor development. According to Micheli *et al*, (2005), pre-coating immobilisation will promote the binding of the antigen with its antibody in order to increase the sensitivity of the analysis.

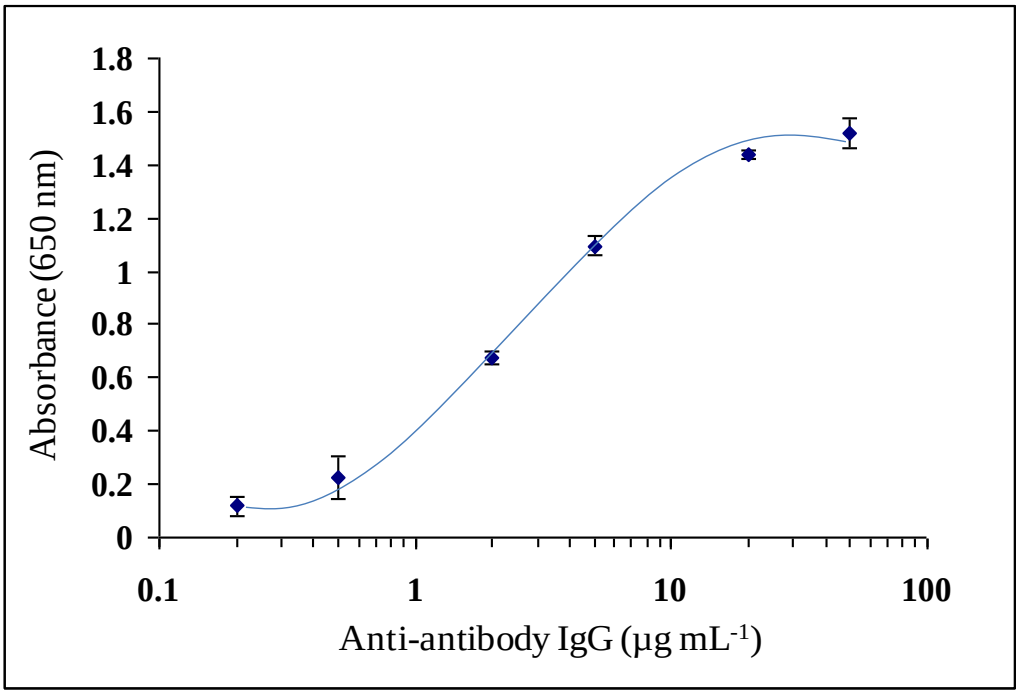


Figure 4.7: Optimisation of anti-antibody IgG (anti-IgG) concentration in a direct non competitive format. Plates were pre-coated by a variable concentration (0 - 50 µg/mL) of anti-IgG followed by coated 1:50 of MAbFms and 1:5 of Fms-HRP. Error bar =SD, n=3.

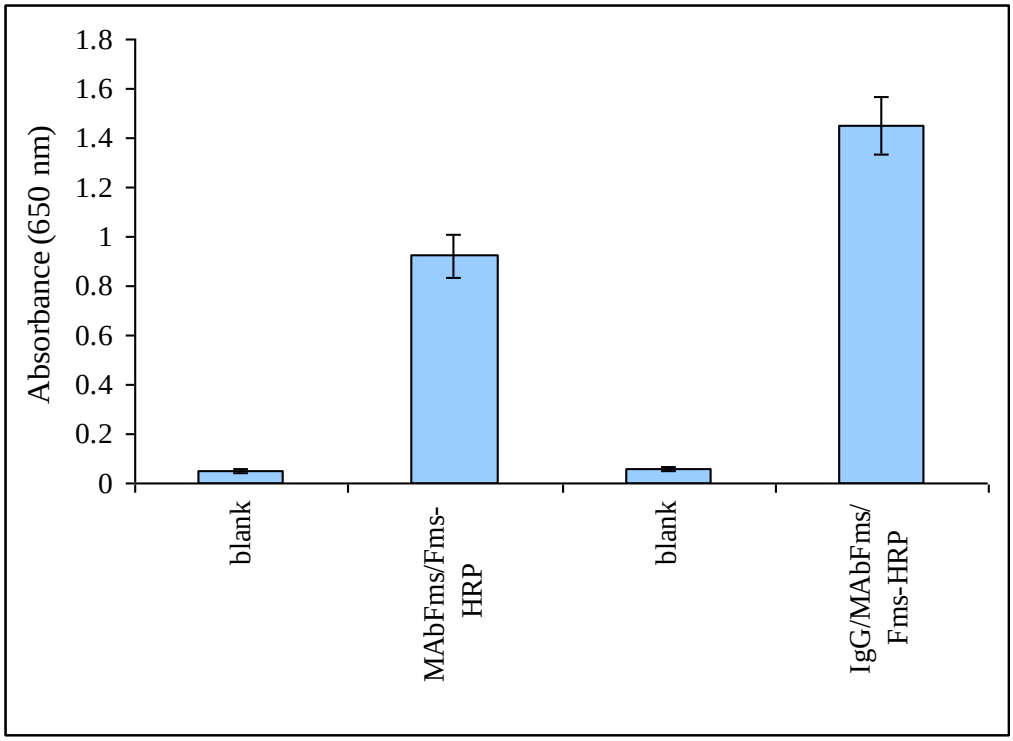


Figure 4.8: The different immobilizations of antibodies in a direct non-competitive format and comparison of absorbance readings between with and without anti-IgG immobilised on the micro-plate well. Error bar =SD, n=3.

4.3.2 Calibration curve for fumonisins detection using an ELISA method

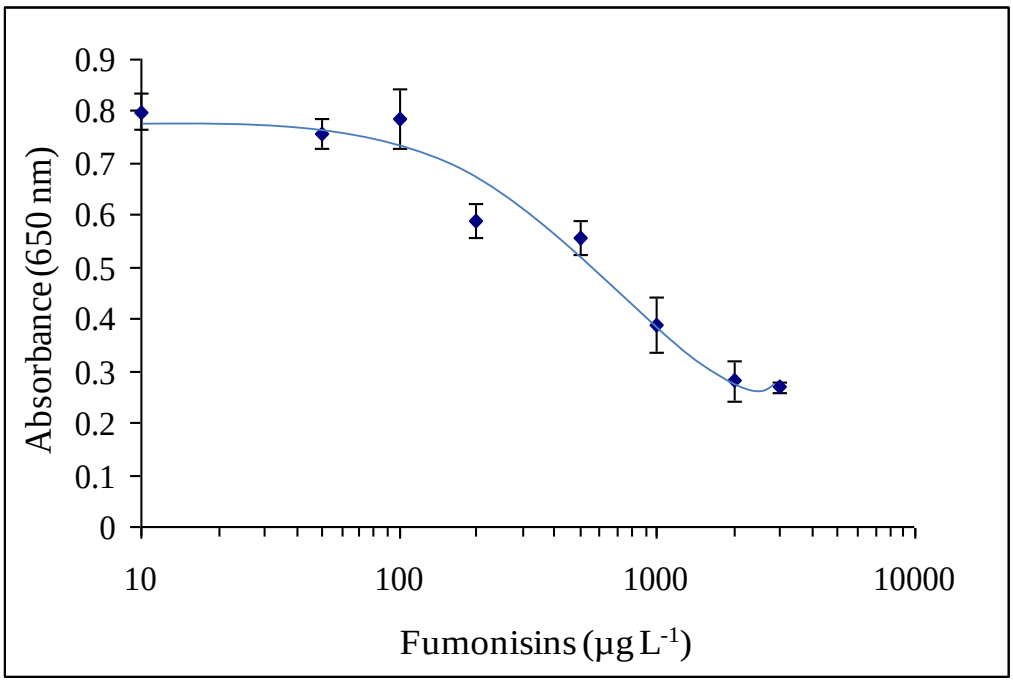
Utilizing the derived optimal concentrations and conditions for the competitive assay, a calibration curve for fumonisins was then carried out with free (0–3000 µg L^{-1}) and HRP labelled fumonisin in a buffer, mixed and added to the wells where they compete for the anti-fumonisin antibody active sites. From the result, shown in Figure 4.9, with the increasing of standard Fms concentrations the signal decreased. The signal dramatically decreased from 100 µg L^{-1} to 2000 µg L^{-1} of Fms. From the graph, the low signal at high concentration of Fms means that the high amount of free Fms was bound to the specific antibody immobilised on the micro-plate well (less or no enzyme reaction). At the concentration less than 100 µg L^{-1} of Fms, the curve shows steadily in high absorbance reading meaning that the maximum amount of Fms-HRP was bound to the specific antibody immobilised (no or very low of free Fms was bound).

These calibration curves were fitted by using a 'non-linear regression plot' (Warwick, 1996). A dynamic range from 100 to 2000 µg L^{-1} (R^2 = 0.95) with a CV = 9.3% was achieved using the linear section of the curve to calculate the data.

After the development of the spectrophotometric ELISA was established and the high orientation binding of antigen in the well was achieved, the method was then subsequently transferred to the gold working electrodes (SPGE) for the immunosensor test. Table 4.2 shows the optimal data of concentrations reagents and conditions obtained after the optimisation was completed. However the data then will be used for the immunosensor assay.

4.3.3 Electrochemical immunosensor test

The direct competitive immunoassay on the electrodes was performed by an electrochemical technique. The technique was evaluated by chronoamperometry at a constant potential of -100 mV for enzymatic activity detection.



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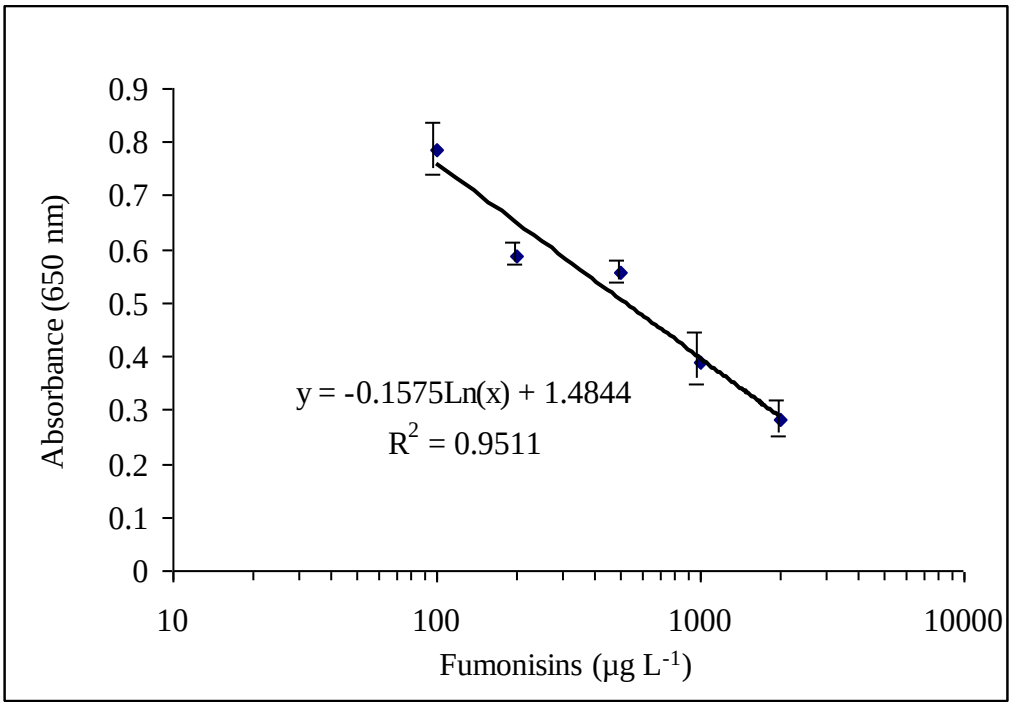

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Figure 4.9: (a) Spectrophotometric competitive immunoassay for calibration curve of fumonisins. Anti-IgG (20 µg mL^{-1}) and MAbFms (1:50 dilution) were pre-coated and coated on the microwell plates, respectively, before adding a mixture of free fumonisins (0-3000 µg L^{-1}) and Fms-HRP (1:5 dilution). Blank: only 1% PVA and Fms-HRP (b) Linear regression a working range of 100 to 2000 µg L^{-1} ((R^2=0.95) (CV= 9.3%). Error bars = SD, n=3.

Table: 4.2: Optimal concentration/dilution of reagents and conditions used for the direct competitive assay of fumonisins detection.

Reagents	Concentrations/dilutions	Conditions
Anti-IgG antibody (anti-IgG)	20 µg mL^{-1}	overnight, 4 ºC
Anti-Fms antibody (MAbFms)	1:50	2 hours, 37 ºC
Fms-HRP conjugate	1:5	1 hours, 37 ºC

The amount of anti-IgG antibody, anti-Fms antibody (MAbFms) and fumonisin labelled with HRP (Fms-HRP) were evaluated and optimised again using screen-printed gold electrodes (by Dupont) based on optimal concentration and condition in Table 4.2. However, in this system, the amount of anti-IgG used is 10 µg mL^{-1}. This amount also produced a high absorbance reading (see Figure 4.7), and this lesser amount will be more cost effective for the sensor development. Thus, re-optimisation of the other reagents concentration was further conducted, for the optimal specific binding reaction.

4.3.3.1 Coating and binding study (non competitive assay)

The performance of MAbFms coating and Fms-HRP binding on the surface was studied by a fixed amount of anti-IgG (10 µg mL^{-1}) and variable amount of MAbFms (1:500 to 1:5 dilution) followed by (1:50 to 1:2.5) dilutions of Fms-HRP. Figures 4.10 and 4.11 represent the data of the optimum level of MAbFms coating and specific binding of Fms-HRP on the electrode surface. These experiments were performed in order to have the optimal current response for the competitive assay of fumonisins detection. From both figures, the results show that the increasing of the current response was proportional with the concentration of reagents of MAbFms and Fms-HRP. However, the signal remained stable at certain concentrations when the binding of MAbFms and Fms-HRP was saturated. The increase in current response

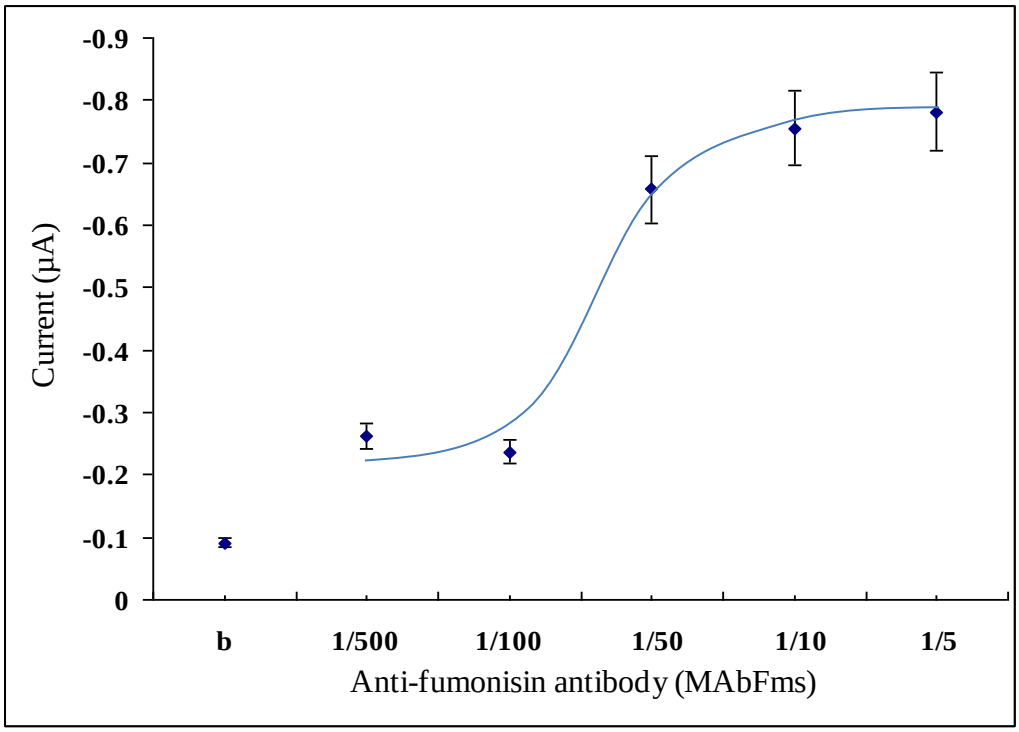


Figure 4.10: Coating signal with the incubation of increasing dilutions of anti-fumonisins antibody. Fixed anti-IgG (10 µg mL^{-1}) and fumonisin-HRP conjugate (1:5) were used. Error bar = SD, n=3.

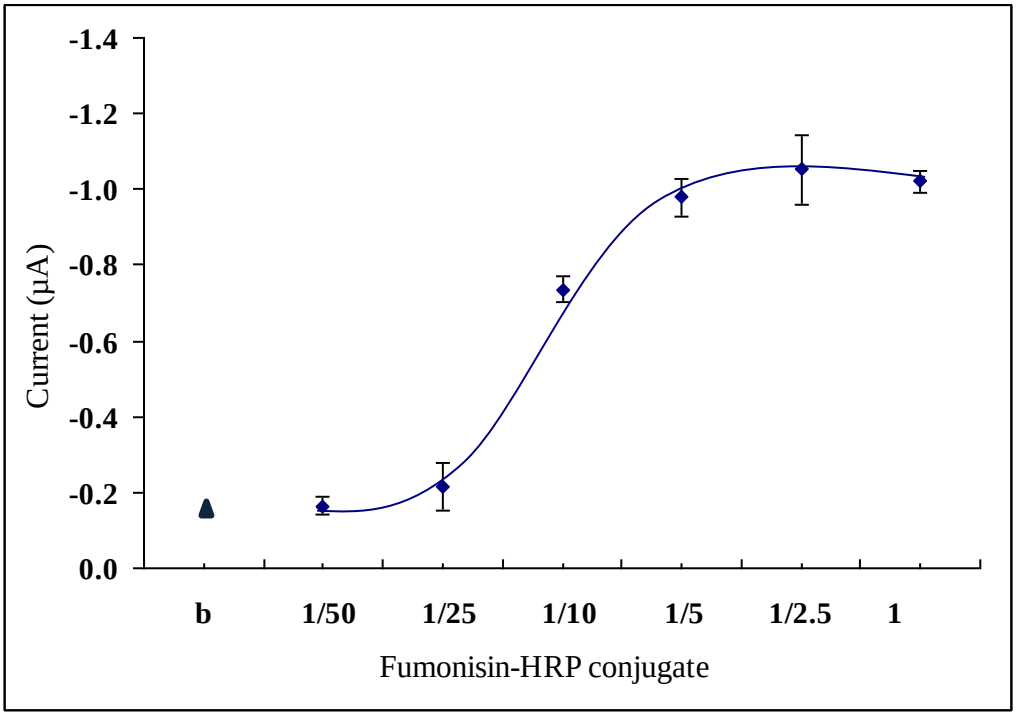


Figure 4.11: Binding signal with the incubation of increasing dilutions of fumonisin-HRP. Fixed anti-IgG (10 µg mL^{-1}) and MAbFms (1:50) were used. Error bar = SD, n=3.

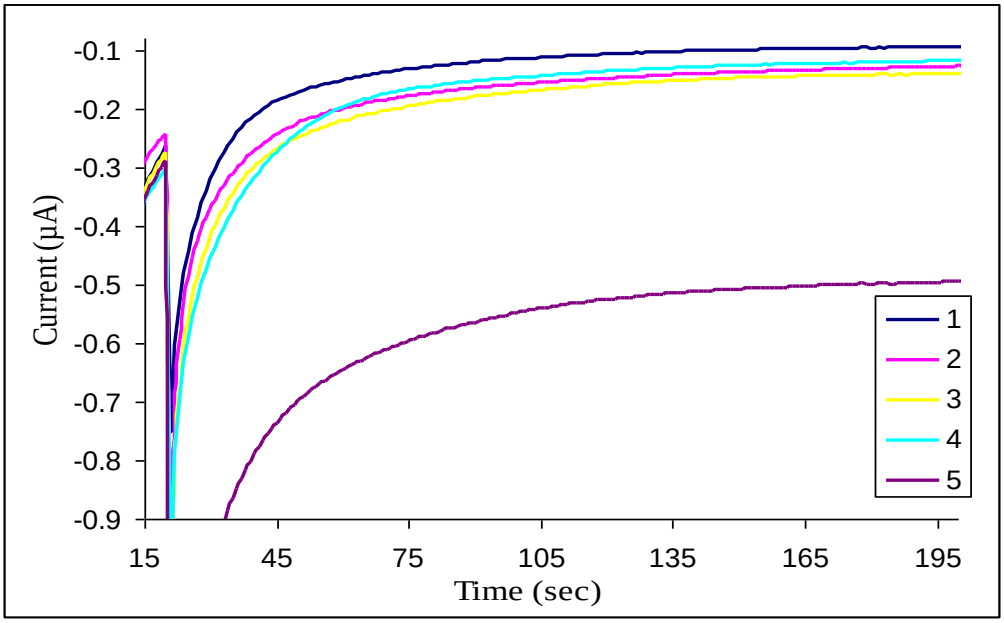
showed the increase of specific binding of antibody coated and labelled enzyme bound on the pre-coated gold electrodes.

In this case, the 1:50 and 1:5 dilutions of MAbFms and Fms-HRP at fixed anti-IgG (10 µg mL^{-1}) immobilised on surface of SPGE, respectively was chosen for the further competitive assay.

4.3.3.2 Steps for immobilisation on the gold surface (passive adsorption)

The effect of the different immobilisation steps taking place for fumonisins detection on the electrochemical signal as a background and assay signal was also investigated. Figure 4.12(a) shows the signal achieved from: (1) without any immobilisation (bare electrode); (2) precoating with anti-IgG (IgG); (3) anti-IgG, blocking with PVA and coating with MAbFms (IgG/PVA/MAbFms); (4) blocking with PVA and adding Fms-HRP; and (5) immobilised with IgG/PVA/MAbFms and adding Fms-HRP. This is an important characterisation step to evaluate the performance of different reagents adsorption as well as to study the effect of the adsorbed reagents on the electron transfer characteristics of the sensor. The detection is based on the reduction current of the HRP towards TMB/H_2O_2 at a -100 mV potential. As seen from the result in Figure 4.12(b), the current reduction was increased (~ -0.6 uA) with the presence of antibodies immobilised and Fms-HRP (+ TMB/H_2O_2) bound on the electrode surface (step 5) compared to bare gold electrodes and the other three steps of immobilisation (between -0.1 to -0.2 uA).

This indicates that the reagents immobilization did increase the background signal slightly but had a minimal effect on the electrochemical characteristic of the sensor and was able to detect the signal achieved from the activity of the enzyme label at high sensitivity. According to Fu, conducting this type of test is important to ensure that the enzyme label is still active and the electrode surface is not blocked due to the chemical modification of the sensor surface (Fu, 2008).



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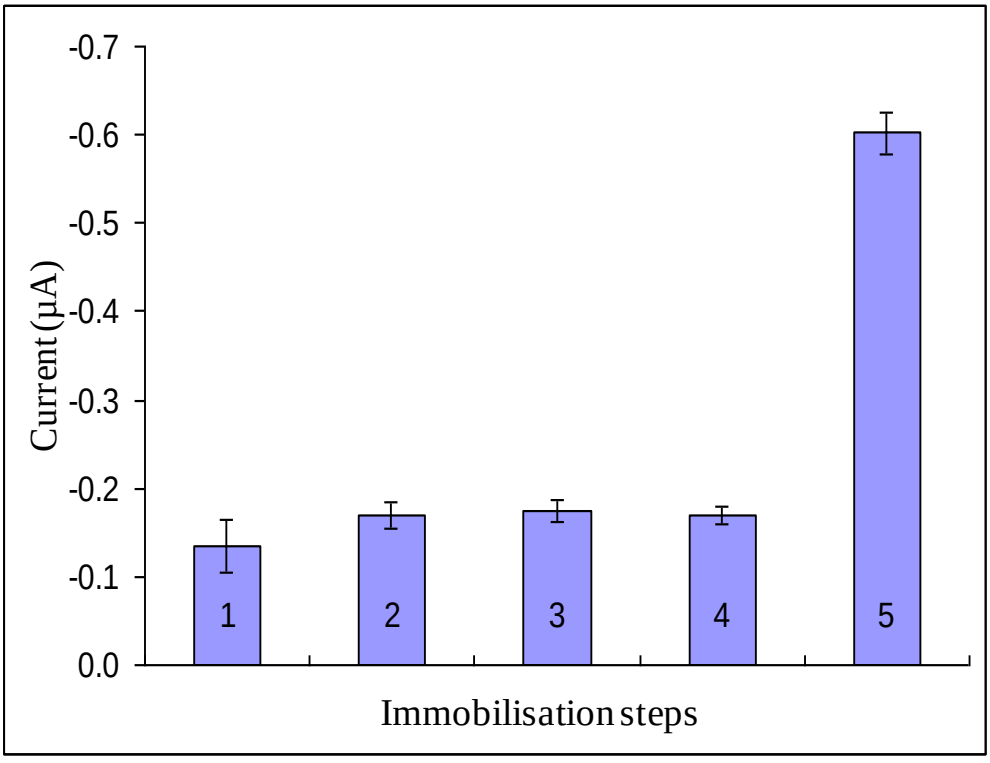

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Figure 4.12: (a) Chronoamperograms of immobilisation steps (passive adsorption) by chronoamperometric detection. (b) Chronoamperometric response of each immobilisation step of the direct non-competitive format on gold surface electrodes. Each gold electrode was immobilised by 1. Bare electrode, 2. Anti-IgG, 3. Anti-IgG/PVA/MAbFms, 4. Anti-IgG/PVA/Fms-HRP and 5. Anti-IgG/PVA/MAbFms/Fms-HRP using TMB/H_2O_2 as the enzyme substrate. Error bar=SD, n=3.

4.3.3.3 Immobilisation using SAM modified gold electrode

The study was also conducted with antibody immobilization via covalent bonding on the modified gold surface of 3,3-dithiodipropionic acid (DTDPA). The modified gold was immobilised with anti-IgG/PVA/MAbFms and adding with and without Fms-HRP. It was compared to passive adsorption on the gold surface. As shown in Figure 4.13, for the reduction current (after incubation of the HRP conjugate) after the addition of TMB / H_2O_2 substrate, the signal found was higher at the SAM modified surface compared to the passive adsorption immobilisation, and the difference of the reduction current increased by about 40% also higher precision with SAM modified (CV=2.1%) compared to passive adsorption (CV= 7.4%). Therefore, the antibody immobilisation using the DTDPA thiol monolayer increased the detection of reduction current on the gold surface.

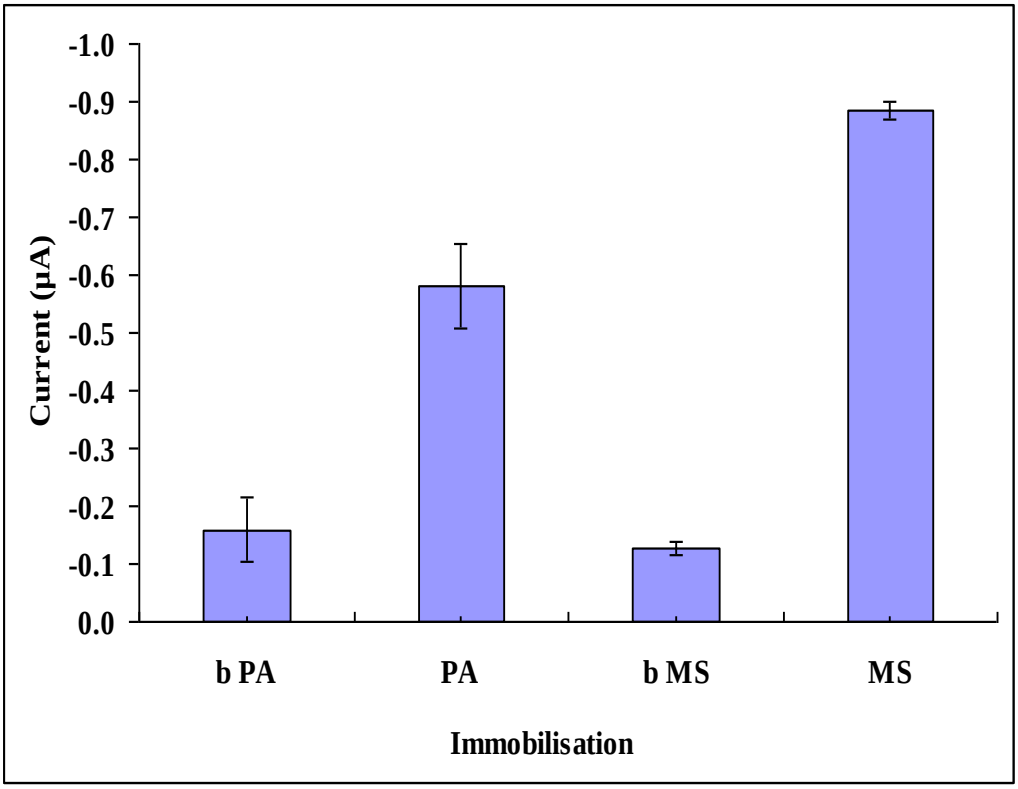


Figure 4.13: The differences of current reduction by passive adsorption immobilisation and covalent bonding immobilisation on the gold surface electrodes. Passive adsorption: first pre-coated with anti-IgG (10 µg mL^{-1}) then coated with MAbFms (1:50 dilution) and to this was added 1:5 of Fms-HRP. Modified Surface: first, the gold surface was treated using 3,3-dithiodipropionic acid before it was pre-coated with anti-IgG (10 µg mL^{-1}) then coated with MAbFms (1:50 dilution) and to this was added 1:5 of Fms-HRP. Note: PA = Passive adsorption; MS = modified surface; b = blank.

The ability of the thiol monolayer to bind with the antibody on the gold was increased. This is due to better orientation of the antibody molecules on the gold sensor surface. Thus, immobilisation via covalent binding with dithiodiproponic acid will enhance the sensitivity of the immunosensor development for fumonisins detection.

4.3.3.4 Competitive binding assays by covalent binding

Based on the heterogeneous immunoassay on the gold surface electrodes, the direct competitive assay for fumonisins analysis was investigated. The study of covalent binding for the competitive assay was also used since it produced a high sensitivity of calibration curve compared to passive adsorption. The competition system was employed within free fumonisin concentrations at a range (0 to 3000 µg mL^{-1}) and Fms-HRP (1:5). A typical standard curve of competition binding antigen was obtained in Figure 4.14 (a) based on signal current (µA) versus fumonisins concentration. The results showed that the current response is inversely proportional to the fumonisin concentrations for both assays. This phenomenon shows the more the fumonisin concentration was increased, the less of HRP label (Fms-HRP) bound to the antibody on the surface and the more the current decreased. Therefore, the result shows the dramatically decreasing of the competitive binding from 50 to 2000 µg L^{-1} and declined steadily after that.

From the data shown in Figure 4.14 (b), the linear regression of a working range for both assays using 50 to 2000 µg L^{-1} with R^2 values are 0.98 and 0.97 on the MS and PA surfaces, respectively. When the curves were fitted by non linear regression as described previously, the limit of detection for the assay on MS is more sensitive than on PA, being 30 µg L^{-1} and 40 µg L^{-1}, respectively. The dynamic range of the competitive binding was increased compared to the ELISA system (100 to 2000 µg L^{-1}). This indicates, the sensor development enhanced the sensitivity of the assay.

As shown in Figure 4.14 also, the competitive binding on the modified gold surface using SAM gave an increase in the current signal and also a low background reading compared to passive adsorption (PA). The increase of the signal to the background

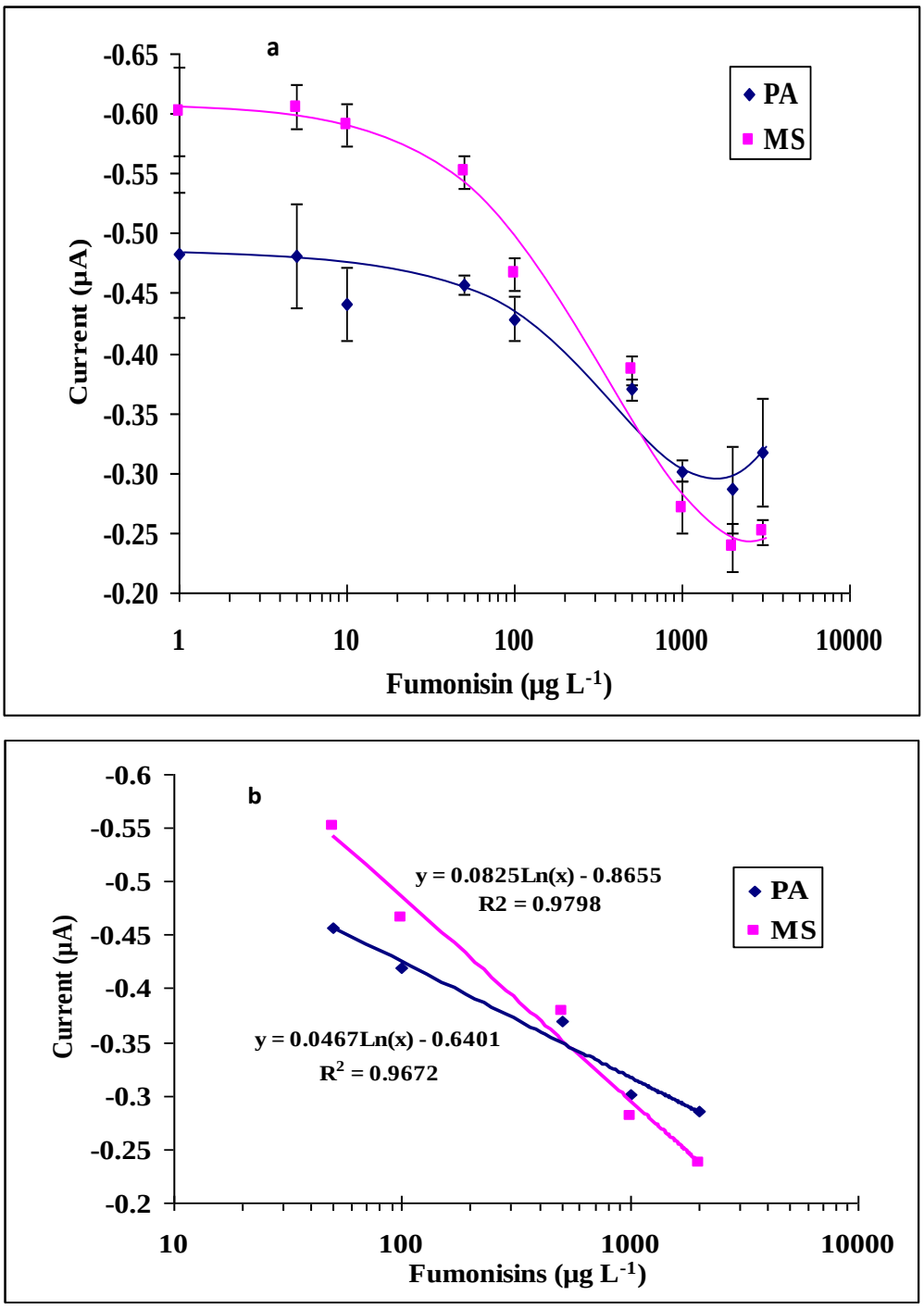


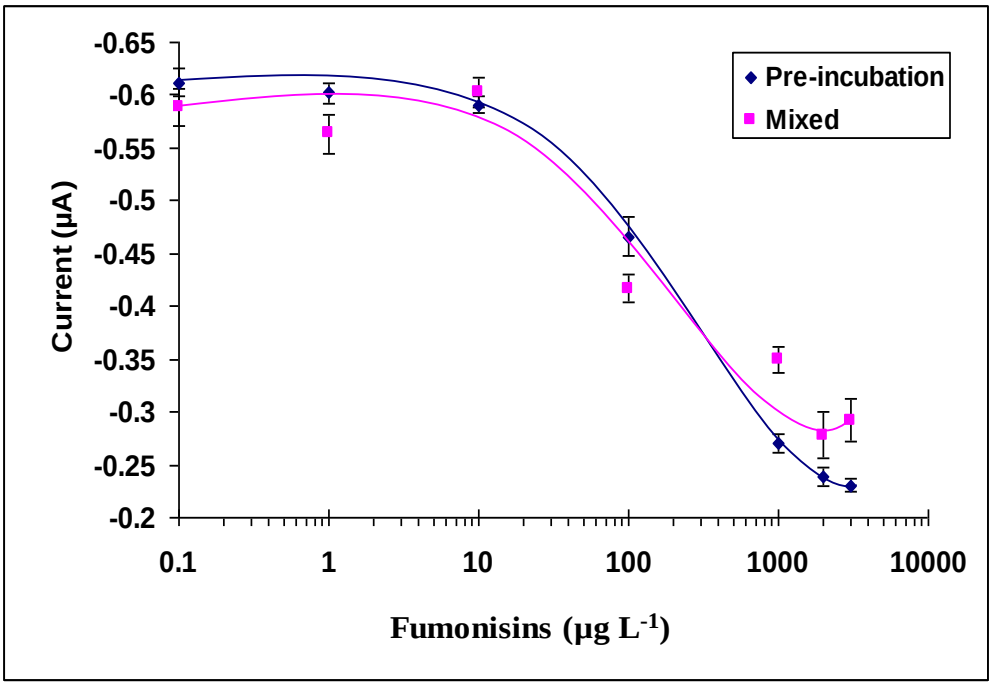
Figure 4.14: The standard curve of the direct competitive immunoassay on the gold surface by passive adsorption and covalent binding (SAM monolayer). Current response (µA) versus fumonisins concentrations were measured by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. The bare and modified surfaces of the screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with mixing of Fms (1-3000) and Fms-HRP (1:5). (a) The curves were fitted by non linear regression. (b) Linear regression of standard curve. Error bar = SD, n=3. Note: PA; Passive adsorption, MS; Modified surface (SAM thiol).

ratio will increase the percent of binding (B/B_o) that will improve the assay sensitivity (Das *et al*., 2007). In this case, the SAM thiol modified surface provides better orientation of the antibodies immobilised on the surface. Therefore, the specific orientations of the antibodies on the binding surface improve the antigen-antibody affinity which increased the S/B of the assay. According to Lu *et al*, orientation was found to greatly affect the antigen-binding activity of the antibodies, since the antigen-binding activity of the oriented antibody fragments was 2.7 times that of the randomly immobilised ones (Lu *et al*., 1995). For further experiments, the immobilisation of antibodies and antigens will be conducted by covalent binding based on the gold surface treated with SAM thiol.

4.3.3.5 Different techniques of incubation in the competition step

In order to improve the sensitivity of the electrochemical immunosensor, different parameters were further investigated. First, the different techniques of incubation in the competition steps were investigated and second, the different incubation times. These optimisations are very important in order to enhance the detection assay (Liu *et al*., 2001). The competitive assay was conducted by adding free Fms onto the modified surface where the specific antibody was immobilised before the addition of Fms-HRP. This method was compared to a different technique of the competition step where free and labelled Fms were mixed first and then added to the modified surface.

The standard curve for the different protocols are shown in Figure 4.15, where the protocol 1 and 2 were evaluated. In protocol 1, a 10 min pre-incubation of free Fms on the electrode surface taken place before the addition of the Fms-HRP and incubated for 50 minute. This obtained a good correlation R^2=0.99 and small value of CV (5.6%). In the case of the second protocol, the free and labelled Fms were mixed onto the modified surface and incubated for 60 minute. This was found to give R^2=0.96 with CV value of 8.4%. The presence of better correlation and a %CV with a more stable signal at protocol 1 produced a better linear regression curve. This pre-incubation method may be promoting the binding reaction of free Fms toxin to the antibody first before the competition with its Fms-HRP conjugate.



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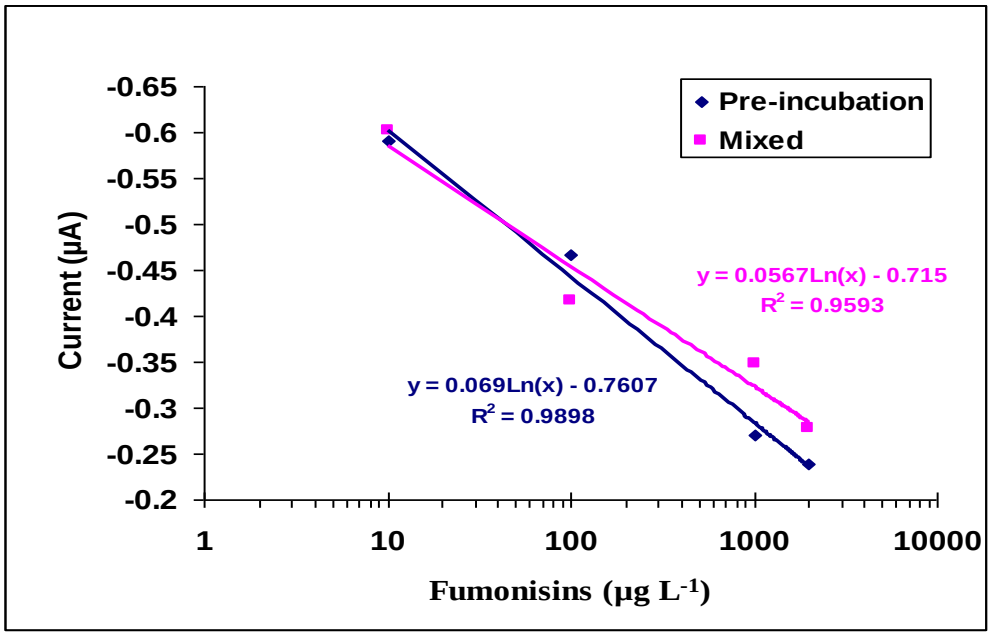

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Figure 4.15: The standard curve of the direct competitive immunoassay on the modified gold surface by the effect of different protocols of incubation. Current response (uA) versus fumonisins concentrations were measured by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. The modified surface of the screen-printed gold electrodes was coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with Fms (1-3000) before of Fms-HRP (1:5). (a) The curves were fitted by non linear regression. (b) Linear regression of standard curve. Error bar = SD, n=3.

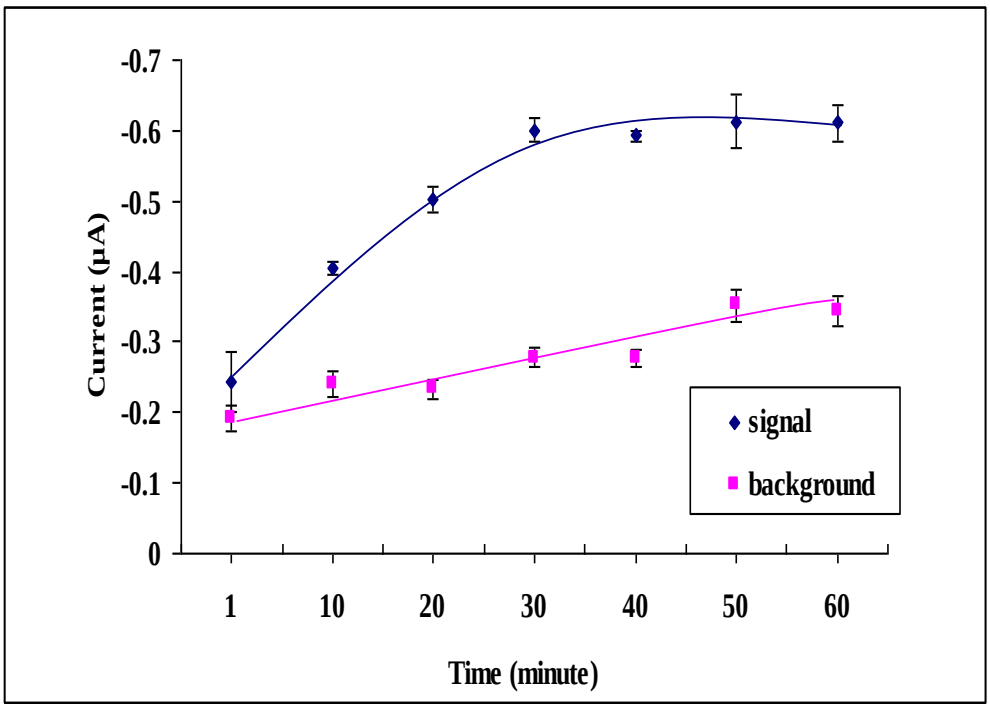
The result also presented a low background signal when pre-incubation was applied, which produced high S/B compared to without pre-incubation. This indicates that the pre-incubation time not only promoted a binding reaction (Harlow *et al*., 1988; Crowther *et al*., 2001; Michelli *et al*., 2005) but also reduced non specific binding on the surface. In this case, the reduced incubation time after the addition of immobilised Fms-HRP in protocol 1, may decrease the steric hindrance caused by enzyme conjugate that binds to the surface.

The increase of the sensitivity was observed when there was pre-incubation of the Fms for 10 min before the addition of the HRP fumonisins conjugate on the surface with the linear range of 10 to 2000 µg L^{-1}. Based on the above result, pre-incubation time was chosen for a further experiment.

4.3.3.6 The effect of incubation time on the competitive assay

For the immunosensor assay in the electrochemical system, the effect of different incubation times (0 to 60 minutes) on non competitive assay was first investigated. The need for incubation for a certain time is for the contact of the antibody/antigen for the forming of immunocomplexes. That is through the addition of the constant Fms-HRP concentration onto the modified surface coated with antibodies immobilised. This procedure was first conducted before the pre-incubation was involved in the competitive assay. The purpose of the experiment was to determine the optimum point of the completed binding of the Fms-HRP to the antibody immobilised on the surface.

As seen in Figure 4.16 (a), the current signal rapidly increased up to 30 min of incubation and tended to be stable after that. The first 1 to 30 minutes incubation is the time where the antigen-antibody interaction is still taking place to form a stable complex. In this case, after 30 minutes of incubation most of the antigen-antibody interaction is completed. In most cases in immunoassay systems it has been found that the complete binding reaction is after 25 to 50 minutes incubation (Liu *et al*., 2001; Micheli *et al*., 2005; Tang and Xia, 2008).



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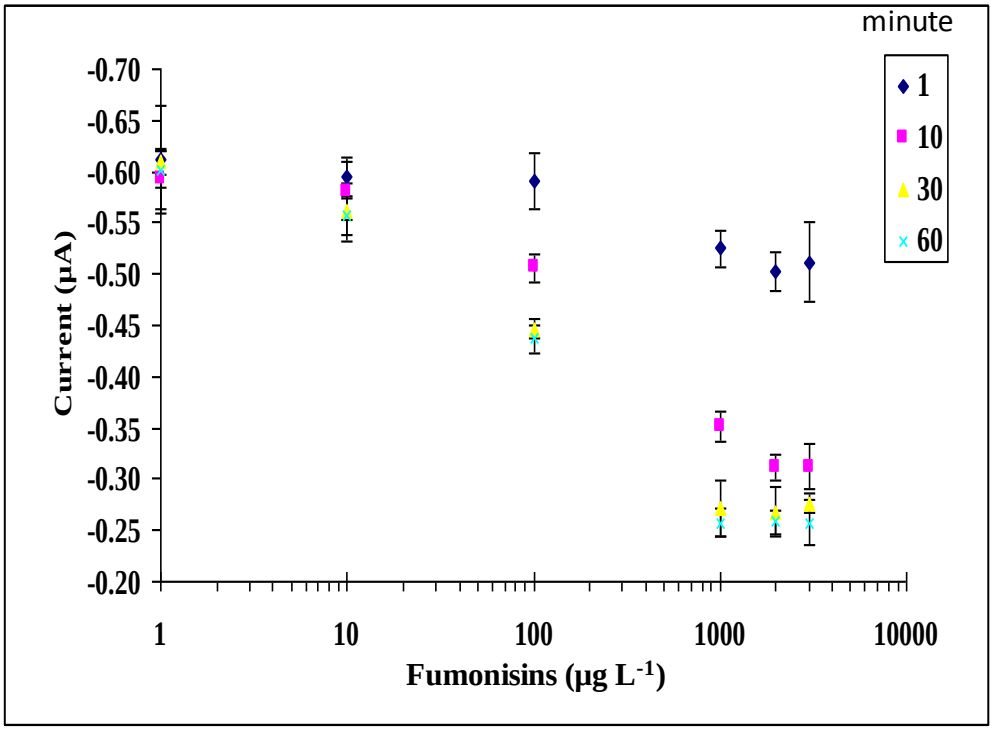

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Figure 4.16: The effect of different incubation times for the competitive assay of fumonisins detection. (a) The graph for the 1 to 60 minutes of incubation time with antibodies immobilised on the modified surface and the adding of Fms-HRP. (b) The graph for the 1, 10, 30, 60 minutes of pre-incubation time of free Fms before 30 minutes incubation after the adding of Fms-HRP labelled on the modified surface coated with antibodies. Error bar=SD, n=3.

However, with increasing the time of incubation the backgroud reading of the assay also increases. This signal is caused by the non specific binding signal. The best result chosen in this experiment and for further investigating is the use of 30 minutes of incubation.

For the competitive assay, the use of different times (1, 10, 30, 60 minutes) of pre-incubation of free Fms before the addition of the Fms-HRP for 30 minutes onto the modified surface for immunosensor system was then studied. The result from Figure 4.16 (b) shows that 10 to 60 minutes of pre incubation presented a good non linear regression curve. From these results, 30 and 60 minutes show that the increased current toward to low concentration of fumonisin. The low concentrations of fumonisins were bound to the MAbFms on the surface for the higher specific binding. This indicates that the limit of detection will increase with the decreasing of fumonisin concentration used in this assay. In this case, the sensitivity of the assay may also increase. However, there was not much difference between 30 and 60 minutes of pre-incubation, therefore after 30 minutes of incubation the assay was becoming stable and was chosen for further works. Using a similar strategy with some modification based on the direct competitive immunoassay, the increase in the sensitivity of immunoassay tests has been reported by Weller *et al*, (1992); Garson *et al*, (1997); Micheli *et al*, (2005).

After two experiments of incubation time optimisation, 30 minutes of free Fms pre-incubated and 30 minutes incubation after the addition of the Fms labelled HRP were selected. This was for enhancing the sensitivity of the assay. In this case the total period of incubation was 60 minute. The effect of the incubation time is a very important part, in order to allow the antibodies to reach the antigens in the incubating solution at the surface of the immunosensor (Liu *et al,* 2001).

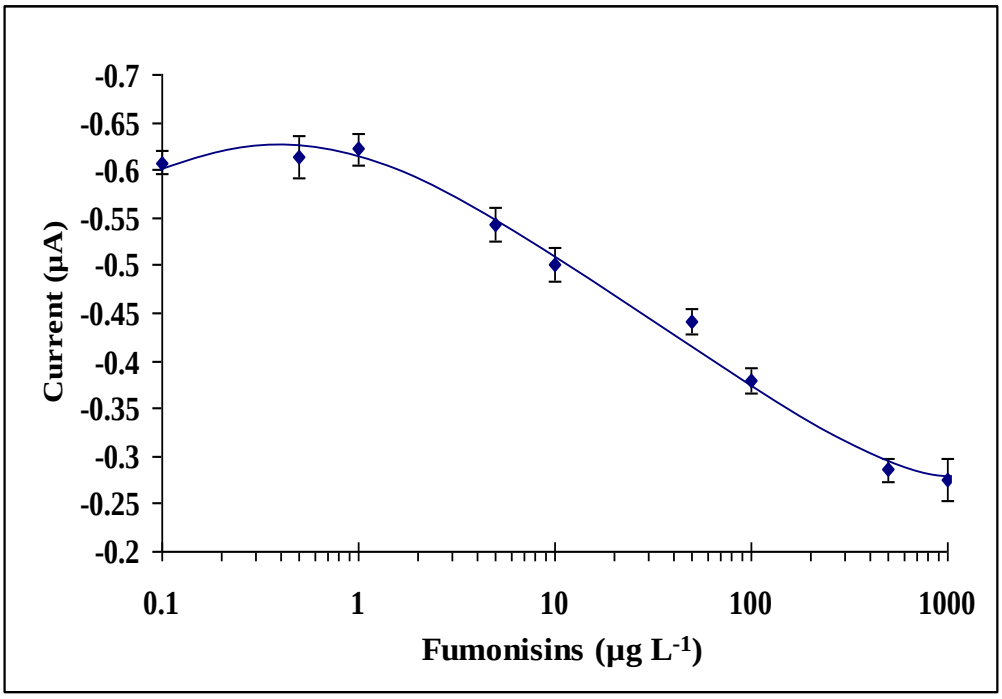
4.3.4 The calibration curve for fumonisins detection

The calibration curve of the competitive immunoassay for fumonisins detection was carried out using a low level of fumonisins standard solution (Neogen kit) which is less than the previous experiment. Referring to the curve produced at 30 minutes pre-

incubation, the linearity of the curve at low level may produce a low detection limit which may obtain high sensitivity of the assay detection. Therefore, the direct competitive experiment was run by measuring a range from 0.01 to 1000 µg L^{-1} of fumonisins on the thiol modified surface.

As seen in Figure 4.17, the decrease of current response was proportional to the fumonisin concentration in the range of 1 to 500 µg L^{-1}. The linear regression equation is µA= 0.053 x $C_{[Fms]}$ – 0.63 with a limit of detection (LOD) of 0.5 µg L^{-1} (estimated to be 3 x the standard deviation of blank-dose signal, n=3, R^2=0.991). In this case, the LOD obtained is less than before optimizing by time incubation in Figure 4.14. Therefore, the lower the LOD means the more sensitive the direct electrochemical immunosensor device is for the detecting of fumonisins. Using this device and technique, we can detect a small amount of fumonisins in the samples. When the amount of sample is more than 500 µg L^{-1}, the dilution factor of the sample is necessary.

There is also no report on an electrochemical immunosensor for fumonisins detection in the literature, so the sensitivity of the results of detection cannot be compared to other immunosensor detection methods as shown in Table 4.3. From Table 4.3 it can be seen that all of the studies are based on optical immunosensor detection. The data show that the LOD achieved in our work is less than the optical immunosensor device and similar found by Sapsford *et al*., (2006). Therefore, the electrochemical immunosensor is more sensitive than the optical immunosensor for detecting fumonisins except from optical detection by Sapsford *et al*., (2006). Data indicate that the electrochemical immunosensors with the direct competitive ELISA format were suitable for fumonisins analysis at the level required by the EU legislation.



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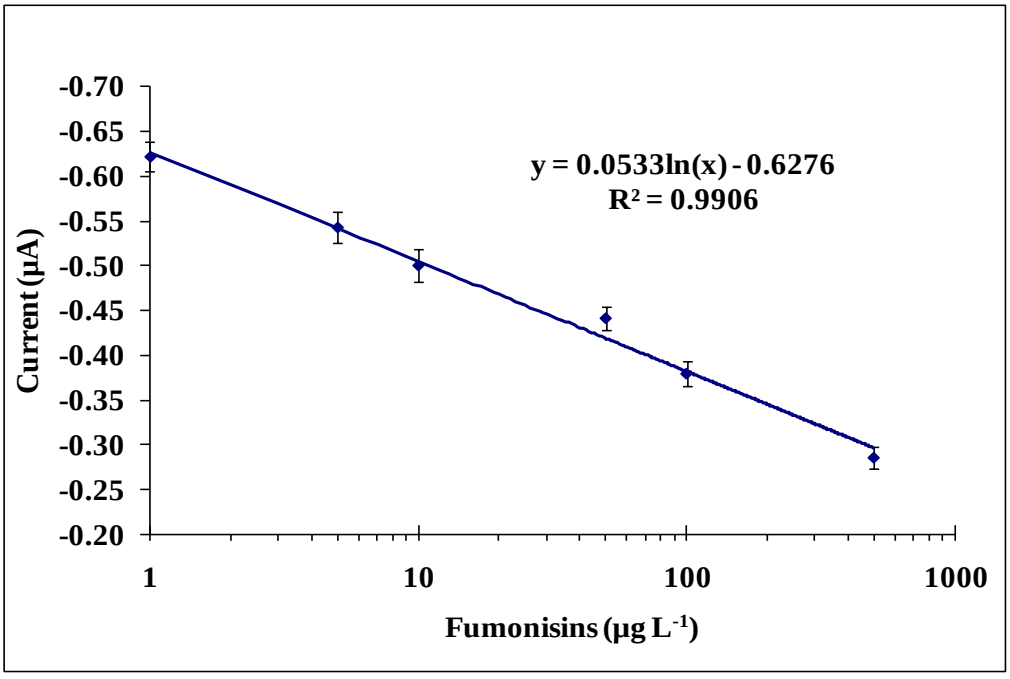

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Figure 4.17: Calibration curve of the direct competitive immunosensor for fumonisins analysis on a thiol modified surface electrode using optimised parameters. Measuring by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Modified screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with Fms (0.01-1000 µg L^{-1}) for 30 minutes pre-incubation time before adding the Fms-HRP (1:5). (a) The curves were fitted by non linear regression. (b) Linear regression of standard curve. Error bar = SD, n=3.

Table 4.3: Comparison of immunosensor detection methods with the developed electrochemical immunosensor for fumonisins detection from 1998 to 2010.

Sensing Methods	**Electrodes**	**Range detection / LOD (μg L^{-1}) in buffer**	**References**
Optical	SPR	50	Mullet *et al*., 1998
Optical	fiber optic	10 – 1000	Thompson and Maragos, 1999
Optical	SPR	NR	Daly *et al*., 2000
Optical	SPR	50	Gaag *et al*., 2003
Optical	Array	0.5	Sapsford *et al*., 2006
Electrochemical	SPGE	0.5	In this work (Kadir & Tothill, 2010)

4.3.5 Cross reactivity of the sensor

The specificity of the developed immunosensor for the detection of Fumonisin B_1 (FmB_1) and Fumonisin B_2 (FmB_2) was investigated in this system. The purpose of the test was to determine the specificity of the sensor for these two structurally related toxins. The results of the competitive assay reaction between MAbFms against all free fumonisins on the sensor are shown in Figure 4.18. The MAbFms showed good specificity against the standard solutions and also good cross reactivity for all fumonisins (FmB_1 and FmB_2). The average values cross reactivity between FmB_1 and FmB_2 was almost 100%. This indicates that the monoclonal anti-fumonisins used in the development of the immunosensor had similar specificity for both toxins and showed similar cross-reactivity for the two structurally related toxins.

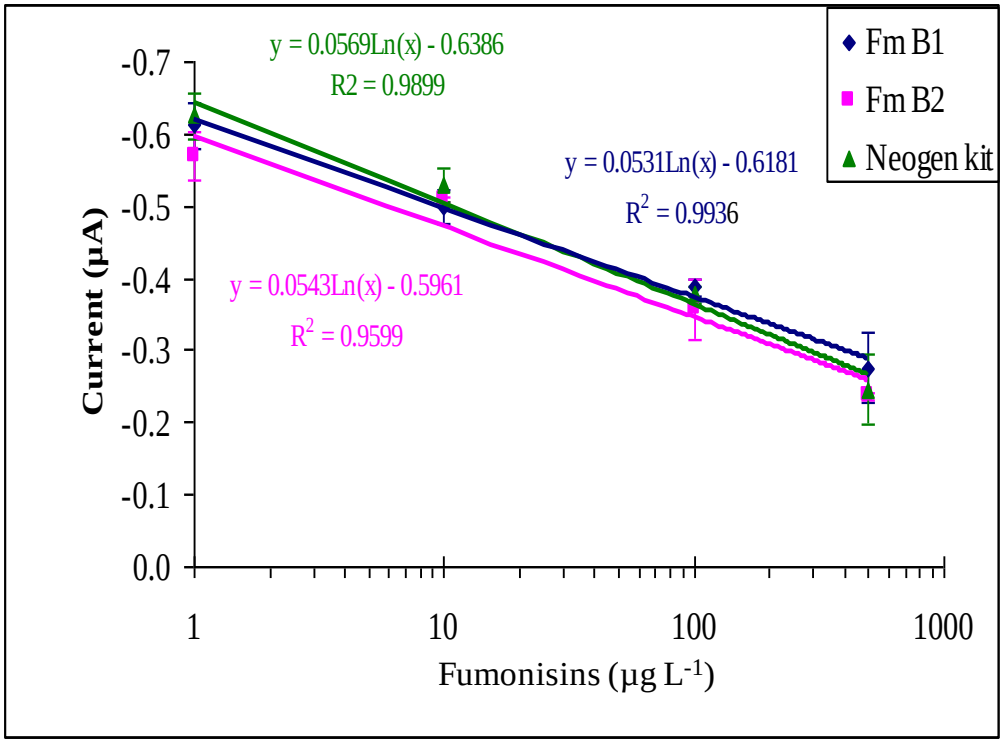


Figure 4.18: Cross reactivity of MAbFms against standard solution of fumonisin in Neogen kit, FmB_1 and FmB_2. Direct competitive Fms immunosensor response on a modified gold working surface electrode by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with Fms (1-1000) for 30 minutes pre-incubation times before adding Fms-HRP (1:5). Error bar = SD, n = 3.

A standard fumonisin solution provided by the Neogen ELISA kit (standard solution for competitive assay in this study) was also tested using the sensor, and the sensor response to this solution was also compared to the response achieved using FmB_1 and FmB_2 toxins. The reactivity of MAbFms with other fumonisin analogs provides an insight as to the antibody actually binding to the fumonisins toxins. In this case, by using MAbFms (in this work) the sensor was able to determine both fumonisins, as was required for an immunosensor able to analyse fumonisins toxins. This indicates the specificity and sensitivity of the developed sensor for the detection and analysis of fumonisins.

4.3.6 Precision and stability of the immunosensor

The precision of the immunosensor system in this case was evaluated by calculating the relative standard deviation (RSD) value (standard deviation/mean x 100). This is because the precision of the system is established based on statistical aspects such as variance, standard deviation and percent relative standard deviation (Epstein, 2002). The satisfactory precision is commonly used based on %RSD, because it is easily perceived (Zhang *et al*., 2007; Tang & Ren, 2008). The relative standard deviation of the analytical technique was conducted by analyzing four concentration levels (10, 50, 100 and 500 µg L^{-1}) with 9 electrodes per run of each level. The immunoassay system was run by using the direct competitive method of fumonisins detection on a SAM modified gold surface of screen-printed electrodes. From the graph in Figure 4.19, it can be seen that the RSDs at each point of the assay were 3.4%, 2.8%, 6.6% and 7.2% at 10, 50, 100, and 500 µg L^{-1} of Fms, respectively.

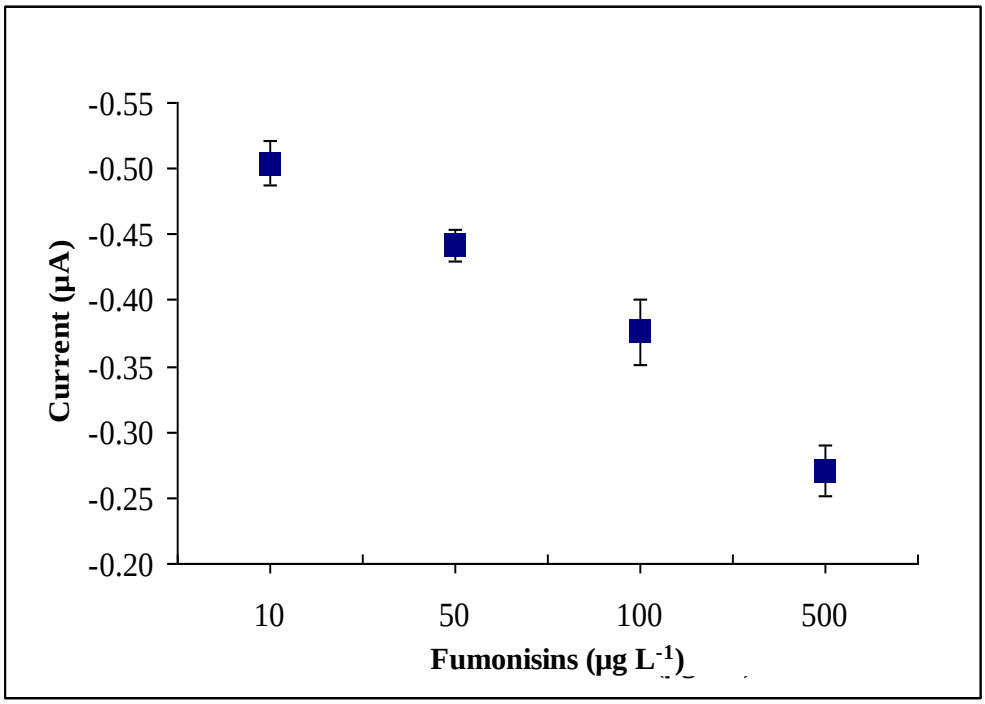


Figure 4.19: The precision data for the Fms analysis on the modified gold surface of SPGE based on four concentration levels (10, 50, 100 and 500 µg L^{-1}). Direct competitive Fms immunosensor response on a modified gold working surface electrode by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with Fms (10, 50, 100 and 500 µg L^{-1}) for 30 minutes pre-incubation times before adding of Fms-HRP (1:5). Error bar=standard deviation, n=9.

The results demonstrated at each point show the %RSD is less than 10%, which is reliable and reproducible. This is based on guidance of industry, where it is said that the RSD should not be more than 10% (FDA, 2001; Jin *et al*., 2008) Thus, the precision of the proposed immunosensor is acceptable.

Additionally, the storage study for stability of the immunosensor assay system was also examined. Without any chemical treatment, the modified gold surface with antibodies coated in PBS buffer was stored at 4ºC for 8 weeks. The test was conducted based on using one level of Fms concentration (50 µg L^{-1}) using chronoamperometry detection under the same condition.

As seen from Figure 4.20, the current response dramatically decreased after four weeks storage at 4ºC and was stable at a low current response after that. The high current response at the first 4 weeks storage indicates better affinity binding of the assay. Therefore the 4 weeks storage stability at 4ºC was acceptable for the immunosensor system in this study. The uses of immunoassay system stabilisers were not applied in this test and therefore to enhance the stability further the use of stabilisers should be examined.

4.3.7 Sensor response in extracted corn samples

To test the performance of the sensor in real sample matrix and conduct a recovery test for fumonisin, ground corn samples were spiked with various concentrations of fumonisins standard solution. The samples were first extracted using a methanol/water mixture (70:30) and then either used without any further treatment or were cleaned and concentrated using a clean-up procedure (C-18 SPE column).

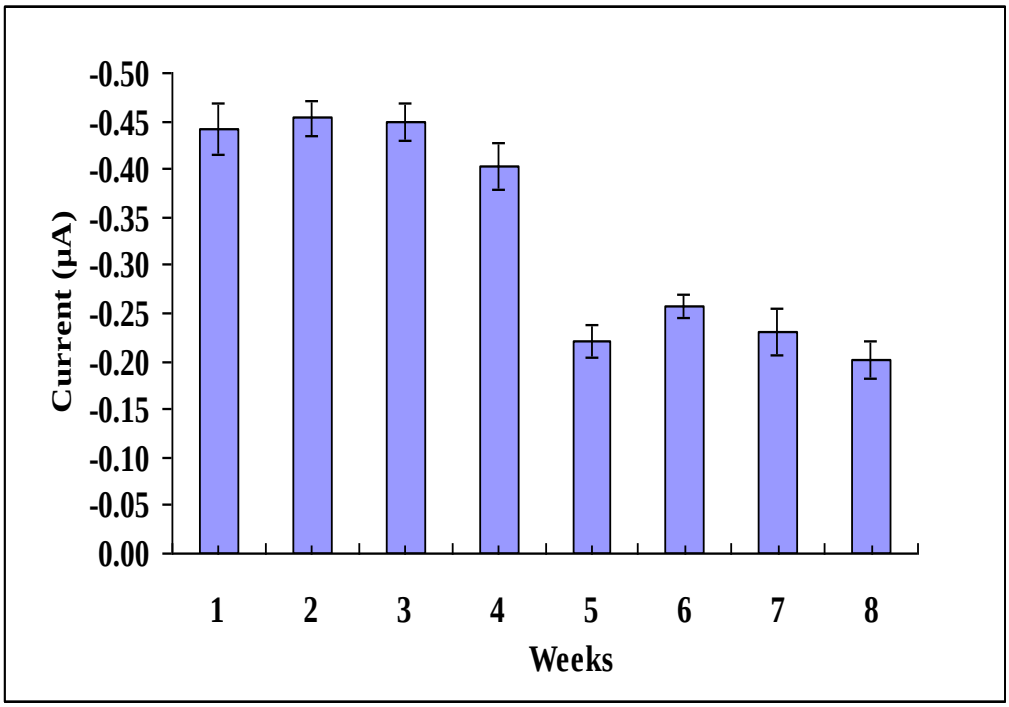
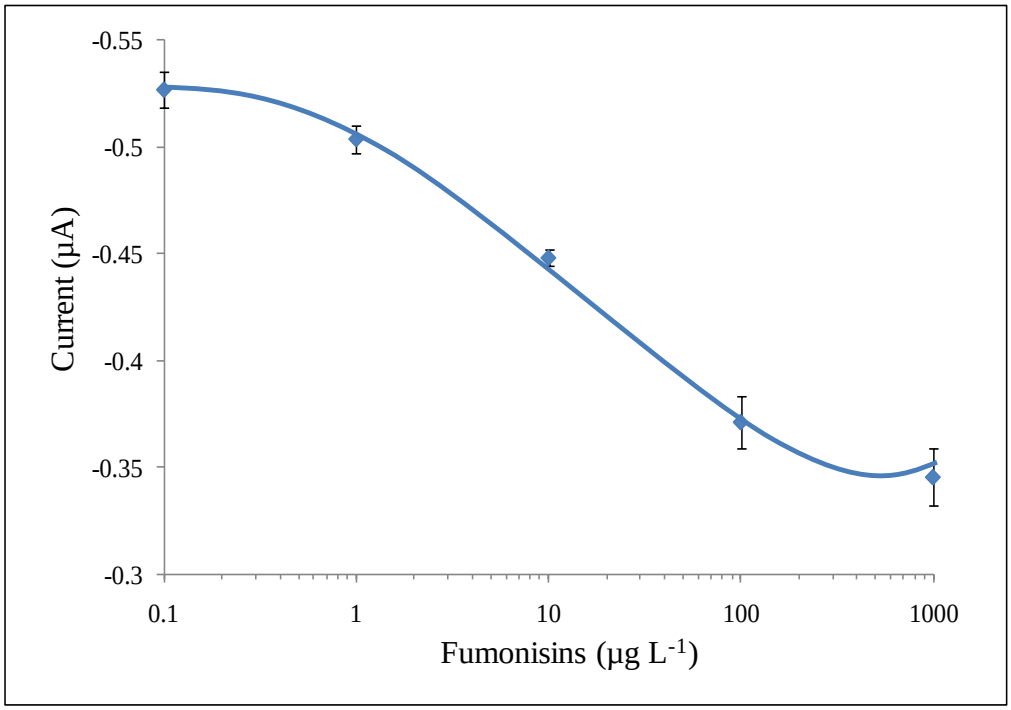


Figure 4.20: Current response of storage study for stability of immunosensor for 8 weeks. Direct competitive Fms immunosensor response on a modified gold working surface electrode by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continue with Fms (50 µg L^{-1}) for 30 minutes pre-incubation times before adding of Fms-HRP (1:5). Error bar=standard deviation, n=3.

Other non-spiked corn (maize) samples were also extracted and determined in order to evaluate the possible matrix effect compared to fumonisins in buffer samples. For the preparation of immunosensor standard curve, corn extract were redisolved with 1 ml of series free Fms standard solution in 0.01 M PBS buffer. The calibration curve results for the corn extract presented in Figure 4.21 show the linear range of 1 to 500 µg L^{-1} with R^2 value of 0.97 and %CV = 5. For the non-spiked samples background signal was found to be similar to the signal normally achieved in buffer. This indicates that there is a minimum matrix effect on the background electrochemical signal of the immunosensor by following the developed procedure.



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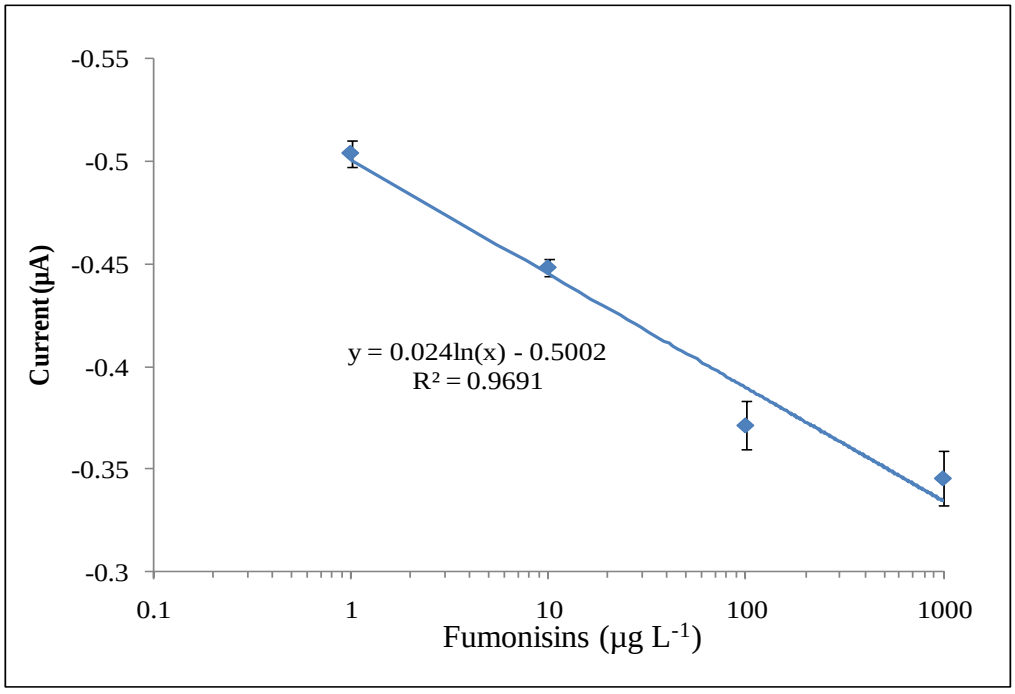

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Figure 4.21: a) The competitive response curve for Fms detection in non infected corn extract using SAM thiol modified SPGE Dupont. (b) Linear regression of standard curve for Fms detection. Measuring by chronoamperometry at potential -100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Modified screen-printed gold electrodes were coated with anti-IgG (10 µg mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continued with Fms (0.01-1000) for 30 minutes pre-incubation time before adding the Fms-HRP (1:5). Error bar = SD, n=3**.**

In the spiked corn samples, the recovery of the fumonisins was determined from the analysed concentrations of fumonisins in the corn samples, compared to the expected values spiked to the sample. Using a calibration curve of fumonisin in a non-spiked corn extract and C-18 cleaned samples, the obtained recovery results for spiked samples were calculated, and these are shown in Table 4.4.

When the two different extraction procedures were compared in terms of recovery, different percentages of recovery were found. The average recovery result for the extraction method without a clean-up step (rapid extraction technique) was much lower than the average recovery with a clean-up and concentration step (using C-18 SPE) (Table 4.4). The lower concentration of spiked fumonisins (50 and 250 μg kg^{-1}) obtained less recovery compared to the higher concentration (500 and 2500 μg kg^{-1}). This may suggest that substances in the samples containing low fumonisins inhibited the toxin signal response. By using a clean-up procedure (C-18 SPE column) which will help in removing these substances, the recovery of fumonisins has increased due to their removal from the sample extract. According to Muscarella *et al*. and in agreement with regulation 401/2006/EC, a recovery in the range of 60–120% for fumonisins is expected for samples containing ~500 μg Kg^{-1} (Muscarella *et al*., 2008). From the achieved results conducted in this study, the use of a clean-up procedure is recommended to improve fumonisin recovery especially at the low contamination level. Also conducting a standard curve for fumonisins using unspiked corn samples extracted and cleaned using C-18 gave very comparable results to fumonisin in the buffer analysis.

The analytical performance of immunosensors was then compared to HPLC. In this case, the calculated recoveries for HPLC were in the range of 86 to 96.1% with an average value of 91.8%. This indicates the immunosensor having a satisfactory agreement with HPLC. The agreement was confirmed with the application of both methods on the detection of Fms in corn samples. However, in the HPLC system, less than 50 ug Kg^{-1} of Fms in spiked samples and non spiked (CTUK) was not detectable, because the lower limits of detection by HPLC are 50 μg kg^{-1} for FmB_1 and 75 μg kg^{-1} for FmB_2. In the case of immunosensor detection, the non-detected samples from HPLC can still be detected through the sensor because the values are higher than 1 μg kg^{-1}.

Table 4.4: A comparison of results of corn samples and spiked corn samples (with fumonisins standard solution), and using two different extraction methods, where the samples were analysed using the electrochemical immunosensor and HPLC method.

Samples	Fms added ($\mu g\ kg^{-1}$)	Measure value ($\mu g\ kg^{-1}$)								
		HPLC			Electrochemical Immunosensor					
		Clean-up using 18			without clean-up			Clean-up using C18		
		Found	%CV	%Recovery	Found	%CV	%Recovery	Found	%CV	%Recovery
Non-infected	0	nd			less than 1			less than 1		
Corn	50	nd			30.05 ± 2.4	9.4	60.1	50.9 ± 3.9	7.6	101.7
	250	240.3 ± 10.8	4.5	96.1	173.5 ±7.9	5.5	69.4	190.1 ± 16.2	8.5	76.0
	500	466.3 ± 32.6	7	93.3	410.9 ± 24.2	5.9	82.2	560.3 ± 25.8	4.6	112.1
	2500	2150 ± 92.5	4.3	86	2055.8 ± 74	3.6	82.2	2616.1 ± 102	3.9	104.6
Average			5.2	91.8		6.1	73.5		6.2	98.6
CTUK	unknown	nd			less than 1			3.7±0.41	11.1	
COMM	unknown	1449.7±119	8.2		1034±126.5	12.2		1526.5±182.9	11.9	
FmB_2 2500		2337.2±217	9.2	93.4	2218.4±200	9	88.7	2533.5±250.2	9.8	101.3

CTUK = Corn Tesco UK; COMM = Corn Open Market Malaysia; FmB_2 = Fumonisin B2. *n*=3.

Therefore, 2500 μg kg^{-1} FmB_2 in spiked corn showed a high recovery for HPLC and also with the immunosensor. The present method here showed a very sensitive sensor device and acceptable for Fms detection.

In the case of non spiked detection, the fumonisins in the COMM sample were in the range of 1034 to 1526 μg kg^{-1} and the lowest was obtained from immunosensor detection without clean-up extraction. This is because recoveries were also lower than the other methods but still reliable for the rapid detection method. The performance of the immunosensor and the results achieved from analysing the corn samples indicate its useful application for the analysis of fumonisins in corn samples. This indicates the developed SPGE immunosensor using electrochemical detection is rapid and reliable for corn samples analysis.

4.4 Conclusions

In this study, we have developed a direct competitive ELISA and immunosensor for the detection of fumonisins in buffer solution and real corn samples. A better orientation of the antibody specific for the fumonisin by precoating the immobilisation antibody before coating with the monoclonal antibody on the ELISA plate was conducted. After optimisation of the reagents using the ELISA method, the direct competitive assay exhibited a linearity range of 100 to 2000 μg L^{-1}. The assay was then subsequently transferred to the screen-printed gold electrodes.

This work also demonstrates the possibility of measuring using screen-printed gold electrodes as electrochemical transducers in two different immobilised antibodies, which are passive adsorption and covalent binding. By using optimal reagents on the covalent binding assay, the high specific binding is based on pre-incubation free Fms for 30 min before competing with Fms-HRP on the antibody site. The calibration curve of Fms in the buffer presented a working range of 1 to 500 μg L^{-1}, more sensitive than the ELISA method. The use of monoclonal antibodies against fumonisins (MAbFms) toxin in general showed a high cross reactivity to FmB_1 and FmB_2, which indicates that the immunosensor will be able to detect both fumonisins.

The working range results for the ELISA (100 to 2000 µg L^{-1}) and immunosensor (1 to 500 µg L^{-1} (ppb)) obtained for Fms detection is compatible for the analysis with the requirement of the EU legislation in food and feed (2 - 4 mg kg^{-1} (ppm)). The high value of recovery of Fms in the spiked corn showed a better performance of the proposed immunosensor which was around 98.6 % and 73.5 % in average for clean-up extraction and direct extraction (rapid), respectively. The reliability of both immunosensor formats for the analysis of Fms in spiked or real samples was verified by comparison of the data with the fully validated confirmatory HPLC results.

CHAPTER 5

DEVELOPMENT OF ELECTROCHEMICAL MICROELECTRODE ARRAY FOR AFB_1 AND FUMONISINS DETECTION

5.1 Introduction

The use of an electrochemical immunosensor using a screen-printed gold electrode for AFB_1 and Fms detection was successfully investigated in Chapters 3 and 4. However, to enhance the detection sensitivity and work towards a multi-array analysis, the use of a microelectrode array was investigated. The microelectrode will reduce the electrode surface area which tends to reduce the quantity *i*Rs by decreasing the current, *i*. The theory has been demonstrated that the 'electrochemical measurements are mainly affected by the noise currents due to the potential drop in solutions *i*Rs, where *i* is the current through electrodes and Rs is the solution resistance between the reference electrode and the working electrode' (Amatore, 2005).

In this work, a microelectrode array was used based on a gold cell-on-a-chip microelectrode with a gold electrode array consisting of 35 microsquare electrodes with 20 µm x 20 µm, 200 µm spacing, a reference (0.4 mm x 0.4 mm) and counter (100 µm L-shape) electrode which were fabricated by standard deposition, etching and lithographic techniques used in microfabrication technology (by Tyndall Cork). The microelectrode array is particularly interesting because such arrays are geometrically different from SPGE and also have various benefits in an electrochemical sensor application. They provide much faster diffusion of electroactive substances and produce different sigmoidal (or steady-state) cyclic voltammograms (Parker *et al.*, 2009). Also they have the ability to improve response time (faster response), greater enhancement of mass-transport which can provide better sensitivity and enhancement of current response to noise ratio (Fungaro & Bretta, 1999). They provide a substantial improvement in the signal response to surface area ratio under steady-state conditions which can solve the extremely low current value problem (Berduque *et al*., 2007).

Surface modification using chemical or biological molecules on the microelectrode array is possible in order to achieve a better control of the electrochemical characterisation, in order to improve the sensitivity and selectivity of the electrochemical sensor detection (Parker *et al.*, 2009). The purposes of modification of the microelectrode surface is for the pre-selection of the target species to improve the binding site, prevent build up of protein layer for reducing signal inhibition and to

increase the amount of bio-recognition for increasing the sensitivity. Therefore, the surface modification must provide selectivity to a target analyte and have to accelerate redox activities (Berduque *at el*., 2005). Methods used for the preparation of modified electrodes are those based on covalent binding (surface hydroxyl groups, etc.), adsorption (thiol chemisorption, etc.) and polymeric coating (polymer with a chemically active moiety, etc.) (Herzog and Arrigan, 2003).

In particular, small electrodes are very useful when working with a small volume of expensive reagents, or small sample volumes (Farell *et al.*, 2004). The sensitivity of the electrochemical reaction has been reported to be enhanced by the use of a small sample volume on a reduced sensor size (dimensions) (Wijayawardhana *et al*., 1999). The reduction of sensor dimensions means a reduction of the diffusion distance (Miu *et al*., 2005). This is also related to the reduction of the incubation time, thus resulting in a rapid detection assay. Another advantage is that using a small volume will decrease the enzymatic product dilution on the electrode surface (Miu *et al*., 2005), which is an essential factor in achieving low detection limits in enzyme immunoassays.

The focus of the study in this chapter was to investigate surface modification of microelectrodes to allow covalent binding of the immunoassay for both AFB_1 and Fumonisins detection. Utilising the optimisation of the direct competitive ELISA and immunosensor was subsequently transferred to electrochemical modified microarrays with minor modification.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

The same materials (reagents, chemicals and buffers) were used as described in Chapter 3 (section 3.2.1) and Chapter 4 (section 4.2.1). Additional chemicals for surface modification of the microelectrode array are 3-aminopropyltrimethoxysilane, 1,4-phenylene diisothiocyanate (PDITC), dimethylformamide (DMF), methanol and 1,2–dichloroethane were purchased from Sigma-Aldrich Ltd (UK). TMB, H2O2 and

all other general chemicals were also purchased from Sigma-Aldrich. All buffers and antibody solutions used were the same concentrations and ionic strengths as reported in Section 3.2.1 or 4.2.1

5.2.2 Apparatus

Incubations for every step of the reactions were carried out by Labsystem iEMS incubator/shaker HT. Electrochemical measurements were performed using AUTOLAB model PGSTAT with multichannel GPES software from Eco Chemie, (Utrecht, Netherlands). A plasma etching instrument (from E.M.Technologies Ltd, Ashford Kent UK) was used for the gold surface cleaning.

5.2.3 Overall method

The flow chart (Figure 5.1) shows the overall view of the electrochemical microelectrode for aflatoxin B_1 and fumonisins analysis.

5.2.4 Microelectrode array (MEA)

A gold cell-on-a-chip microelectrode was used in this study as designed and fabricated by photolithography at the Central Fabrication Facility, Tyndall National Institute (Cork, Ireland) as described by Parker and co-workers (Parker *et al*., 2009). The electrode device was fabricated with a working electrode, counter electrode, and reference electrode, and is referred to as the cell-on-a-chip electrode. The material used for the electrode device was gold. The microelectrode array device consisted of 35 microsquare electrodes with 20µm x 20µm, 200µm spacing, a reference (0.4 mm x 0.4 mm) and counter (100 µm L-shape) electrode (Figure 5.2).

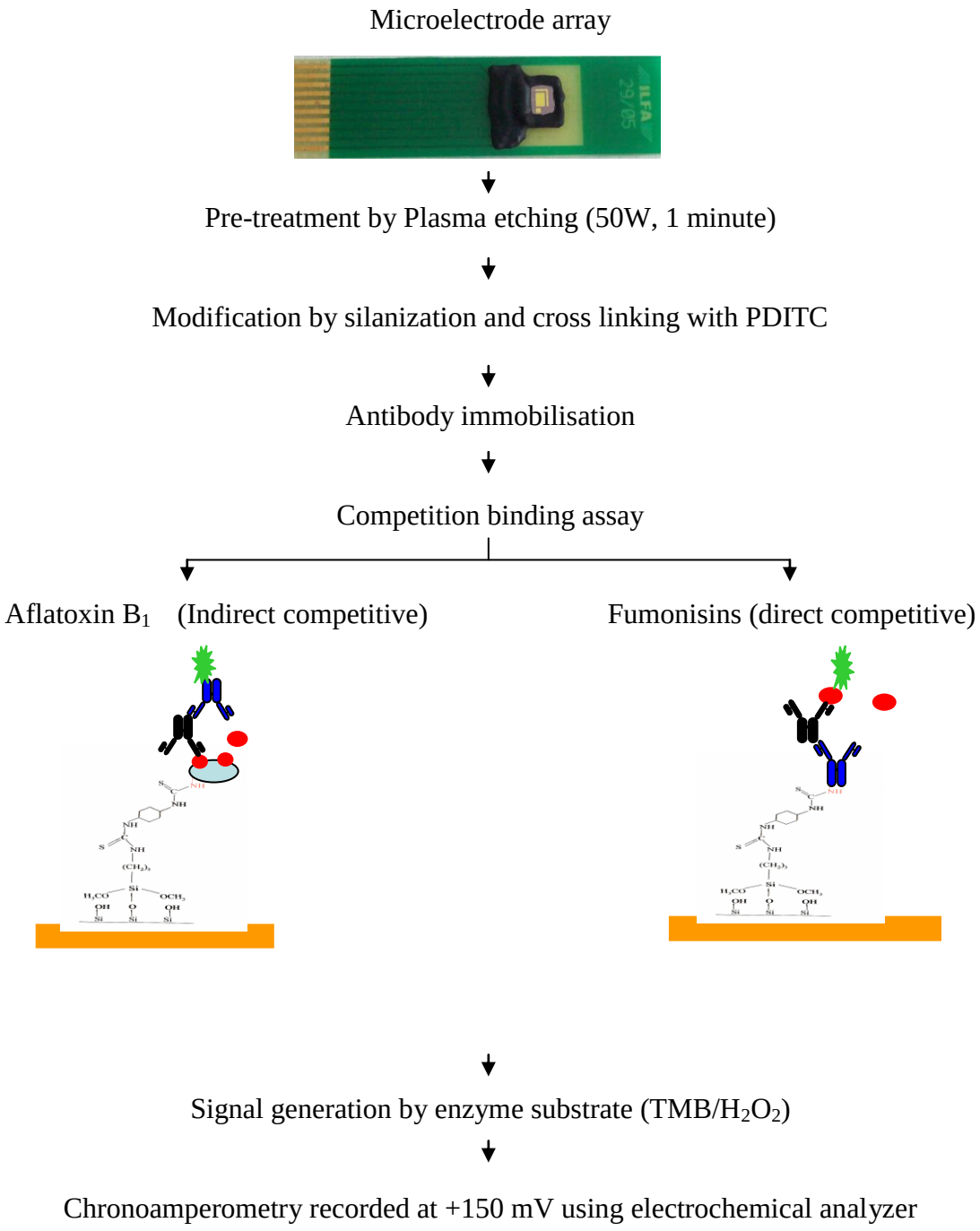


Figure 5.1: Schematic diagram of electrochemical immunoassay development using microelectrode array for mycotoxins analysis.

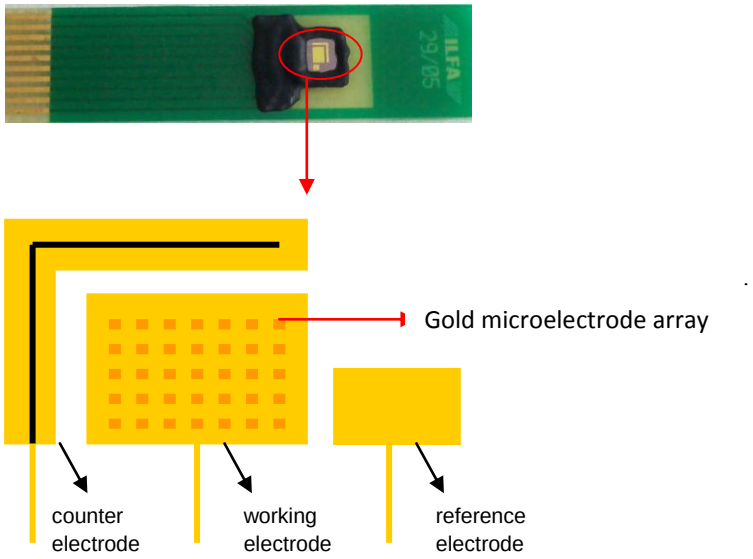


Figure 5.2: Microelectrode array silicon based cell-on-a-chip electrode designed and fabricated at Tyndall (Cork, Ireland). The sensor chip with working electrode area (containing gold microelectrode array), reference and counter electrode.

5.2.4.1 Surface modification for antibody immobilisation

Surface modification was performed by silanization and cross linking with PDITC before the antibody was immobilised on the surface. The modified surface was for the covalent binding of the recognition system to the insulating surrounds of the microelectrodes. The gold chips surface was modified by being pre-treated first with oxygen plasma using a plasma etching for 50 W, 5 minute exposure with a continuous oxygen flow before the silanization procedure. Then, the chips were immersed in 3% 3- aminopropyltrimethoxysilane in a 19:1 dilution of methanol : water (HPLC grade) for 2-4 hours at room temperature (25 ºC) (silanisation procedure), followed by washing with methanol and deionised water. After that, the electrode chip was heated at 120ºC for 15 minutes.

Further, the electrode chips were then used to deposit a cross linker by immersion in 1 Mm 1,4-phenylene diisothiocyanate (PDITC) in dimethylformamide (DMF) containing 10% pyridine for 2 hours. Lastly, the chips were washed with DMF and 1,2 dichloroethane followed by drying under nitrogen gas (Figure 5.3).

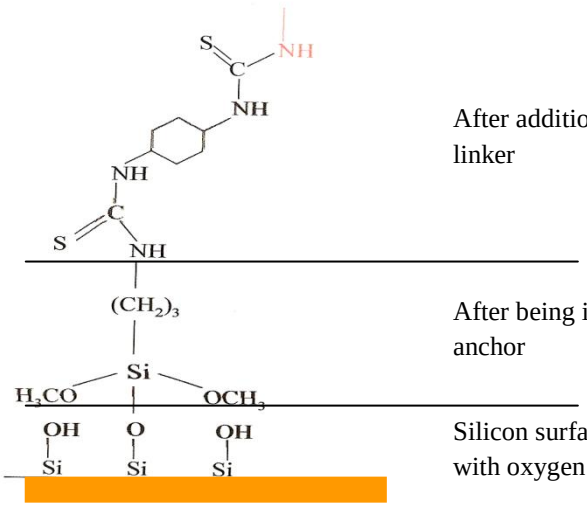


Figure 5.3: Schematic diagram of pre-treated chemical modified surface using an amino-silane (3-aminopropyltrimethoxysilane) anchor and cross linked with 1,4-phenylene diisothiocyanate (PDITC) for covalent immobilisation on the surface.

5.2.5 Scanning Electron Microscope (SEM) analysis

The characterisation of the microelectrode surface was analysed using SEM. The SEM (scanning electron microscope) images and elemental scans were taken using a Philips XL30 SFEG (scanning field emission gun) (Philips, Guildford, UK). The surface of the new and used microelectrode arrays were observed, in order to monitor and ensure silicon and gold surface was cleaned for further use.

5.2.6 Electrochemical measurement

A computer controlled four channel Eco Chemie Autolab electrochemical analyzer multipotentiostat (Eco Chemie, The Netherlands) with four connector electrodes was used for the electrochemical measurement. This machine provides four cells to connect to the 4 Pomona-clip connectors for measuring the signal simultaneously at

the same time. The chips were clipped via their gold basal track into a connector. The data acquisition was recorded via the supplied GPES version 4.9 software to a PC.

5.2.6.1 Voltammetric studies

Experiment was performed where the gold microelectrodes were used as working electrodes and using the on chip reference and counter electrodes. Before each experiment, the chips were treated with oxygen plasma for 1 minute at 50 watt to remove remaining interference particles.

Cyclic voltammetry

The electrochemistry of the microelectrode arrays was investigated by CV analysis using potassium ferrocyanide. The electrochemical behaviour of potassium ferrocyanide (0.005 M) in 0.1 M KCl on the chips (bare, modified, used and re-used) electrode was performed by CV at 10 mV s^{-1}.

The parameters for the CV measurements were carried out by:

Number of cycles:	5
Start potential:	-1 V
First vertex potential:	+ 1V
Second vertex potential:	-1V
Step rate:	0.00198
Scan rate:	0.00999 to 0.03999 V s^{-1}

Chronoamperometry

In this study, the chronoamperometry measurement was chosen for the current response of enzymatic activity. Amperometric measurements were obtained with a single fixed potential procedure with a current acquisition interval of every second. The sensitivity range was set from nAmps to 10 µAmps.

A mediator substrate of 5 mM TMB containing 0.075% H_2O_2 was selected by applying a reducing current of HRP enzymatic activity and monitored by amperometry analysis. This chronoamperometric measurement was also used to evaluate the potential of the microelectrode as a TMB sensor. The different potential ranges from -500 to 500 mV were studied.

5.2.7 Immunosensor preparations

Competitive formats for the detection of AFB_1 and Fms were studied. The protein or antibody were covalently immobilised following the steps listed below (5.2.7.1 and 5.2.7.2).

5.2.7.1 Indirect competitive immunoassay for Aflatoxin B_1 detection

The antibody immobilisation and competition step of immunoassay for AFB_1 was performed based on an indirect competitive format. This immunoassay format was investigated for comparison with the previous immunosensor with SPGE.

After modification of the surface, about 1 µl of AFB_1-BSA conjugate (1 µg mL^{-1}) in coating buffer (0.1 M carbonate buffer pH 9.6) were immobilised on the modified surface then incubated overnight at 4ºC. The chips were then washed using PBS-T (twice) followed by PBS (once). The surface was then deactivated using 0.1% NH_4OH before being incubated for 1 hour at room temperature (25 ºC) (stop reactive compound). The assay was then blocked with 1% PVA blocking solution (1 µl) before being incubated at 37°C for 1 hour, followed by washing. The competition reaction involved the addition of various AFB_1 standard solutions (0-10 µg L^{-1}) with a fixed anti-aflatoxin antibody ($MAbAFB_1$) (10 µg mL^{-1}) in PBS for a 30 minute pre-incubation at room temperature in an eppendorf tube before 1 µl of mixture was transferred to the electrode for 30 minutes at 37ºC incubation. After the washing step, a 1 µl of anti-antibody labelled with HRP (anti-IgG-HRP) (1.0 µg mL^{-1}) in PBS was then added onto the electrode and incubated for 30 minutes at 37ºC, then the unbound material was washed out.

5.2.7.2 Direct competitive immunoassay for Fumonisins detection

The general protocol of the adsorption procedure in the initial study is described below. About 2 µl of 10 µg mL^{-1} goat anti-mouse IgG (H+L)) primary layer (capture species) in a carbonate buffer was strictly immobilised on the modified gold surface of the electrodes then incubated overnight at 4ºC. The electrodes were then washed using PBS-T (twice) followed by PBS (once). The surface was then deactivated using 0.1% NH_4OH before being incubated for 1 hour at room temperature (25 ºC). The blocking reagent was added on the electrode surface with 1 % PVA in PBS before incubation for 1 hour at 37ºC. About 2 µl of 1:50 MAbFms antibody capture was coated on the gold working electrode then it was incubated for 1 hour at 37ºC. To the assay was then added 1 µl of free Fms (0 to 4000 ppb) for 30 minutes incubation before adding 1 µl of 1:2.5 of antigen labelled HRP (Fms-HRP) onto the modified gold surface. The electrode was then incubated for 30 minutes at 37ºC. During each step of the assay, washing procedures were carried out between immobilisation (precoating/coating), blocking, competition and detecting, by rinsing each electrode twice with PBS-T and once with PBS. The signal generated was performed by dropping 5mM TMB/0.075% H_2O_2 solution onto the electrode surface.

All the microelectrodes used during incubation at 37ºC had to be in high humidity to avoid evaporation (for the reaction of antibody binding in solution on the surface).

5.2.7.3 Enzymatic reaction

For the signal generation, TMB and H_2O_2 enzyme substrate was used. The preparation of the enzyme substrate is as described below. About 1 mg of TMB was dissolved with 150 µl deionised water for the stock solution. The enzyme substrate also needed a combination with hydrogen peroxide, diluting 10 times stock 30% hydrogen peroxide to 3%. From both stocks, about 20 µl of each stock solution was taken and made up to 1000 µl using 10 mM citrate buffer (pH 5.0) in 0.1 M KCl at 37^{o}C. A 5 µl aliquot of the TMB/H_2O_2 solution was deposited onto the immuno-microelectrode

array immediately prior to current detection. The stock solution and substrate was freshly prepared and stored in dark conditions before use.

5.2.7.4 Amperometric detection

The chronoamperometry was performed by attaching the microelectrode array to the electrochemical analyzer (Potentiostat, Autolab). In this case, a preconditioning pre-potential was carried out first for 5 sec. at a potential +250 mV before measuring at a potential of +150 mV. This preconditioning method has been reported to enhancing the current response of the immunoassay (Parker *et al*., 2009).

5.2.8 Gold surface cleaning for reusable microelectrode arrays

After the MEAs were used, the MEAs were soaked in deionised water (DI) overnight. Then, the gold surface was cleaned by the addition of 10 µl of 3:1 H_2SO_4 H_2O_2 (piranha solution) on the surface and rinsed thoroughly with DI water. The MEAs then treated with plasma etching with 50 watts power, 5 minutes exposure with continuous oxygen plasma flow using a plasma etching instrument (from E.M.Technologies Ltd, Ashford Kent UK).

5.2.9 Sample preparation for aflatoxin B_1 and fumonisins in sample matrix

The protocol used for the sample preparation of AFB_1 and fumonisins in peanut and corn were similar to those described in Chapter 3 (section 3.2.) and Chapter 4 (section 4.2.), respectively.

5.2.9.1 Standard curve for sample extract

The development of a self-prepared standard curve for aflatoxin B_1 (AFB_1) in peanut extract, ranging from 0 to 10 µg peanut extract L^{-1} were conducted. Finally, 5 g of

ground samples (free toxin) were placed into a 150 mL flask and a 25 mL of 70% methanol was added. The samples were then shaken vigorously for three minutes manually. Extract samples were then filtered through filter paper (Whatman No.1 or equivalent). After that, the filtered sample was diluted with 1:1 of free AFB_1 in 0.01M PBS pH 7.4 to give a standard series of 0, 0.001, 0.01, 0.1, 1 and 10 µg L^{-1} which were used for the immunoassay standard. A 1 µl of diluted samples were used for testing on the MEAs surface.

The development of the standard curve for fumonisins (Fms) in corn extract were conducted following the procedure as described by the Veratox ELISA kit (Neogen Corporation, UK). A 5 gr of ground corn sample (free toxin) was used, mixed with 25 mL of 70% methanol and 30% water and then shaken vigorously for 3 minutes. The extract was filtered through a Whatman #1 filter paper to remove the solid material and the filtrate was then collected. The filtered sample was diluted with 1:10 of free fumonisin in 0.01M PBS pH 7.4 to give a standard series of 0, 0.1, 1, 10, 100, 1000 and 2000 µg L^{-1} which were used for the immunoassay standard.

5.3 Results and Discussions

Each gold microelectrode in this experiment consisted of a gold microelectrode array, a working electrode with a reference (0.4 mm x 0.4 mm) and a counter (100 µm L-shape) electrode. By using SEM at 65x magnification image, the gold microelectrode array on the working electrode consisted of 35 micro-square electrodes with 20 µm x 20 µm, 200 µm spacing, as clearly shown in Figure 5.4.

5.3.1 Plasma cleaning of microelectrode

Before the electrodes were used for immobilisation, it is necessary first to clean the gold surface from contaminants. Using a plasma machine the technology provided a dry cleaning process to the gold electrode surface by a continuous flow of ionized oxygen in vacuum chambers. In this case, SEM at 2000x magnification was employed for observing the quality of the gold surface, for either a new or used working electrode surface.

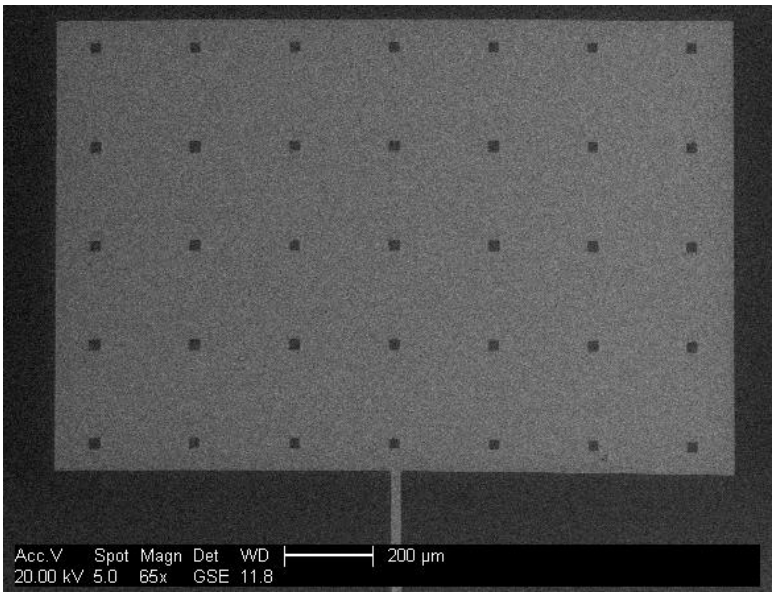


Figure 5.4: The images of 35 micro-square on the working electrode of microelectrode array by SEM at 65x magnification image.

In Figure 5.5 (a) and (b) the small particles on the surface of used working electrode can clearly be seen. These small particles are normally residual organic and chemical matter that may have come from the environment (new) and also from immuno reagents remaining after use (used electrode). However, the residues were removed after the surface was treated with O_2 plasma for 1 minute at 50 watts (Figure 5.5, c and d). According to Alberici *et al*, the downstream O_2 plasma cleaning is performed as an ashing reactor which strongly removes organic contamination. This is 'no matter whether the substrate is silicon or silicon nitrite' (Alberici *et al*., 2004). In this case,

removal of the residue from the gold surface will increase the yields in binding activities on the surface and enhance the sensitivity of the sensor.

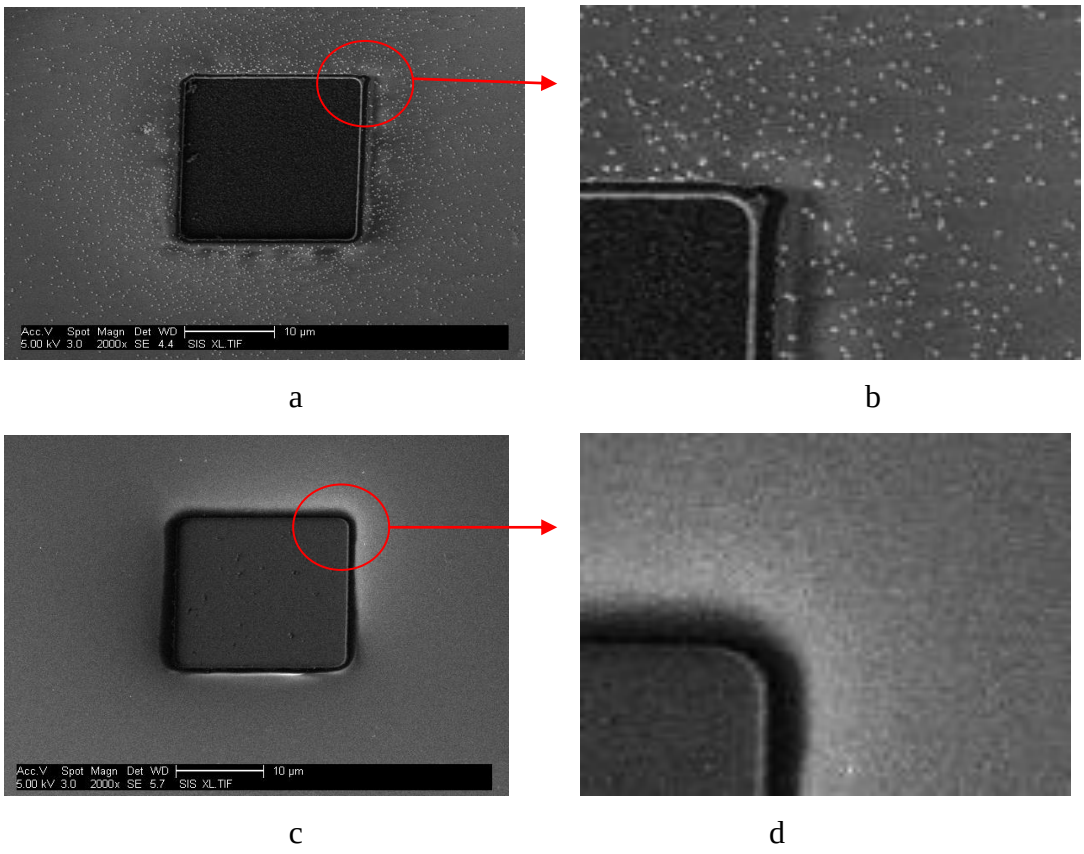


Figure 5.5: SEM images of part of a used working electrode before and after being treated with oxygen plasma. a) used microelectrode, b) used microelectrode with residual organic matter, c) used microelectrode after being treated with O_2 plasma and d) cleaned used microelectrode. Typical scanning electron micrographs (2000 x magnification).

5.3.2 Electrochemical characterisation for microelectrodes

The microelectrode array (MEA) was then characterised by cyclic voltammetry (CV). The cyclic voltammetry (CV) with ferrocyanide electrode was used first for determining the cleanliness of the microelectrode array surface.

Figure 5.6 shows increasing the scan rate increased the sigmoidal peak shape of voltammograms. The expected reduced sigmoidal (steady state) voltammograms were

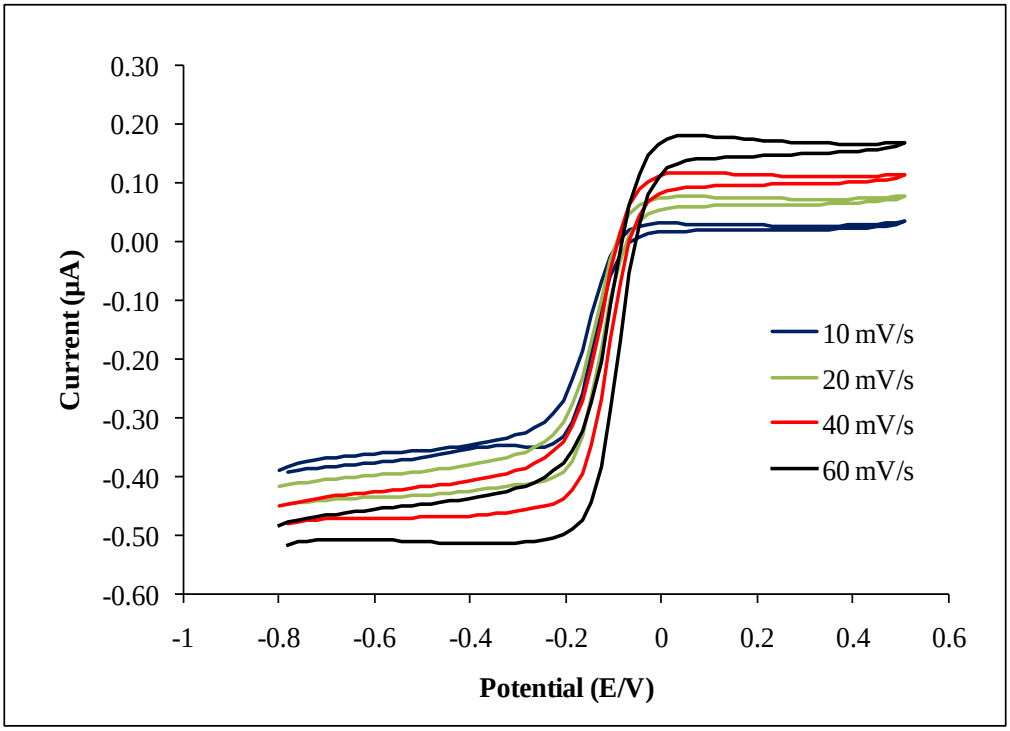


Figure 5.6: Cyclic voltammograms of 5 mM ferrocyanide in 0.1M KCl on bare microelectrode array at scan rate 10, 20, 40 and 60 mVs^{-1}. Cyclic voltammograms of 5 mM ferrocyanide in 0.1M KCl at different scan rate (10 to 60 mVs^{-1}) for characterisation of bare microelectrodes after treated with oxygen plasma.

obtained after running at the slower scan rate. It was a different peak shape compared to SPGE (larger redox peak shape). In the article by Kudera *et al*., when the diameter of electrode is decreased from macro scale down to micron scale, radial diffusion of the electroactive species towards the electrode surface becomes more dominant rather than linear diffusion (Kudera *et al*., 2001). According to Berduque *et al.*, (2007) the sigmoidal shape is formed from MEA, which is an indicative hemispherical diffusion layer on the surface. With this diffusion layer a much faster diffusion of electroactive substances occurs compared to planar diffusion from SPGE.

This indicates that the sensor is able to improve response time (faster response), and has a greater sensitivity and increased response per unit electrode surface area (Berduque *et al*., 2007). In this study, the well-defined steady state responses of the arrays with microelectrodes demonstrated that these arrays fulfil the prerequisites for microelectrode arrays.

5.3.3 Surface modification

Using a small electrode surface will limit the amount of bio-molecules that can be immobilised. This is relative to the sensitivity of the electrode. In order to improve the sensitivity of the electrode, the surface modification of the sensor surrounding the silicon layer around the microelectrode was investigated. So, in this study, the modification of the chips with the amino silane anchor (APTES) and cross-linker PDITC immobilised on the sensor for covalent binding was carried out. According to Parker *et al* (2009) the increase of binding of bio-recognition material on the surface will increase the bio-capture capacity and sensitivity of the electrode.

The APTES was immersed at room temperature for 4 hours to the surface by forming a covalent on Si−O−Si bond with the amino group extending from the surface. From the previous study, the reaction of amino silane on silica surface was present to anchor through formation of a surface Si−O−Si bond at room temperature (Kanan *et al*., 2002). Manning *et al* (2003) and Manning and Redmond (2005) described the use of this excellent method for amine modified probe protocol to silanise support materials. Then the PDITC was immersed for 2 hours at room temperature for a bi-functional cross-linker (1,2 homobifunctional cross-linker) to attach an amine modified from the antibody to the surface. This covalent attachment of an antibody is similar in manner to what has previously been demonstrated about covalent attachment of amino-terminated probe oligonucleotide microarrays by Manning and Redmond (2005). According to them, the amino derivatised surface, coupled with excess PDITC, converts the amino groups into amino reactive phenylisothiocyanate groups, which are then used to couple to the antibody. Any remaining phenylisothiocyanate groups can be blocked for deactivation by using NH_4OH.

The schematic of surface chemistries used, based on APTES and PDITC for covalent attachment of antibodies, is illustrated in Figure 5.7. The purpose of modification is to improve the binding of the biomolecule recognition mainly on the silicon area; therefore, an assessment of the surface coverage on the gold electrode surface was carried out by electrochemical measurement.

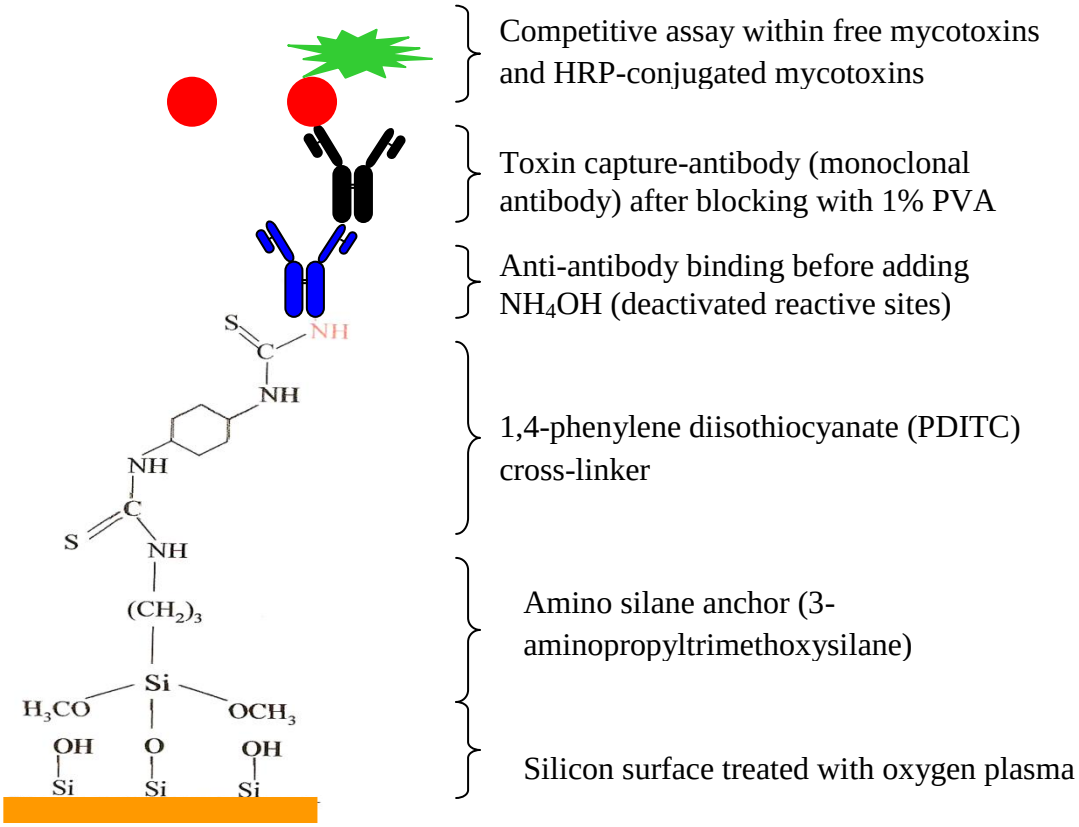


Figure 5.7: Schematic of surface modification based on an amino silane anchor and PDITC cross-linker for covalent binding of direct competitive ELISA format for fumonisins detection.

5.3.3.1 Cyclic Voltammetry (CV) after surface modification

The most commonly used technique to assess the extent of electrode surface coverage after modification is based on cyclic voltammetry of ferrocyanide. In this study, to extend the understanding of the electrochemical properties of the modified electrode, we compared the redox potential of ferrocyanide at unmodified microelectrodes using cyclic voltammetry.

Figure 5.8 shows a representative CV of modified and unmodified electrodes based on an amino silane and PDITC cross-linker attached on the gold electrode surface. At a scan rate 10 mVs^{-1}, the expected sigmoidal voltammogram potential at the modified

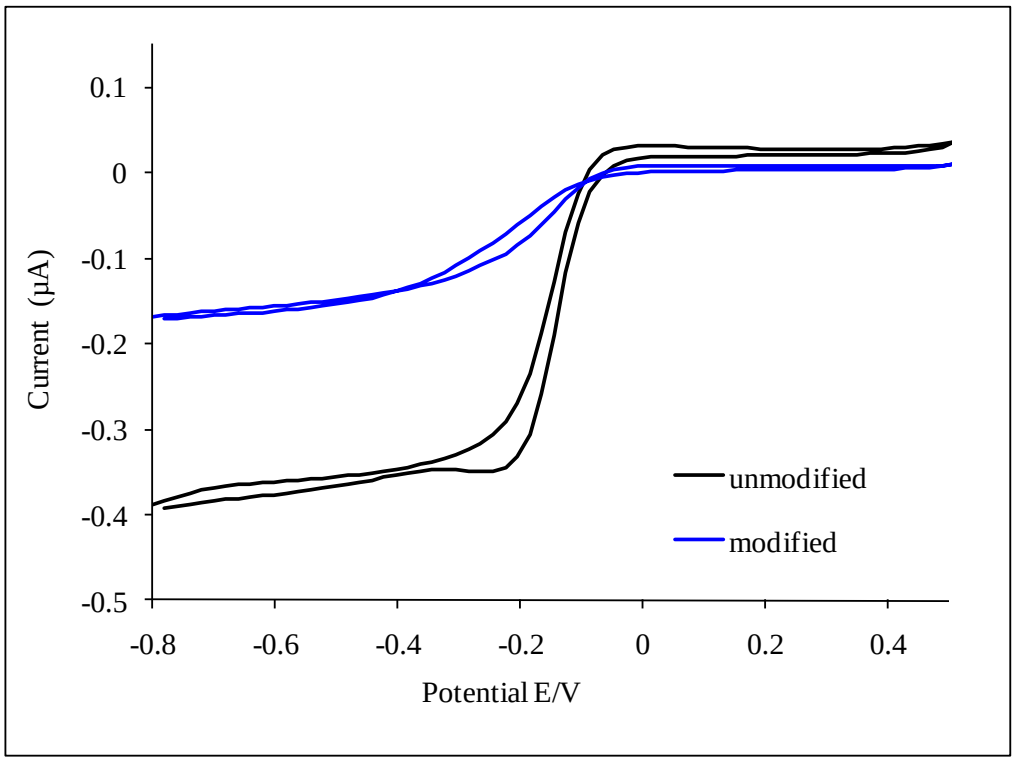


Figure 5.8: CV of modified and unmodified surface based on an amino silane and PDITC cross linker attached on the gold electrode surface after being treated with oxygen plasma using 5 mM ferrocyanide in 0.1KCl at 10 mVs^{-1}.

microelectrode array was reduced the peak shift. This indicates low current transfer kinetics compared to the unmodified electrode.

This behaviour indicates that by reducing the CV redox peak after modification, the attachment of some amino silane anchor and PDITC cross-linker is taking place on the electrode surface as well as on the surrounding silicon area. These results led to the conclusion that a small electron transfer takes place between the chemical molecules and bare surface (Kudera *et al*., (2001).

The electron diffusion transfers are still permitted, suggesting the modification with facilitators to be an appropriate method to improve the interfacial contact with protein molecules. In this case, surface modification is important to improve the binding of the capture antibody through covalent immobilisation.

5.3.4 Potential determination of modified microelectrode

Similarly to SPGE, the potential determination of the microarray electrode was investigated based on enzymatic activity and using chronoamperometry detection. The indirect immunoassay format was immobilised onto a modified surface working microelectrode to determine the signal and background response. The HRP activity was applied as a signal response at different potentials (-500 to 500 mV).

In this case the value of the signal over the background (S/B) was plotted and calculated as a function of optimal potential as shown in Figure 5.9. A high value of S/B was achieved at +150 mV, since more positive potentials give small ratio values. In the case of negative potential detection, -200 mV is the highest value compared to other negative potential. However, +150 mV was chosen as a suitable potential for the chronoamperometry detection of TMB/H_2O_2 on modified MEA.

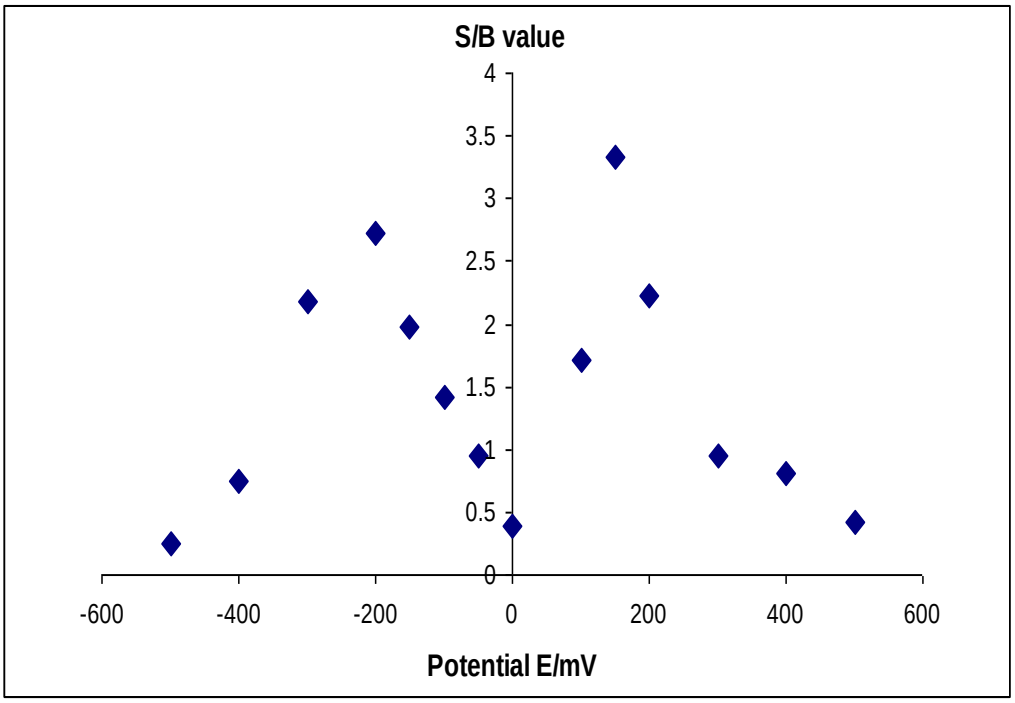


Figure 5.9: The value of signal over background ratios of potential on modified MEA. Current response for enzymatic reaction using TMB/H_2O_2 substrate based on the direct non competitive format on modified MEA at different potential. Experiments were conducted using a modified electrode based on amino silane anchor and PDITC cross-linker and binding with anti-antibody IgG, blocked with 1 % PVA followed by MAbFms and continued with Fms-HRP. Backgrounds were conducted without Fms-HRP.

5.3.5 Immunoassay development

The modified microarray electrode sensors were used for subsequent immunosensor development for the detection of AFB_1 and fumonisins. For the detection of AFB_1, an indirect immunoassay format using a monoclonal antibody (primary antibody) ($MAbAFB_1$) was carried out as the sensing molecule for the microarray electrode. In the case of fumonisins detection, a monoclonal antibody (MAbFms) was also used as the sensing material based on the direct immunoassay format. The competitive reaction was developed and characterised the same as in our previous work, using the SPGE. One of the main benefits of using a microelectrode compared to a screen-printed gold electrode in sensor application is enhanced mass transport in faster diffusion of the electrochemical species. Using the successful indirect and direct immunoassay, the immobilisation procedure from SPGE was transferred to the modified MEA for AFB_1 and Fms measurement, respectively.

5.3.5.1 Development of microsensor for aflatoxin B_1

Non-competitive assay

In order to ensure optimal orientation of the monoclonal antibody, covalent immobilisation was used for the microarray through the PDITC cross-linker chemistry. The first test conducted was to determine the performance of the antibody coating and antigen binding on the modified microelectrode surface by using a chronoamperometry test. The preliminary work was done using a non competitive indirect immunoassay method. The test was performed by a fixed amount of AFB_1-BSA conjugate (1 µg mL^{-1}) and $MAbAFB_1$ (0 to 200 µg mL^{-1}) being immobilised before being followed by anti-IgG-HRP (1 µg mL^{-1}) on to modified surface.

From the result shown (Figure 5.10), the optimal amount of $MAbAFB_1$ coating to the specific binding of AFB_1-BSA on the modified MEA was present. It can be seen that the increasing of the current response is proportional to the concentrations of $MAbAFB_1$ immobilised on the MEA, which dramatically increased from 1 to 20 µg mL^{-1} of $MAbAFB_1$ (high dynamic range). This suggested that a good orientation of antibody-antigen binding on the modified microelectrode array was present.

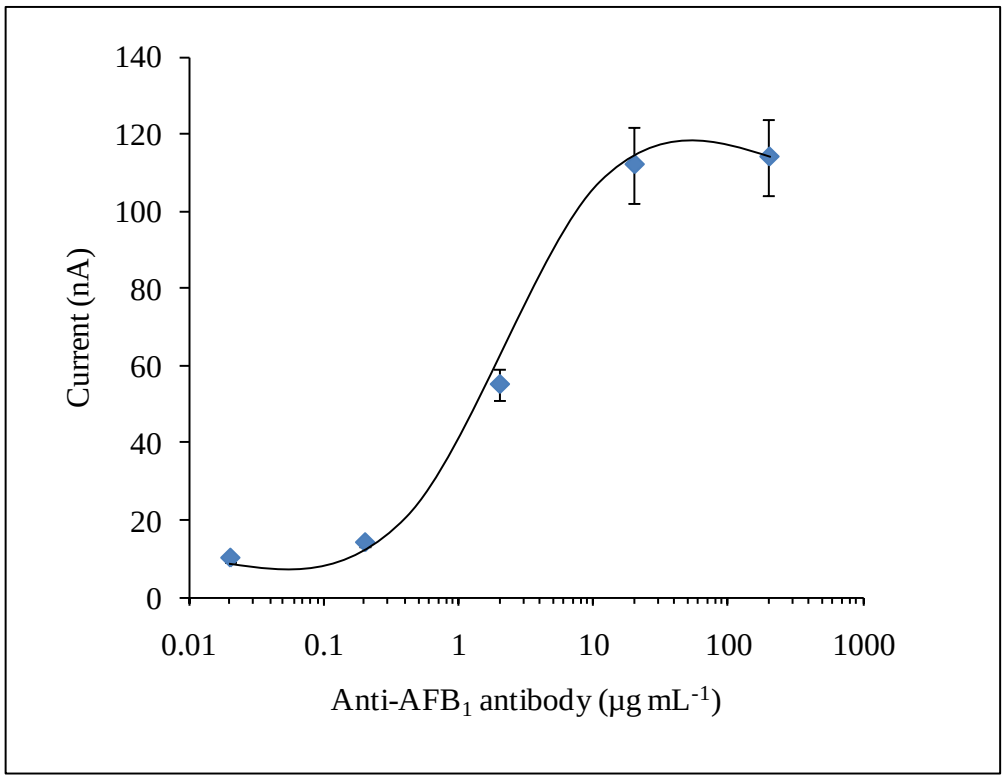
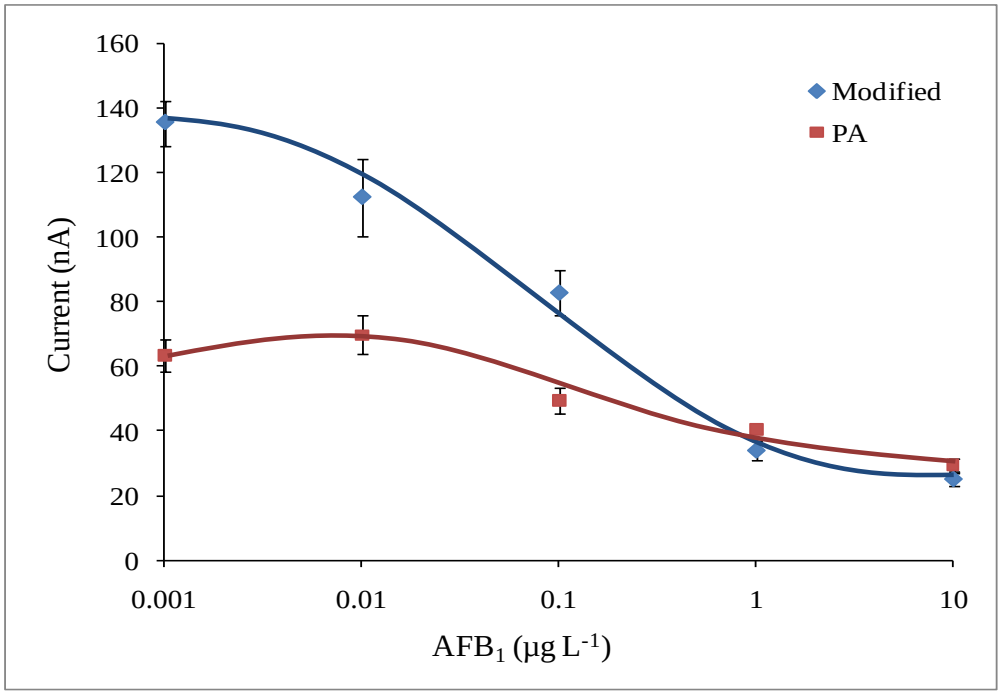


Figure 5.10: Coating signal with the incubation of increasing concentrations of anti-AFB_1 antibody. Fixed AFB_1-BSA (1 µg mL^{-1}) and anti-IgG-HRP conjugate (1 µg mL^{-1}) were used. Error Bar=SD, n=3.

From this Figure, the maximum reading was observed between 20 to 200 µg mL^{-1}, However, a 10 µg mL^{-1} amount of $MAbAFB_1$ was chosen when the fixed immobilised AFB_1-BSA conjugate (1 µg mL^{-1}) and anti-IgG-HRP (1 µg mL^{-1}) was used in the assay. This amount still produced a better specific binding while a high reading was still obtained. The same amount as in SPGE was selected, for a comparison study with SPGE and also for the cost effective assay.

Competitive assay

The development of a competitive immunoassay method for the AFB_1 detection on microsensors was carried out by passive adsorption and covalent immobilisation. The system was employed using an indirect format within a free AFB_1 solution at concentration range (0 to 10 µg L^{-1}). A typical standard curve of competition binding antigen was obtained in Figure 5.11.



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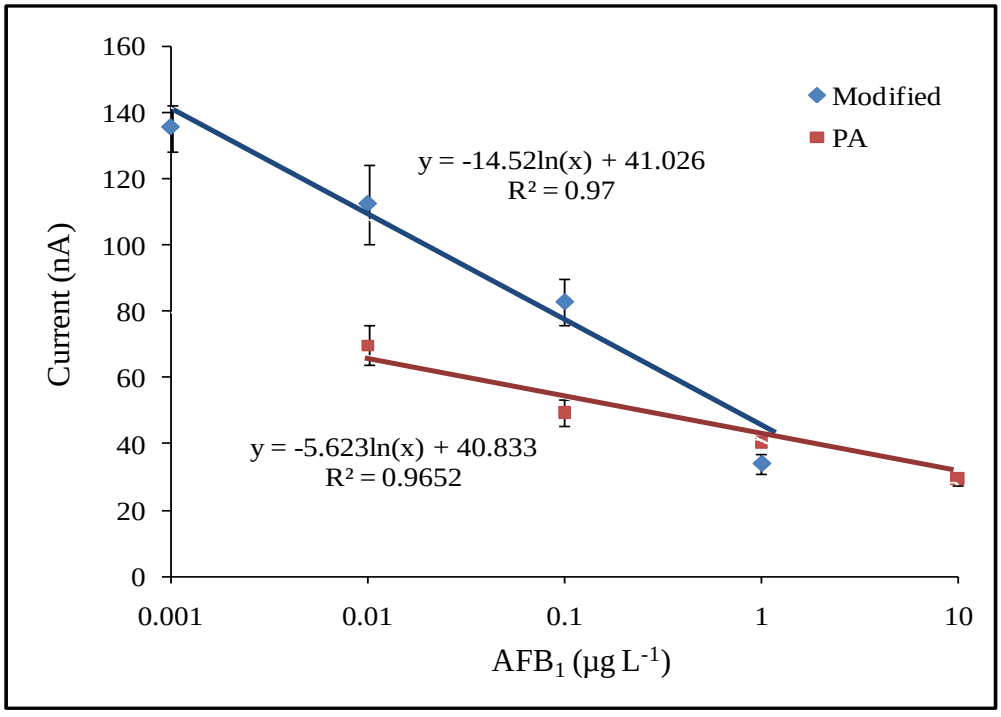


b

Figure 5.11: (a) Calibration curve of AFB_1 based on indirect competitive immunoassay by passive adsorption and covalent immobilisation using MEA. (b) Linear graph of AFB_1 using MEA. Indirect competitive AFB_1 immunosensor response on a modified MEA by chronoamperometry at potential +150 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Modified MEA were coated with AFB_1-BSA conjugate (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (10 µg mL^{-1}) and free AFB_1 (0 to 10 µg L^{-1}) before being continued with anti-IgG-HRP (1 µg mL^{-1}). Error Bar=SD, n=3.

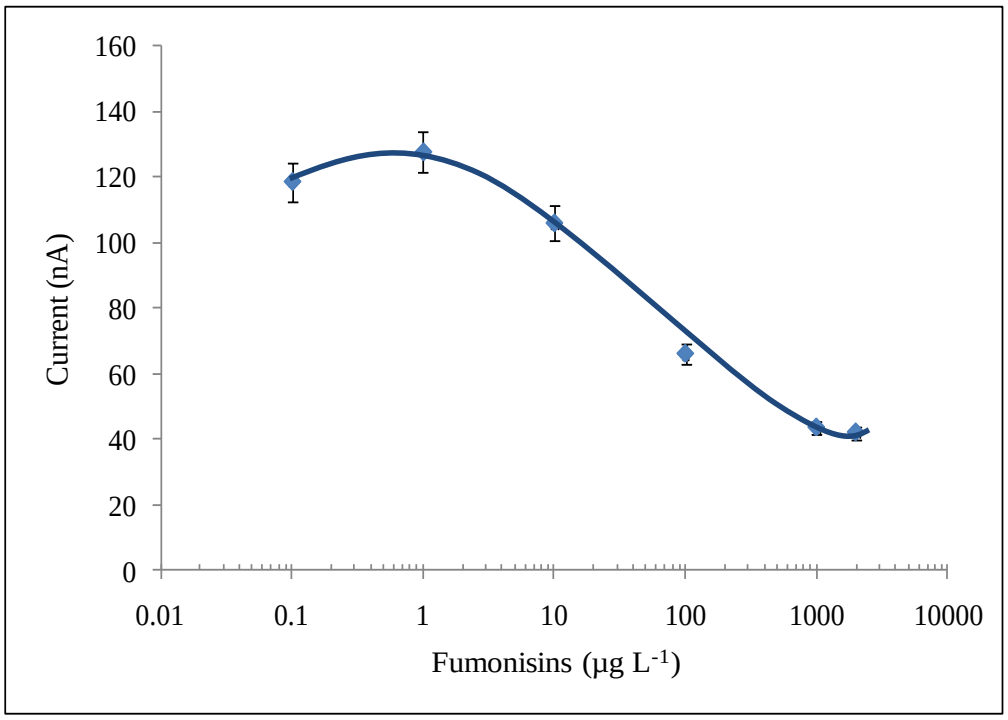
For both curves in Figure 5.11(a), the current response was inversely proportional to the AFB_1 concentrations. However, a higher signal response with a high S/B was obtained when the competitive assay was on the modified surface.

This shows that the antibodies had covalently immobilised with better orientation binding antibody-antigen on the modified electrode. The calibration plot gave a limit of detection of 0.001 µg L^{-1} AFB_1 while the linear regression value obtained was R^2 which is 0.97 (Figure 5.11(b)). The percent of coefficient of variation (%CV) for the immunoassay on the modified electrode was 7% (*n*=3).

In case of the passive adsorption method, the antibody-antigen reaction on the gold surface also gave a sigmoidal curve, but with less current detected, and the data is still reliable. The linear regression was found to be R^2 = 0.96 with the %CV = 6.2%. However, the limit of detection was found to be less than on the modified surface (less sensitivity – 0.01 µg L^{-1}). According to Parker *et al*, (2009) antibodies were passively adsorbed on the gold working MEA while the covalent binding was to the surrounding silicon layer. This suggests that immobilisation was taking place but with lower efficiency. The LOD result using the modified surface shows that the microelectrode arrays are more sensitive than using a screen-printed gold electrode.

5.3.5.2 Development of microsensor for fumonisins

Due to the limited available supply of the electrode, the binding performance of the non competitive assay for immunoreagents used for fumonisins detection was not conducted. Therefore, the optimal concentrations of reagents and conditions from a SPGE sensor was transferred onto the MEA. The development of a direct competitive immunoassay for fumonisins detection was performed by covalent immobilisation on the MEA. The competition method was carried out by coated goat anti-mouse IgG antibody (10 µg mL^{-1}) before being immobilised with anti-fumonisin antibody (MAbFms) (1:50 dilution) followed by free Fms (0 to 2000 µg L^{-1}) before continuing with Fms-HRP (1:5). A typical standard curve of competition binding antigen was obtained in Figure 5.12.



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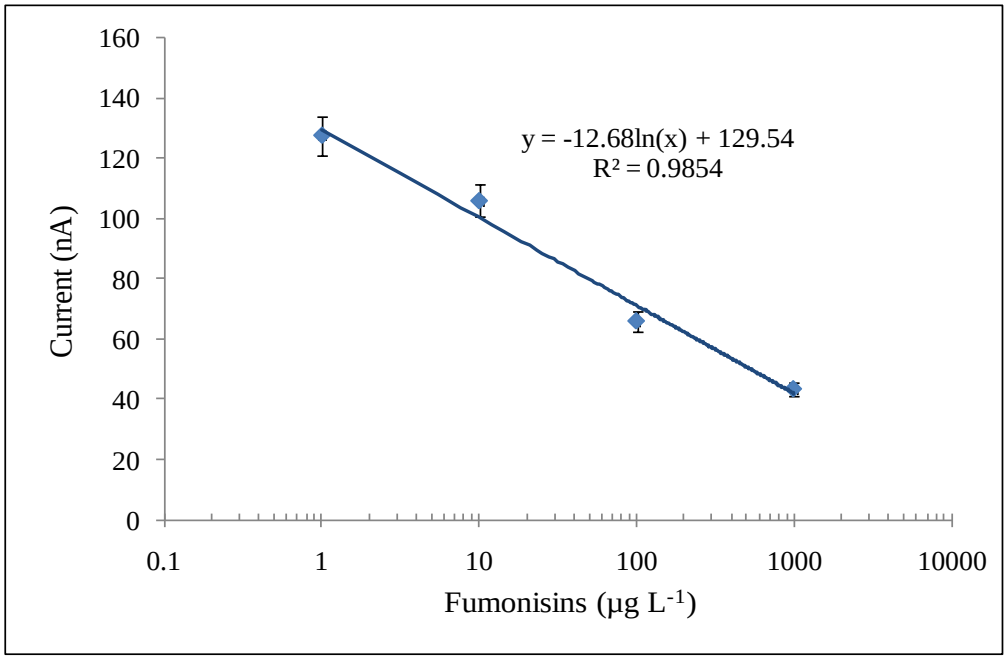


b

Figure 5.12: (a) Calibration curve of fumonisins using the modified MEA and detection using chronoamperometry. (b) Linear graph of Fms using MEA. Direct competitive Fms immunosensor response on a modified MEA by chronoamperometry at potential 150 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Modified MEA were coated first with anti- IgG (10 µg mL^{-1}), then blocked with 1% PVA before the addition of MAbFms (1:50), followed by free Fms (0 to 2000 µg L^{-1}) before being continued with Fms-HRP (1:5). Error bar=SD, n=3.

The current response was also inversely proportional to the fumonisin concentrations. The signal was dramatically decreased from about 1 to 1000 µg L^{-1} and slowly became stable after that. This phenomenon shows that with the optimal concentration of reagents and conditions for the Fms detection in this study, the analytical sensitivity achieved was 0.5 µg L^{-1} of free fumonisins in buffer. The precision was determined by carrying out three replicate measurements at each concentration. Therefore, Figure 5.12(b) show the linear graph of the working range of Fms detection is between 1 and 1000 µg L^{-1} with the linear regression, $R^2 = 0.98$ with the %CV ~ 5.5%.

Few comparable publications for microelectrode immunosensors are available. The most directly comparable report in terms of method and application is the report from Parker *et al*., (2009). Parker *et al*., (2009) produced an immunosensor for the detection of AFM_1 by immobilising an anti-AFM_1 antibody to the same microelectrode array surface and exposing the antibody against a labelled HRP. The reported working range and detection limit for this immunosensor is 10 to 100 ng L^{-1} and 8 ng L^{-1}, respectively, and comparable to the limits observed in this project. Dill *et al*. (2004) also used a direct competitive assay for the detection of α 1 acid glycoprotein on a microelectrode surface and found the detection limit was 5 ng L^{-1}.

5.3.6 Immunosensor using reused microelectrode array

Due to the cost of electrode devices and limited supplies, a reusable electrode device was investigated to save on analysis costs. The clean up process and treatment with O_2 plasma were carried out before the electrodes were reused. Cleaning is important in order to discard all the contaminants or organic residue on the used surface. The electrodes were soaked in deionised water overnight to remove the salts. Then the sensing surfaces were cleaned with piranha solution (3:1 H_2SO_4 and H_2O_2) and rinsed thoroughly with distilled water to discard all the organic residues. The piranha solution was used for the gold surface cleaning because it is the most commonly used method (Balasubramanian *et al*., 2006). Lastly, these electrode surfaces were treated with O_2 plasma to remove and clean all the residual particles on the surface (see

section 5.3.1). These processes were undertaken to ensure that the used electrodes could be reused after cleaning.

5.3.6.1 Cyclic voltammetry for used MEA

The efficacy of cleaning the sensor surface was confirmed by evaluating through cyclic voltammetry testing. The cyclic voltammetric of 5 mM ferrocyanide in 0.1M KCl was tested to characterise the used MEA before using it for device analysis. Figure 5.13 shows the different results of cyclic voltammograms of used and new MEA before and after cleaning. At a scan rate of 10 mVs^{-1}, the steady-state (sigmoidal) voltammograms of the used microelectrode array (used before cleaning) showed a reduced peak shift compared to a new bare microelectrode. This behaviour indicated that the used electrode surface was covered with chemicals or biological molecule particles, which were used in the immunoassay analysis. These results led to the conclusion that particles were inhibiting electron diffusion transferred onto the electrode surface.

After the surface was cleaned (used after cleaning), the sigmoidal redox potential was increased and the peak shape indicates an increase in electrocatalytic activity (Hocevar *et al*., 2005), indicating that the residual particles were removed and this was beneficial for enhancing the microelectrodes response.

However, not much difference in steady state voltammograms resulted between a cleaned used electrode (used after cleaning) and a new electrode. This may suggest that there is no difference in sensitivity of microelectrode response. This characterisation was also undertaken to ensure that the used electrodes could be reused like a new electrode.

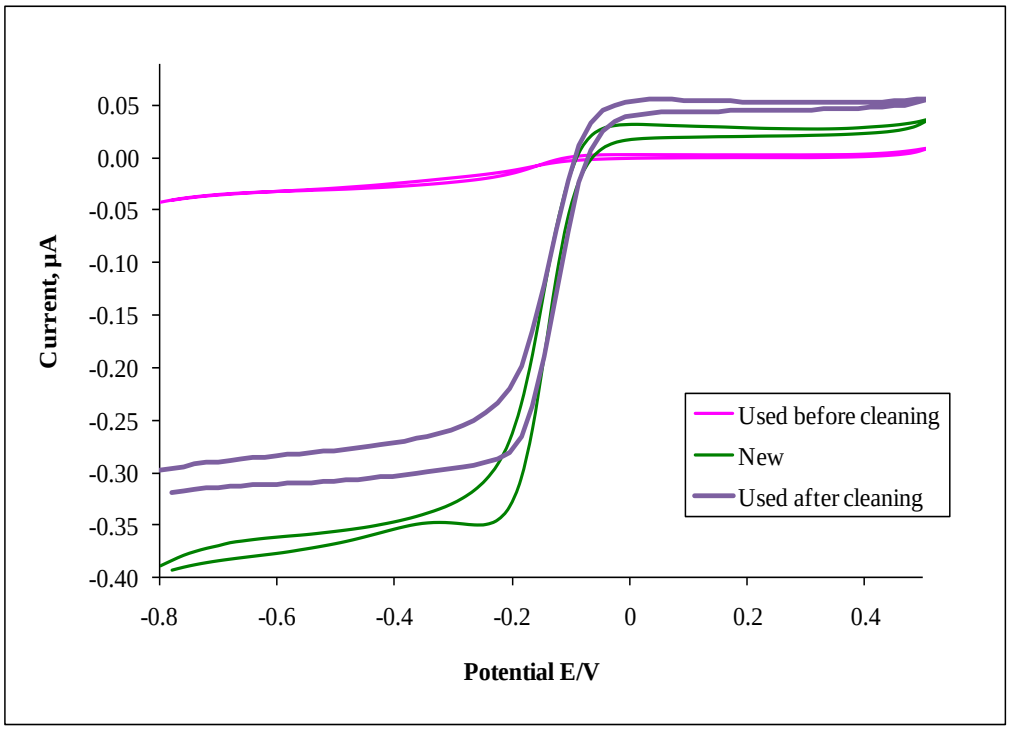


Figure 5.13: Cyclic voltammograms of new and used MEA before and after cleaning, recorded in a solution of 5mM ferrocyanide in 0.1 KCl at 10 mV^{-1}.

5.3.6.2 Aflatoxin B_1 detection using used MEA

The used MEA were tested for AFB_1 detection using chronoamperometry, in order to compare the sensitivity of the assay to the new electrode. Used electrodes were then modified with amino silane anchor and PDITC cross linker before being immobilised with the antibody. The performance of the electrodes after repeating 3 times of cleaning was then evaluated, based on a calibration curve of AFB_1 detection.

Figure 5.14 show the different response of calibration curve obtained. The inversely proportional current response for the first time of cleaning (C1) was still good reproducibility as compared to the new electrode (%CV = 5.2%). No significant difference in current response and the sensitivity of analysis was observed, giving a LOD of 0.001 µg L^{-1}. After the second and third time of cleaning (C2 and C3), the current response was slowly reduced from 140 nA to 100 nA which refers to the maximum signal at 0.001 µg L^{-1} concentration.

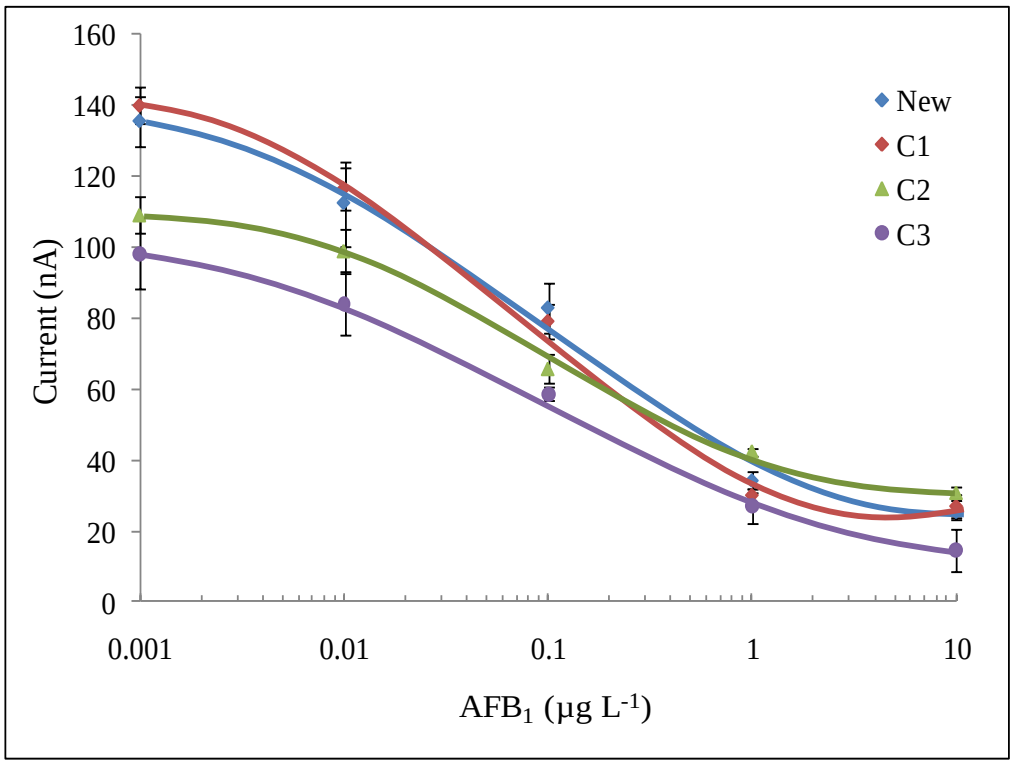


Figure 5.14: Calibration curve of AFB_1 based on indirect competitive immunoassay by covalent immobilisation using new and used MEA. Indirect competitive of AFB_1 immunosensor response on a modified MEA by chronoamperometry at potential +150 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Modified used MEA were coated with AFB_1-BSA conjugate (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MAbAFB_1$) (10 µg mL^{-1}) and free AFB_1 (0 to 10 µg L^{-1}) before being continued with anti-IgG-HRP (1 µg mL^{-1}). Error Bar=SD, n=3.

This means less assay sensitivity compared to the new and first cleaned electrode. However, the electrodes were still producing reliable data, showing a high dynamic range (better orientation binding), and present linear concentration range between 0.01 to 2 µg L^{-1}.

The result suggested that MEA can be reused for 2 times with a high sensitivity assay to detect AFB_1 by 0.001 µg L^{-1} and above. Then, the electrode still can be reused again for a third and fourth time but with reduced level of detection, which is (0.01 µg L^{-1}) and above.

For the overall discussion of MEA in buffer, the target when using MEA is the possibility of enhancing the sensitivity of the assay higher than SPGE. For AFB_1 detection, the working range of standard curve can detect a level more than 0.001 µg L^{-1} which means it is more sensitive than ELISA and SPGE. In terms of the signal/background ratio, good value is present in MEA detection which is 3 times better than SPGE. That means a low background reading was achieved during the detection. The potential to increase the sensitivity and achieve a low detection level of antigen is still under investigation, and will involve further optimisation of conditions and concentration reagents and analytes. Reusable MEA for fumonisins detection was not shown in this chapter. It is expected that the performance of used electrode would be similar in performance to that in AFB_1 detection.

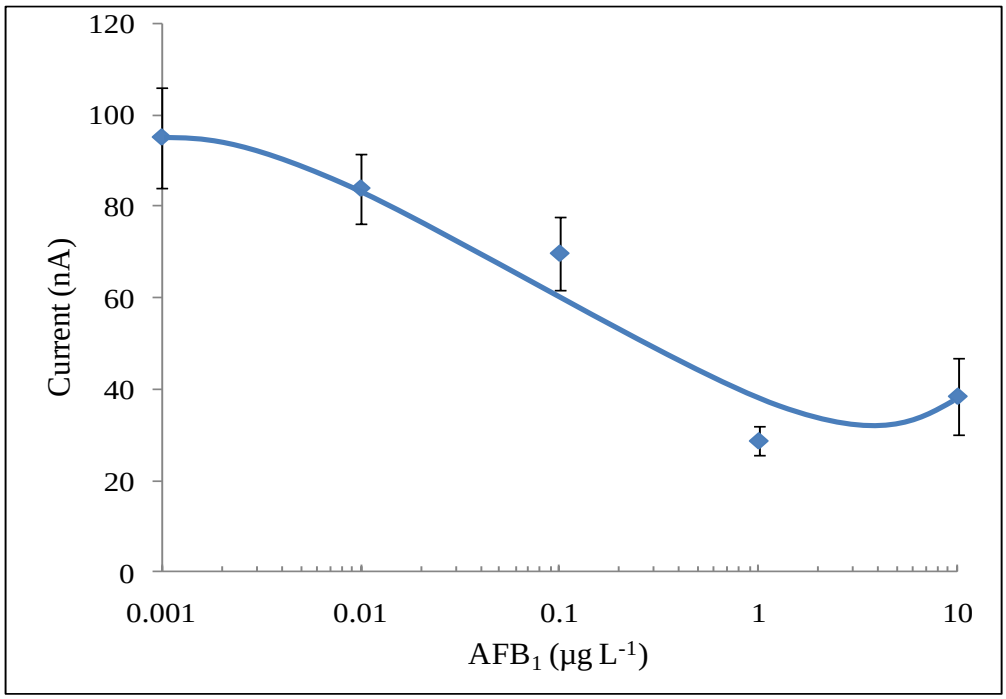
5.3.7 Sample matrix analysis

The microelectrode showed very good performance for AFB_1 and fumonisins detection in pure buffer solution. Further investigation was carried out to assess the performance in a sample matrix. The detection of AFB_1 and fumonisins in a real sample analysis was constructed by first developing a calibration curve in a sample solution.

5.3.7.1 Aflatoxin B_1 analysis in peanut matrix

The purpose of this study was to determine the performance of the MEA sensor to detect AFB_1 in a peanut extract compared to buffer. The measurement of the AFB_1 in the peanut matrix was also based on the calibration curve developed. Calibration curves for peanut were produced by spiking the blank peanut extract with an AFB_1 standard solution. The extraction was carried out as reported in the sample extraction procedure section (5.2.8.1).

In Figure 5.15 the standard curve of AFB_1 prepared in blank peanut extract also showed a decrease with the increasing of the AFB_1 concentration in the peanut



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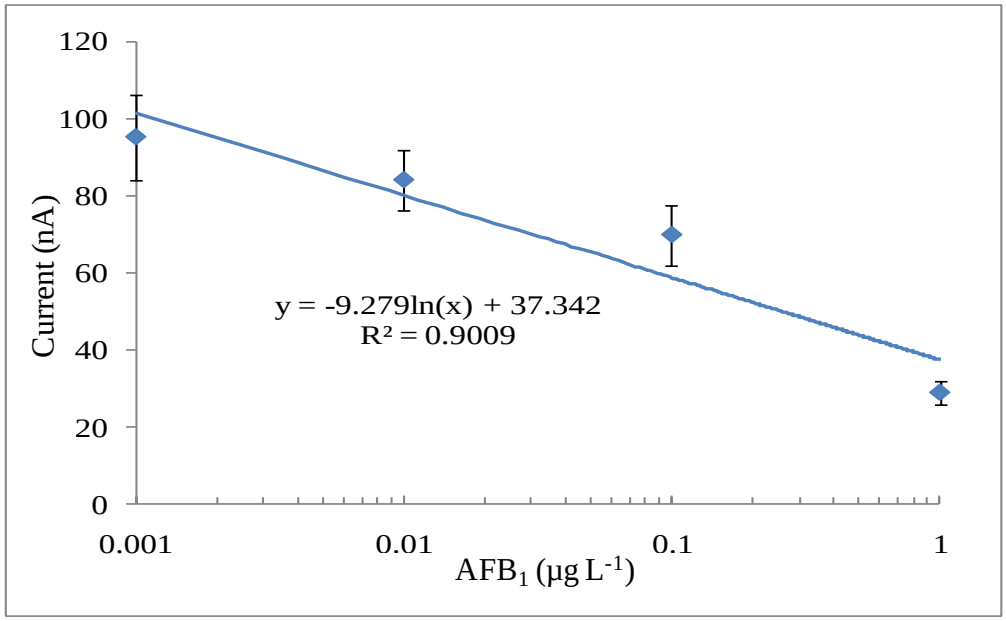

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Figure 5.15: Standard curve for AFB_1 detection using modified MEA in peanut extract. Indirect competitive AFB_1 immunosensor response on a modified MEA by chronoamperometry at potential +150 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Modified MEAs were coated with AFB_1-BSA conjugate (1 µg mL^{-1}), blocked with 1% PVA followed by anti-AFB_1 antibody ($MabAFB_1$) (10 µg mL^{-1}) and free AFB_1 (0 to 10 µg L^{-1}) before continue with anti-IgG-HRP (1 µg mL^{-1}). Error Bar=SD, n=3.

extract. The phenomenon is similar to the previous test of the competitive immunoassay format. The performance of the AFB_1 detection in a real matrix was evaluated by the non-linear and linear regression of the equation which is found LOD to be 0.001 µg L^{-1}, and the correlation coefficient was R^2=0.90 with the %CV ~10%. The results were compared to the buffer performance and this showed that the matrix effect resulted in a lower R square value and a high value of %CV. However, the detection limit was still similar to the analysis in buffer.

The different response is due to the fact that peanut extracts can interfere, depending on factors such as organic matter content and the nature of the peanut sample. Causes considered for the signal reduction (from ~140nA to ~90nA, maximum signal) included an electrode problem (different batches of sensors used) (Parker *et al.,* 2009), and inhibition of the enzymatic label by compounds present in the extract (Kroger *et al*, 1998). While such matrix effects are undesirable, they are common to most environmental biosensor systems (Kroger *et al*, 1998).

Finally, the study of the extraction efficiency of AFB_1 was carried out by unspiked (0 µg kg^{-1}) and spiked peanut samples with a known amount (4, 40, 100 and 250 µg kg^{-1}) of the toxin. Then the samples were extracted, diluted to 1:1 with PBS and assayed on the modified MEA. The performances of the MEA sensor for the detection of AFB_1 in the peanut samples were evaluated and compared to the ELISA kit (Ridascreen^{TM}), and the values are reported in Table 5.1. The AFB_1 concentration was calculated from the current response using a calibration curve established in the peanut extracts (Figure 5.15) included in the test kit (Ridascreen^{TM}).

The recovery of the fortified peanut sample ranged from 79.2 to 91.3% for the MEA sensor, while the ELISA kit detection obtained from 72.8 to 90.3%. The average of %CV for the microsensor and the ELISA kit detection to the spiked sample was 8.3 and 6.9%. From the average value result of recovery, this indicates that the MEA immunosensor worked satisfactorily with the ELISA kit. The satisfactory agreement was confirmed with the application of both methods to the detection of AFB_1 in the peanut samples.

Table 5.1: Application of microsensor for determination of AFB_1 in spiked peanuts and unspiked peanuts compared to ELISA kit method.

Sample	AFB_1 added (µg kg^{-1})	Measure value average (µg kg^{-1})					
		ELISA kit (Ridascreen kit)			Microelectrode arrays		
		Found	%CV	%Recovery	Found	%CV	%Recovery
	0	< 1			< 0.001		-
	4	2.91 ± 0.24	8.2	72.8	3.2 ± 0.4	12.8	79.2
	40	32.36 ± 1.73	5.3	80.9	33.7 ± 2.5	7.5	84.2
	100	90.3 ± 4.52	5.0	90.3	88.0 ± 7.5	8.6	88.0
	250	214.5 ± 20.15	9.4	85.8	228.3 ±10.4	4.6	91.3
Average			6.9	82.5		8.3	85.7
Unknown Sample							
PSM		7.1 ± 0.9	12.6		8.12 ± 0.18	2.3	
POMM		223.2 ± 19.7	8.8		245 ±17	7.2	
PTUK		< 1			0.07± 0.01	10.2	
POMUK		< 1			0.23 ± 0.04	10.5	

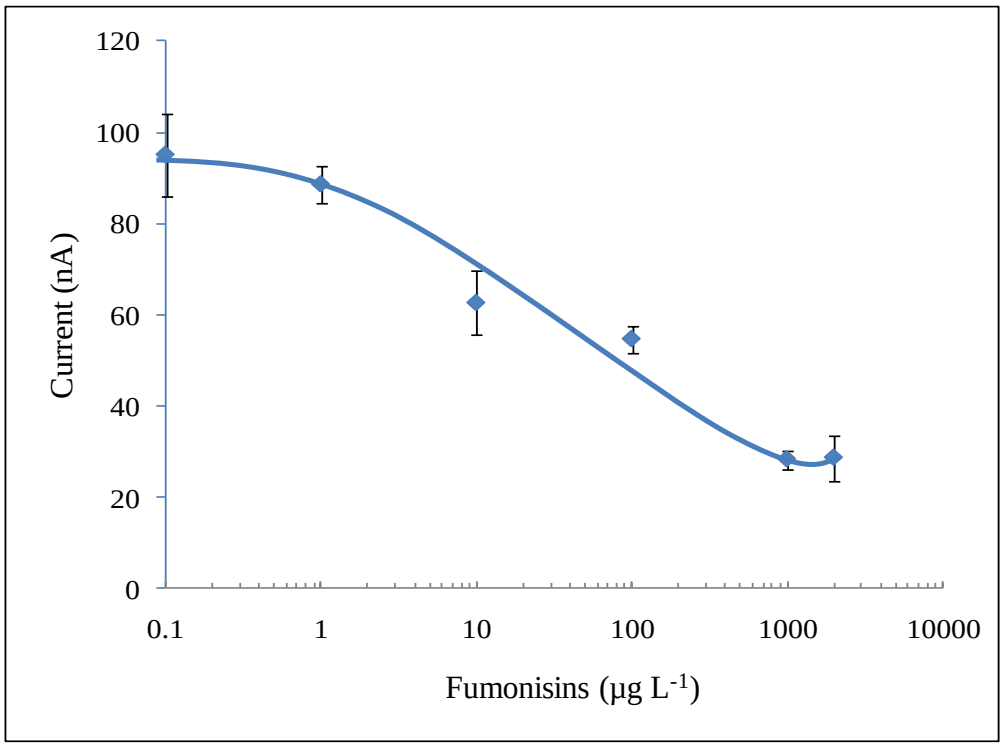
PSSM = Peanut Supermarket Malaysia; POSM = Peanut Open Market Malaysia; PTUK = Peanut Tesco UK; POMUK = Peanut Open Market UK. *n*=3.

The device's response to unspiked peanuts (peanut collected from the UK and Malaysia) was also present and calculated in Table 5.1. The results show the amount of AFB_1 detected in the peanut sample showed the accurate values of SD and %CV. These data show that there is no significant difference between the results given by two different methods of samples PSM and POMM. The accuracy of the value may suggest the applicability of the microsensor device for use in field-based work.

5.3.7.2 Fumonisins in corn matrix

In the case of fumonisin detection in corn samples, the calibration curve of Fms in a corn extract was developed first. To obtain the calibration curve, a series of different concentrations of Fms (0 to 2000 µg L^{-1}) was prepared by spiking into the blank corn extract. This proposed method was applied to test the performance of the microsensor for the detection of Fms in the corn sample matrix. The current response for the Fms spiked into the corn extract (Figure 5.16) was generally similar to that obtained in buffer (Figure 5.12). The working range of detection was 1 to 1000 µg L^{-1} with LOD was 0.5 µg L^{-1} the linear regression was found to be $R^2 = 0.96$ with the %CV = 5.2% also slightly similar to that in buffer.

By using a standard curve on the corn extract, the corn samples spiked at four levels of Fms from 50 µg kg^{-1} to 2500 µg kg^{-1} gave recoveries ranging from 76.1% to 101.3% (average 87.3±11.4%) with the average of coefficients of variation (CV) < 6% (Table 5.2). In the case of the ELISA kit detection, the average recovery of 500 µg kg^{-1} and 2500 µg kg^{-1} was 80.9% with %CV of = 7% . Also in the case of the ELISA kit detection, an amount of less than 400 µg kg^{-1} could not be measured because the range developed from the standard curve was 400 to 6000 µg L^{-1}. The higher percentage of recovery found was shown with the higher level performance of the extraction method for the microsensor and the ELISA detection in the corn sample matrix, showing that the performance of the microsensor detection is much better as it can detect Fms in corn less than 400 µg kg^{-1}.



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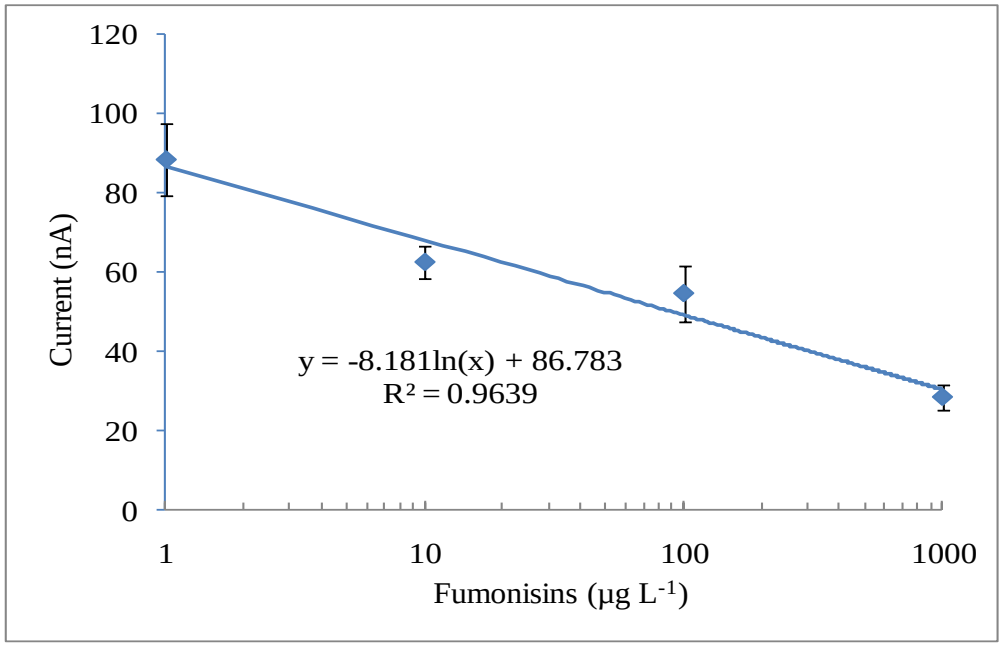

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Figure 5.16: Standard curve for fumonisins detection using modified MEA in corn extract. Direct competitive Fms immunosensor response on a modified MEA by chronoamperometry at potential +150 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as substrate. Modified MEA were coated first with anti IgG antibody (10 µg mL^{-1}), blocked with 1% PVA before adding MabFms (1:50), followed by free Fms (0 to 2000 µg L^{-1}) before continuing with Fms-HRP (1:5). Error bar=SD, n=3.

Table 5.2: Application of microsensor for determination of fumonisins in spiked corns and unspiked corns compared to ELISA kit method.

Sample	Fms added (µg kg^{-1})	Measure value average (µg kg^{-1})					
		ELISA kit (Neogen kit)			Microelectrode arrays		
		Found	%CV	%Recovery	Found	%CV	%Recovery
	0	< 400			< 1	-	-
	50	< 400			50.7 ± 3.1	6.03	101.3
	250	< 400			190.3 ±8.5	4.5	76.1
	500	412.6 ± 45.1	10.9	82.5	460 ± 35.6	7.73	92
	2500	1984.8 ± 62.5	3.1	79.3	2016 ± 59.8	2.97	80.6
Average			7	80.9		5.29	87.3
Unknown Sample							
CTUK		< 400			3.7 ± 0.18	15.0	
COMM		1102.3 ± 157	14.2		1219.7 ±88.4	7.2	
FmB_2 2500		2068.1 ± 133	6.4	82.7	2142 ± 239.4	11.18	85.67

CTUK = Corn Tesco UK; COMM = Corn Open Market Malaysia; FmB_2 = Fumonisin B_2 *n*=3.

This suggests the electrochemical detection using an MEA sensor is more sensitive than the spectrophotometric ELISA kit.

In the case of the unknown amount of Fms in corn samples (Table 5.2) which were tested, the study was found to have a different value of Fms in different corn samples collected (see Table 5.2). When the FmB_2 (2500 µg kg^{-1}) were spiked in blank corn in this system (modified MEA), the FmB_2 was detected and the recovery test obtained about 85.7 %. This indicates that the FmB_2 was also specifically binding to the antibody used in this assay, which is the same as we know in the previous chapter (Chapter 4) (SPGE sensor).

5.4 Conclusions

The gold MEAs, fabricated using the photolithographic technique, were applied for the development of an immunosensor for AFB_1 and Fms detection in peanut and corn, respectively. The electrode supplied was first treated using O_2 downstream oxygen plasma before characterisation using cyclic voltammetry. The sigmoidal peak of CVs after plasma showed a good performance of new and used MEA and the possibility of providing sensitive detection in sensor application.

Characterisation of the surface modification on an electrode using CV makes it possible to distinguish the different layers of surface attachment chemistry. The reducing of the sigmoidal peak indicates that the surface was covered with an amino silane anchor and PDITC cross linker. The surface modification successfully improved the specific binding of the capture antibody through covalent immobilisation.

The competitive immunoassay of the AFB_1 and Fms solution on the modified MEA was then performed by transferring the chemistry successfully from the SPGE. The sensor devices were reproducible and very sensitive as they could detect more than 0.001 µg L^{-1} and more of AFB_1 in buffer and peanut matrix. In the case of Fms detection, the lowest level that could be detected was 1 µg L^{-1} in buffer and corn

extract. The proposed gold immunosensor of the MEA system shows it is a high performance device for detecting AFB_1 and Fms in peanut and corn matrix based on a high percent of recovery achievement.

In terms of producing a signal background ratio, good value is present in the MEA detection which is 3 times over different compared to SPGE. That means a low background reading was achieved in detection which also influenced the sensitivity of the MEA assay. The sensitivity of the MEA for the detection of aflatoxin M_1 in milk was better than using a screen-printed electrode (Parker *et al*., 2009). This shows that a microelectrode could be used as a replacement for SPE technology.

CHAPTER 6

OVERALL DISCUSSION, CONCLUSIONS AND FUTURE WORKS

6. Overall Discussion, Conclusions and Future Works

6.1 Introduction

In this thesis, the most important point is the applicability of using screen-printed gold electrodes and microelectrode array performance based on an electrochemical affinity principle for AFB_1 and Fumonisins analysis. The development of an electrochemical immunosensor system in the present study shows that it can be applied for the detection of both mycotoxins in buffer and also in samples (peanut and corn). The first stage of the biosensor development was discussed in Chapter 1, where the potential of the use of the electrochemical immunosensor technique was demonstrated for both AFB_1 and Fumonisins detection. From the literature obtained, immunoassays in sensor application methods potentially have advantages over the other procedures because of their specificity, sensitivity and applicability of the immunosensor to the buffer and also real samples. With the advances in microsensor technology and the functionalization of nano-particles and utilising current instrumentation with new modelling technology, high ability and more stable receptors and the miniaturization of microelectrodes, the focus will be on developing sensitive, simple, rapid, environmentally-safe and inexpensive methods.

6.2 Optimisation and immunoassay development

The ELISA method, using spectrophotometric output, has been developed and optimised before subsequently being transferred to the working electrode for the immunosensor development. It is critical for the successful development of any sensor device to ensure the binding efficiency of biomolecular recognition and substrate reaction on the surface for the detection system. For AFB_1, two different immunoassay methods (indirect and direct format) were used in this study based on the application of the monoclonal anti-aflatoxin B_1 antibody ($MAbAFB_1$) from Abcam UK. The monoclonal antibody was affinity purified specifically for AFB_1 detection and was provided with the appropriate antigen conjugates designed in conjunction with the solid surface based assay system. The monoclonal antibody was chosen because it is highly specific to the AFB_1 and also it showed an increase in the

specificity and sensitivity of AFB_1 detection assay (Wang *et al*., 2001; Ammida *et al*., 2004; Anna *et al*., 2006).

For the enzyme/substrate system, HRP with TMB substrate solution was chosen. The reaction was sensitive and rapid when a low HRP concentration was used in a competitive assay. As compared to other labelled enzymes such as alkaline phosphatase it is smaller in molecular weight (~44000 Daltons), more stable and less expensive. Hence, the antibody provides the specificity to locate the protein of interest, and the HRP enzyme, in the presence of a substrate, produces a detectable signal. Horseradish peroxidase is commonly used in techniques such as ELISA.

Immobilisation of $MAbAFB_1$ and other reagents supplied (indirect: anti-antibody labelled with HRP and Aflatoxin B_1 conjugated with BSA; direct: anti-antibody IgG and AFB_1labelled with HRP) on the 96 Nunc plates were the first and most important step in the ELISA method. The most effective microtitre plate used in this study was polystyrene Nunc microtiter plate because these produce a higher binding of biomolecules on their surface (Anna *et al*., 2006). The immunosorbent coating on the Nunc microtitre plate of the assay was particularly important to ensure the surface binding capacity was saturated with the strongly specific binding solid phase.

Any uncoated molecules on solid phase that were poorly bound were removed with washing procedures. Generally, a lack of proper washing would result in decreased specific antibody interaction and increased non specific antibody binding. With a stable immunosorbent, three washes (twice with PBS-0.05%Tween 20 (non ionic detergent) and once with PBS) after each immobilisation and reaction was sufficient to remove loose and unbound antibody reagents. This was to ensure that only interactions of specific affinity occurred. To prevent non specific binding or interaction on the well, the buffer of HRP conjugate was included with 0.1% blocking agent (PVA). When the Tween 20 and blocking reagent were used in the washing and conjugate buffer, respectively, a ~ 90 % of problematic non specific binding reaction in the assay was solved (Qu *et al*., 1998).

6.2.1 Optimisation for indirect competitive immunoassay

Initially the work for titration of the indirect immunoreagent was conducted following first the work described by Ammida *et al*. (2004) and the recommendations given by Crowther (2001) with modification as a foundation for the development of the ELISA method. The optimum concentrations of immunoreagent were established by titration using a checkerboard assay. This required the optimisation of reagent concentration and each assay parameter to maximise response and sensitivity and to minimise non-specific binding. The method has been developed and optimised by coating with AFB_1-BSA then $MAbAFB_1$ binding before labelling with horseradish peroxidase (HRP) labelled antibody (anti-IgG-HRP). From the titration, the enzyme reaction measured was proportional to the amount of specific binding of anti-IgG-HRP to the amount of $MAbAFB_1$, even though the AFB_1-BSA coating conjugates revealed good $MAbAFB_1$ recognition and binding. The results show that the procedure was suitable for producing higher binding, specific for detecting AFB_1.

The influence of several parameters such as coating buffer, blocking agent and time incubation were optimised to improve the performance of the immunoassay. The coating buffer was also equally important: the most suitable for hydrophobic binding was determined to be 0.1 M sodium carbonate buffer at pH 9.6. The use of high pH with the carbonate buffer for protein conjugate or antibody adsorption on the microwell plate increased the binding response and gave results better than pH 7.4 (PBS). A high pH value can have an influence on the steric structure of protein, thus having an effect on immobilisation, and the AFB_1-BSA can maximally adsorb on to the wells using this pH (Chen *et al*., 2008). The carbonate buffer (pH 9.6**)** has most commonly been used in immunoassays such as ELISA for the detection of mycotoxins (Ammida *et al.*, 2004; Alarcon *et al*., 2004; Micheli *et al*., 2005; Ammida *et al.*, 2006; Piermarini *et al*., 2007).

The effect of blocking agents was also tested using a typical protein polymer (milk diluents and gelatine) and non protein polymer (PVA and PVP) to bind to any vacant solid phase binding sites. This was determined by the abilities of the polymers to prevent non specific binding of an anti-IgG-HRP conjugate to the surface of the microtitre wells. The PVA at 1% was significantly better – at least two-fold better –

for reducing non-specific binding and more effective than other blocking reagents. In the experiment, 1 hour at 37 ºC of incubation were better conditions estimated to coat the microplate well with PVA. That considerably lowered the background reading and was, therefore, used in all further experiments. The result also showed no interference with the specific binding of the conjugate to antigen-coated microtitre wells. This is strongly evidenced when Micheli *et al*. (2005), Ammida *et al.* (2006), Parker, (2008), Parker *et al*., (2009) and Parker & Tothill, (2009) used the polyvinyl alcohol as a blocking agent in their immunoassay test for low non specific binding and increased the sensitivity immunoassay developed for mycotoxins detection.

Incubation time can also influence the increasing of specific and non specific binding in immunoassay tests. In this study, the optimisation of incubation time is based on the competition step of $MAbAFB_1$ with free AFB_1 and AFB_1-BSA coated on wells. The study found that the sensitivity of the immunoassay could be increased by pre-incubating the AFB_1 and $MAbAFB_1$ for 30 min at room temperature (25 ºC) before the addition of the AFB_1-BSA coated on wells to allow competition reaction. After 60 min incubation at 37 ºC, the immunoassay achieved the optimal competition performance. Using incubation of more than 60 min increased the binding response and also increased the non specific binding which increased the background. Therefore, a time of 60 min was chosen as a compromise for a sensitive immunoassay with an acceptable signal.

By the use of the optimal parameters of concentrations and conditions, the indirect competitive immunoassays for the development of AFB_1 detection were 1 µg mL^{-1} of AFB_1-BSA, 10 µg mL^{-1} of monoclonal antibody and 1 µg mL^{-1} of anti-antibody labelled HRP.

6.2.2 Optimisation for direct competitive immunoassay

The direct competitive method for AFB_1 detection was also employed in this study. First, the wells were precoated with anti-antibody IgG (anti-IgG) before being immobilised with $MabAFB_1$ and the competition between free AFB_1 and AFB_1-HRP. In this case the AFB_1 conjugated HRP was selected from the commercial ELISA kit

(AFB_1 Ridascreen kit), not synthesised in the lab, as there is no commercially available product. The combination of coating AFB_1-HRP with antibodies concentration, which resulted in an absorbance in the range 0.05 to 0.3 OD, were considered a low absorbance reading. Micheli *et al.* (2005) also used toxin conjugated HRP from the Ridascreen kit and the result was found in the range 0.06 to 0.25 OD. According to Kolosova *et al*., chemicals contained in an AFB_1-HRP solution, such as additives or blocking agents, may interfere with the binding which then produces low readings (Kolosova *et al.,* 2006). However, with the use of this enzyme conjugate it was found that the signal did increase with the increase of $MAbAFB_1$ in the non competitive assay test.

Due to the limited supplies of AFB_1-HRP and the expensive cost of the Ridascreen kit, the optimisation binding of antibodies and antigen was employed using a different concentration of reagents by single titration on the microplate. This procedure was initially conducted following the method used by Micheli and co-workers (Micheli *et al.,* 2005) with small modifications. The coating buffer and blocking agent for the optimisation assay were similarly used as in the indirect immunoassay method. A captured $MAbAFB_1$ and AFB_1-HRP labelled enzyme was initially optimised by the optimal concentration of 20 µg mL^{-1} and 1:10 dilution, respectively, without preactivation support of the immunoassay on the wells surface. When the precoating consisted of an immobilisation using anti-antibody IgG on the surface, the amount and orientation of the antibody specific for the analytes was increased by about 10 to 20% binding. The optimal concentration of anti-IgG used in this system is 10 µg mL^{-1}. Most of the direct competitive immunoassay has been developed based on the presence of precoating immobilisation on the microplate wells because a better random orientation of specific binding was found (Alarcon *et al*., 2006; Neagu *et al*., 2006).

The competitive assay has been developed based on the optimal concentration of reagents and conditions (selected time incubations). Pre-incubation of free AFB_1 for 10 min in the competition step before the addition of AFB_1-HRP was the best method to achieve better sensitivity of the assay. The free AFB_1 were first bound to the $MAbAFB_1$ site in order to achieve maximum binding before competing to the AFB_1-

HRP for 50 min. The orientation of specific reaction gave results which were better than the direct immobilisation of the mixture of free and labelled $AFB_{1.}$

6.2.3 Competitive assay for indirect and direct immunoassay for AFB_1

In order to have the maximum response as a result of enhancing the sensitivity of the competitive assay, the optimum concentration and conditions were applied. Pre-incubation of the competition step (indirect: 30 min of mixture AFB_1 and $MAbAFB_{1;}$ and direct: 10 min of AFB_1 on the coated wells) has improved the sensitivity of the immunoassay developed. Although there are many variants of ELISA, they all depend on the same basic elements: coating and capture, plate blocking, detection, and signal measurement. Both of the ELISA systems are capable of detecting more than 0.1 µg L^{-1} of AFB_1. The sensitivity of the assay is still not significantly different in either of the systems. The investigation and characterisation for both immunoassays have continued and been transferred to the screen-printed gold electrode (SPGE) for immunosensor development. Critically, the development of the ELISA demonstrated that the commercially sourced antibody was suitable for use as a sensing material for the electrochemical immunosensor.

6.3 Electrochemical Immunosensor for Aflatoxin B_1 detection

6.3.1 Electrochemical Characterisation

The initial characterisation of the ELISA immunoreagent was conducted in order to identify the suitability of the immunoreagents for use in the immunosensors. Then the optimum concentration and conditions of the developed ELISA were subsequently transferred to the gold screen-printed electrode for the construction of the electrochemical immunosensor device. Portable electrochemical detectors were used because they are inherently sensitive and selective for electroactive species (Ramirez *et al*., 2009). They are also fast, precise and low-cost (Mehrvar & Ardi, 2004; Chen *et al.,* 2006)). An electrochemical immunosensor based on three electrodes (working, reference and counter) has been developed by screen-printing technology and used for

Aflatoxin B_1 (AFB_1) detection. The screen-printed gold electrode (SPGE) was fabricated in house using Ercon ink. A second electrode which was used in the bulk of this work and another one were designed and fabricated in a collaborative work with DuPont (UK) (Richard, 2009). In this study, the gold working electrode was chosen because of its good conductive material (Ramirez *et al*., 2009), protein is easily adsorbed through physical adsorption and there are strong protein bonds through covalent bonds (Abad *et al*., 2002). Both of the gold working electrodes have been characterised with cyclic voltammetry (CV) by scanning $K_4Fe(CN)_6$ at different scan rates. This CV application is for qualitative diagnosis of chemical reactions that precede or succeed the oxidation and reduction process (Wang, 2006). SEM was also used to visualise the different features of the gold sensor surface appearance. From these experiments a great difference was found in the particle structure and morphology of the gold particles using the Ercon and the DuPont gold ink. This could have an effect on the immobilisation efficiency on the electrode surface and the variations that can result from this study. The different structure and composition of Au for both electrodes produced different peak to peak separations, and both obtained more than 59 mV values, which means the redox reaction is quasi-reversible. Thus, the peak separation can be used to determine the number of electrons transferred which are controlled by charge transfer and mass transport (Wang, 2006).

The detection method for the electrochemical immunosensor was conducted based on the changes of reduction current from the redox of HRP label antigen or antibody. Cyclic voltammetric and chronoamperometry was applied for monitoring the final step of HRP catalysed by the use of 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB)/hydrogen peroxide (H_2O_2) mediator/substrate system. TMB has been reported to be a good mediator for the electrochemical detection of low levels of HRP when TMB/H_2O_2 is used as the substrate system (Salam & Tothill, 2009, Parker *et al*, 2009) compared to hydroquinone (Volpe *et al*., 1998). In this study, the optimal concentration of 5 mM TMB and 0.075% H_2O_2 was the effective concentration for the HRP substrate. The HRP activity can be measured by determining the reduction current generated by TMB. By chronoamperometry detection, the high current response was achieved when the electroactivity reaction was involved in the presence of HRP (Fu, 2008). The generated current is proportional to the amount of HRP conjugate bound to the electrode surface through the AFB_1 antigen and indirectly the

magnitude of the current is also directly inversely proportional to the number of AFB_1 being tested.

When amperometry was used for the detection of reduction current in this study, the measuring potential using step chronoamperometry for screen-printed electrodes was conducted. With the different material of electrodes, Butler *et al.* (2006), Ammida *et al*. (2006), Fanjul- Bolado *et al.* (2005) and Volpe *et al*, (1998) used different constant potentials for measuring the current. For the gold electrode sensor (SPGE), the applied constant potential was selected through the study of the electrochemical behaviour of the bare gold electrode with a complete reaction of the non competitive immunoassay at different potentials. The high value of the difference between signals over background from selected potentials was chosen as an optimal potential. For the gold screen-printing the −100 mV vs. on board screen-printed Ag–AgCl pseudo-reference electrode was discovered to give the greatest signal.

6.3.2 Immunosensor development using competitive immunoassay

The developments of the competitive immunoassay on the gold working electrodes were based on the transfer of the optimal indirect and direct immunoreagents onto the surface of the electrode. The optimisation of the immunoreagent using electrochemical SPGE has been developed and found to be similar to the ELISA. Then, the first immunosensor design with indirect and direct ELISA format was employed using physical adsorption on the SPGE Ercon. The sensitivity of both the assays were increased three times compared to the developed ELISA. However, the indirect method was chosen for further competitive reaction investigation because a better current response (higher signal) with high dynamic range was achieved. Stable, simple and rapid immobilisation on the surface through physical adsorption is the best choice for an immunosensor system. The mechanism of the passive adsorption interaction between an antibody and an antigen molecule on the transducer surface usually refers to hydrophobic interaction, electrostatic interaction, van der Waals force, and hydrogen bonding. The hydrophobic or hydrophilic interaction was also possible, it is based on the mixture of gold with the organic polymer (hydrophobic surface) of the electrode. However, the competitive assay with passive adsorption

immobilisation was still lacking orientation antibody/antigen binding on SPGE Ercon. Many literature reviews have reported that the random orientation of specific binding can influence the sensitivity of the assay, and covalent immobilisation was proposed for enhancing the poor surface coverage (increase the orientation binding) of the sensor (Lee *et al*., 2005; Bilitewski, 2006; Silva *et al*., 2008). Due to the limited supplied of the SPGE Ercon sensor, it was difficult to continue with the study with covalent immobilisation, and therefore covalent binding was conducted using the DuPont sensors.

Many reported on the exploiting of the self assembled monolayer (SAM) thiol modified on a solid surface for covalent immobilisation in the immunosensor system (Park *et al*., 2004; Subramaniam *et al*., 2006; Escamilia-Gomez *et al*., 2007). This because the SAM thiol performance had the potential to increase the performance of biomolecular interaction on the surface. Normally the cyclic voltammetry based on the redox behaviour of a reversible couple was used for looking into the density and sensitivity of the self assembled monolayer on the electrode surface. According to Escamilla-Gomez *et al,* the thiol group was chemically adsorbed onto the gold surface by the formation of a thiolate bond (Escamilla-Gomez *et al*., 2008). The self assembled monolayer (SAM) coating on the gold electrode surface is normally used to provide an effective coupling with the bio-sensing component on the transducer surface and also to protect the surface for non specific binding. It is therefore important to test the integrity and properties of such a layered structure on the gold surface. The main strategy is try to enhance the immobilised antibody as much as possible onto the sensor surface to increase the antigen binding capacity which results in increasing sensor sensitivity. After selection from three different thiol group (long chain, short chain and short chain-double), the short chain-double of 3,3-dithiodipropionic acid (DTDPA) was found to be the most suitable thiol group on the SPGE DuPont surface.

The SAM's thiol modified surface for covalent antibody immobilisation and compared to passive adsorption was applied using the DuPont gold working electrode. The sensitivity of the assay for both immobilisations was not significantly different. The calculated data can be seen in Table 3.2 of Chapter 3. According to Seurynck-Servoss *et al,* different types of immobilisation, such as covalent or physical

adsorption, have presented similar binding depending on the types of surfaces (Seurynck-Servoss *et al*., 2007). However, a higher current response with a low background reading was presented from the immunoassay on thiol modified surface. This evidence showed the better random orientation specific binding of the indirect competitive assay on the thiol modified surface compared to the passive adsorption. Usually the orientation binding on the modified sensor surface is measured by the activity of the antigen binding to the immobilised antibody. Then, the higher antigen binding site in the Fab region might be from the correct antibody orientation on the modified surface.

In this study, the development of the AFB_1 immunosensor using a DTDPA SAM thiol surface was a new immunosensor method finding.

6.3.3 Immuno gold nano-particle sensor for competitive immunoassay

Gold nano-particles have been applied in many applications including electrochemical immunosensor systems especially for sensitivity improvement (Guo and Wang, 2007; Zhang *et al*., 2009). The advantages of using gold nano-particles have been described in Chapter 1 (Introduction). These gold nano-particles have also been used in electrochemical immunosensors for AFB_1 detection (Owino *et al*., 2008; Sun *et al*., 2008). However, a new approach towards the function of gold nano-particles was performed in this study and will be compared with those methods. A new signal amplification strategy based on gold nano- particle labels anti-AFB_1 antibody ($MAbAFB_1$) and horseradish peroxidase (HRP) as signal amplifiers in electrochemical immunosensors for AFB_1 was developed in order to increase the sensitivity of the assay. The combination of reaction between gold nano-particles, $MAbAFB_1$ and HRP was described as immuno gold nano-particles labelled with HRP. According to Luo *et al*., (2006), due to their large specific surface area and high surface free energy, nano-particles can adsorb biomolecules strongly and play an important role in the immobilization of biomolecules in biosensor construction.

The new immunoassay formatting for the application of immuno gold nano-particles in this study was described in Chapter 3. After optimisation of immuno gold nano-

particles labelled HRP, then the indirect competitive sensor was constructed by immobilising the AFB_1 conjugate and immuno gold nano-particles labelled HRP on the gold electrode surface. With the same optimal concentration of 1 µg mL^{-1} AFB_1-BSA coated and 10 µg mL^{-1} of $MAbAFB_1$ captured antibody (coated on nano-particle) as in ELISA and SPGE, the 100 µg mL^{-1} of HRP immobilised on gold nano-particles for enzyme labelled have increased the current response. The current response was increased two-fold, compared to conventional immunosensors. The target for amplifying the signal was achieved in this system. The high amount of HRP adsorbed on the immuno gold particle might increase the enzyme reaction and bring about a greater reduction of current toward TMB/H_2O_2 on the electrode surface. This is because, the increase of current response is proportional to the amount of HRP conjugate on the surface. Then, the application of gold nano-particles on the surface might enhance the conductive surface area for electron transfers especially between the nano-particle surface and the electrode surface (Luo *et al*., 2006).

The development of the calibration curve through gold nano-particle immunosensors for AFB_1 in buffer was performed also by covalent adsorption and passive adsorption. Both systems have a potential to increase the sensitivity which produced low detection limits to 2 and 1 ng L^{-1} for passive and covalent, respectively. This limit of detection is lower than for conventional immunosensors and ELISA developed. In this case, the lower current response at covalent immobilisation treated might be due to the high capacity of the surface with thiol and protein immobilised causing electrical blocking (Ivanova *et al*., 2006). However, high antigen binding to correct antibody orientation was presented which produced a higher R^2 value and lower %CV compared to passive. Good reproducibility of the gold nano-particle immunosensor device was referred from the low value of %CV average and SD for both systems, which is less than 10%. This is a new assay format which uses a nano gold electrochemical immunosensor based on the screen-printed gold electrode for AFB_1 which has not been reported in the literature. This is also an easy method of preparing an enzyme conjugate and cheaper than purchasing a ready conjugated enzyme label. This amplified electrochemical immunosensor was then compared to other immunosensors for AFB_1 detection in the current situation (Table 6.1).

Table 6.1: Comparison of the proposed electrochemical immunosensor with other immunoassay for AFB_1 detection from 2006 to 2010.

Immobilisation /fabrication	**Working range (µg L^{-1})**	**LOD (ng L^{-1})**	**References**
Indirect/SPCE (PA)	0.03 & 0.09	10	Ammida *et al*, 2004 and 2006
Indirect/SPCE (PA)	0.15-2.5	150	Pemberton *et al*, 2006
HRP/anti-AFB_1/NG/AET/ME	0.5-10	100	Liu *et al*, 2006
Indirect/Screen Printed-microplate (PA)	0.05-2	30	Piermarini *et al*, 2007
HRP/anti-AFB/NG/TiO_2/RTIL/Nafion /GCE	0.1-12	50	Sun *et al*., 2008
AFB_1-BSA-HRP/PTH/NG/GCE	0.6-2.4	70	Owino *et al*., 2008
Indirect/Cys/SPGE (Covalent)	0.1-10	60	Chu *et al*., 2009
Indirect/SPGE (PA)	0.01-10	6	This work
Indirect/DTDPA/SPGE (Covalent)	0.01-10	5	This work
NG-anti-AFB_1-HRP/AFB_1-BSA/SPGE (PA)	0.005-1	2	This work
NG-anti-AFB_1-HRP/AFB_1-BSA/DTDPA /SPGE (Covalent)	0.005-1	1	This work

SPCE: Screen-printed carbon electrode, PA: Passive adsorption, NG: Nano gold, AET: aminoethanethiol, ME: Microelectrode, TiO2: Titania nano-particle, RTIL:1-ethyl-3methyl imidazolium tetrafluoroborate, GCE: Glassy carbon electrode, PTH: polythionine, DTDPA: 3,3dithiodipropionic acid, Cys: cysteamine, SPGE: Screen-printed gold electrode, LOD: Limit of detection

The Table shows the different sensitivity of the developed assays by different approaches of the electrochemical immunosensor for AFB_1 detection in buffer. As seen from the Table, the high sensitivity assay was developed from our work. Using a gold electrode surface and the application of gold nano-particles the sensitivity of AFB_1 detection has been improved. With this method also the immobilisation and incubation step was reduced by one step compared to the conventional method.

6.4 Electrochemical immunosensor for fumonisins detection

6.4.1 Optimisation using ELISA method

The development of an immunosensor for Fumonisin detection was performed based on the direct immunoassay on the gold working electrode. Optimisation of the direct immunoreagent using the ELISA system with spectrophotometric detection was first conducted before the subsequently transfer to the gold electrode. All the reagents used in this experiment were from a commercial supplier and some from the Veratox ELISA kit. A monoclonal mouse anti-fumonisins (MabFms) was chosen for the detection of total fumonisins in the sample. While Fumonisins labelled HRP (Fms-HRP) for enzyme reaction was selected from the Veratox kit (kit for total fumonisins) it is suitable for measuring total fumonisins. Our study here was focused on the detection of total fumonisins either in buffer or real corn samples. The optimisation test was carried out by the checkerboard technique in a Nunc 96-well microplate based on the method described by Anna *et al*. (2005). The coating buffer (carbonate buffer pH 9.6), blocking agent (1% PVA), incubation time (2 hours maximum) still referred to the method shown in the immunoassay of AFB_1 developed. All those parameters were basically used for developing ELISA or the immunosensor system in producing an optimal response. The purpose and function of the chosen parameters were already explained in section 6.2.1.

The maximum reading of 1.8 OD showed high affinity binding of Fms-HRP to MAbFms site on the well. However, the optimal specific binding by 1.02 OD with affinity concentration 1:50 and 1:5 dilution of MAbFms and Fms-HRP respectively, was selected. When the immunoassay was pre-coated first with anti-mouse IgG 20 µg

mL^{-1} on the well, the absorbance reading was increased by the increasing of the antibody/antigen binding.

6.4.2 Immunosensor development

Based on the present concentration of the immunoreagent above, the direct competitive assay for the development of Fms detection on the gold working electrode was conducted.

Highly specific binding on the gold working electrode was achieved by pre-incubation free Fms for 30 min before competing with Fms-HRP on the antibody sites. By using pre-incubation method, the increase of S/B was achieved. The specificity binding of the immunoassay was demonstrated by promoting of binding of the free Fms with coated antibody. The specific binding at the correct orientation antibody when using covalent immobilisation (DTDPA thiol on surface) was also increased and decreased at low and high concentration of free Fms, respectively. This is inversely proportional to the Fms with a low background reading and a high current response compared to passive adsorbtion. Combinations of these two techniques in this work for direct competitive immunoassay on a gold modified surface have exhibited a linearity range that is more sensitive than the ELISA method. The limit of detection obtained was also lower (more sensitive) than in an optical fiber optic immunosensor (Thompson and Maragos, 1999) and optical SPR (Gaag *et al*, 2003). The resulting data has been discussed in Chapter 4. From the literature review, it has been seen that no report on electrochemical immunosensor detection for fumonisins analysis has been investigated until now. The development of a gold modified immunosensor for total fumonisins in this work may be the first one and still need improvement for future work.

6.4.3 Specificity, reproducibility and stability of the immunosensor

The specificity of the gold immunosensor has been tested and good cross reactivity of MabFms against FmB_1, FmB_2 and Fms from the Neogen kit was found. Moreover, the

high cross reactivity was found to be an inherent property of the antibody raised for the total fumonisins (Gascon *et al*., 1997). This presence in the assay shows that the MAbFms had specificity to the structure of those fumonisins. Thus, the monoclonal anti-fumonisins antibody used is suitable for detecting total fumonisins in buffer or samples. The reproducibility and stability of the immunosensor system was evaluated and examined.

The CVs% at each of the points of assay (*n*=9) were 3.4%, 2.8%, 6.6% and 7.2% at 10, 50, 100, and 500 µg L^{-1} of Fms, respectively. A figure of 10% or less is considered satisfactory (Murray *et al.*, 1993). The formula used for the calculation of CV% is slightly different from the conventional formula (Standard Deviation divided by the mean and multiplied by 100). This is the ability of the assay to consistently reproduce a result when samples are taken from the same specimen (Liu & Eisenbarth, 2007). Thus, the proposed immunoassay method could be used with acceptable precision and reproducibility (Jin *et al*., 2008).

The stability of the antibody coated is also required for a high performance immunoassay to maintain effective detection. The antibody coated on the modified gold was stored at 4 ºC; it could keep its initial response for eight weeks, and the amperometric response was stable for 3 weeks (21 days) and slowly decreased after that. That is 90 to 100% current response retained and down to ~ 60% after 5 weeks storage. The antibody was coated by covalent attachment. The reason for the slow decrease of response might be due to the fact that the electrode materials were leached from the base surface, or due to the partial deactivation of the immobilised antibody incorporated in the composite (Sun *et al*., 2008). The stability of other screen-printed immunosensor developed using a similar technique (antibody coated by covalent immobilisation) was 14 days (SPGE), 19 days (glassy carbon electrode) and 25 days (SPCE) as found by Radi *et al*. (2009), Su *et al*. (2008) and Wu *et al*. (2006), respectively. This work reported here was similar to that performed by Radi and co-workers (2009); however, the increasing of stability may be from the strong covalent binding between the antibody and the thiol modified surface. They could prevent the antibody from leaking out from the surface. Bi-thiol functional linkers such as DTDPA may act as spacers between the target surface of immobilization and the antibody molecules which afford minimal effect on the immunoprotein activity and

may prevent the denaturing of biomolecules thus causing an increase in the shelf life of biosensors.

6.5 Development of Microsensor electrode for mycotoxin detection

6.5.1 Characterisation and surface modification

The sensitivity of the immunosensor for AFB_1 and Fms detection using the gold screen-printed technology conducted in this work was compatible with the requirements of EU legislation. However, the requirement for a robust, rapid, less chemical-using and multi-array test for several analyte detection for AFB_1 and Fms can be answered by using microelectrode technology. In this study, the microelectrode array (MEA) fabricated by photolithography from Tyndall (Cork, Ireland) was selected because the electrode is suitable for electrochemical detection containing a reference electrode, counter electrode and working electrode. The working electrode was designed consisting of a 35 micro gold chip array producing hemispherical diffusion for better electron diffusion.

Use of arrays of microelectrodes enables the harnessing of the enhanced mass transport benefits of microelectrodes while producing greater current signals and so allowing less sensitive instrumentation to be used if needed. They are geometrically different from SPGE, and include the ability to enhance the current response while retaining the properties of a single microelectrode (Morita *et al*., 1996). Parker *et al*. (2009) have described the characterisation of the same MEA used in this work using atomic force microscopy and scanning electron microscopy for electrochemical analysis of aflatoxin M_1 detection. In this study the characterisation of MEAs was investigated by examining their performance in a solution of ferrocyanide in KCl using cyclic voltammetric analysis after O_2 plasma cleanup. The characteristic microelectrode array sigmoidal shaped cyclic voltammograms (CVs) was clearly visible for the microsquare arrays indicating hemispherical diffusion to the MEA surface (Parker *et al*., 2009; Huang *et al*., 2009). According to Berduque *et al.*, the CVs show characteristic sigmoidal CVs as expected for microelectrodes with sufficient interelectrode spacing (Berduque *et al.,* 2007). This will benefit the

developed sensor because of the improved sensor response time (faster response), and it is the electroactive species towards the electrode surface that becomes more dominant rather than linear diffusion (SPGE) (Kudera *et al*., 2001).

Covalently immobilising the antibody onto the gold surface of the microelectrode array with PDITC, as described by Parker *et al*., (2009), was a new method of immobilisation compared to the developed screen-printed electrode immunosensor which relied on passive absorption and thiol covalent immobilisation onto the gold surface. Protein (antibody or antigen conjugate protein) attachment chemistry, which is applicable to silicon chip substrates, has been developed through (i) substrate cleaning protocols (O_2 plasma), (ii) silanisation protocols (3-aminopropyltrimethoxysilane), (iii) linking molecules that minimise steric hindrance during hybridisation (1,4-phenylene diisothiocyanate) and (iv) methods for covalent attachment of the protein molecules. Covalent attachment of protein using this method enhances the chemical stability of the immobilised layers and enables the implementation of more stringent pre- and post-hybridisation wash conditions. This is an appropriate method to improve the interfacial contact between the protein molecules and the gold surface (Kudera *et al*., 2001). The aim of the surface modification was for attachment of the bio-recognition material mainly on the surrounding silicon layer, therefore an assessment of the surface coverage on the gold electrode surface was carried out by voltammetry. The most widely used method to assess the extent of electrode surface coverage is based on cyclic voltammetry of ferricyanide or ferrocyanide (Lucarelli *et al*., 2004), and the reducing current at modified electrode with respect to an unmodified surface suggesting some modification of the electrode surface as well as the silicon surface.

6.5.2 Competitive immunoassay for AFB_1 and Fms detection

The electroactive species on the MEAs were measured by chronoamperometric detection based on selected constant potential suitable for the MEA. Initial optimisation was carried out to determine the optimum potential for TMB/H_2O_2 detection on the microelectrode array since the microelectrode array consists of a gold working, auxiliary and reference electrode, whereas for the screen-printed electrode

the working is gold, auxiliary is carbon based and the reference electrode is Ag/AgCl. The suitable constant potential was +150 mV which is very similar to that used by Parker and co-workers (+168 mV) (Parker *et al.*, 2009). For the enzyme activity on the modified MEA the determination of signal and background responses was evaluated by using TMB/H_2O_2 similar to the SPGE.

AFB_1 detection

Better sensitivity was obtained when the indirect method was applied on the SPGE. All the optimal indirect immunoreagent used in SPGE was transferred to the MEA for the detection of AFB_1. Then, the sensitivity of the assay could also compared to the SPGE developed. Transferring the receptor chemistry from the screen-printed electrode to the microelectrode array was a success with detection significantly superior to the screen-printed electrode. The best method in this case was covalent immobilisation with the correct antibody binding to the antigen. The sensitivity improved from 5 ng L^{-1} for the screen-printed gold electrodes to 1 ng L^{-1} for the microelectrodes, both in buffer and matrix samples. However the sensitivity of the assay gave similar results when compared to the immuno gold nano-particle sensor.

Fumonisin detection

All the optimal direct competitive immunoreagents used in SPGE were transferred to the modified MEA for the detection of Fms.. The sensitivity was increased compared to the ELISA developed; however, the dynamic range found at MEA (1-1000 ng L^{-1}) was wider than in SPGE (1-500 ng L^{-1}) in buffer and matrix samples.

6.5.3 Reusability of the microelectrode array

The screen-printed electrode is the cheapest technology reviewed in this conclusion due to the low cost of electrode production. As compared to the microelectrode technology the costs are significantly higher, with the microelectrode arrays estimated at 5 euros each by the Tyndall Institute. In this study, the reusable test of MEA was applied due to its being a cost effective sensor product. The strong immunoreagent reaction between the modified MEAs and the antibody against analytes were exploited in order to construct a reusable microsensor array. The most important part

before the electrode can be reused is the cleaning process. This is because the silicon surface contains a chemical modified surface with a strong binding of immunoreagent that might be influencing the reproducibility of the sensor. The removing of the contaminant on the silicon surface using a piranha solution before following with O_2 plasma is a part of the cleaning process used in this study. According to Min *et al.*, a piranha solution is used to remove all organic contamination from the surface of gold or silicon (Min *et al.*, 2008). Then, the O_2 plasma is very effective in removing hydrocarbon and adsorbed water vapour from the surface (Tada *et al*., 2002). According to Tada *et al*, the plasma cleaning also provides a soft etching on the substrate surface that might influence the surface layer. The performance of the cleaned electrode was then analysed by CVs to examine the wetting properties of the gold surface. The cyclic voltammogram of redox reaction on the surface was totally changed from the small or no redox peak (uncleaned electrode) to the sigmoidal peak (after cleaning) similar to the new electrode. The achievement of the steady state peak of electroactive reaction on the reused electrode surface indicated that the surface was free from contamination and with sufficient interelectrode spacing.

The most commonly reused electrode for an immunosensor system has been involved in flow injection capacitive immunosensor systems such as Surface Plasmon Resonance (Fu, 2007) and QCM (Zhang *et al*., 2008), and responded significantly well. However, in the case of the electrodes used in electrochemical detection, the performance response for the direct solution deposited onto the electrodes surface is still under investigation. Therefore, the performance of reusable electrodes was conducted by repeating the same electrodes four times in the competitive assay for developing a calibration curve of AFB_1. For the second assay after the first cleaning, the orientated specific binding of the antibody antigen was evaluated, and obtained significantly similar results on linear range of detection, limit of detection and low non-specific binding. The current response and the assay sensitivity were reduced by 40% and 10-fold after the second and third cleaning, respectively. This indicates that the performance of the electrodes was reduced after the second cleaning. In this case, the cleaning test of the MEAs for a reusable immunosensor, based on chronoamperometric detection, was best after the first time.

Another benefit when MEA was applied in this study was that it could be postulated that since diffusion alone is the source of mobility of the analyte in the screen-printed electrodes, ELISA and microelectrode arrays, a reduction of the test volume has yielded a shorter incubation times and faster assay times (compared to SPGE). In conclusion, employing gold microelectrodes rather than gold based screen-printed electrodes, allows new applications of high sensitivity and low detection limits to be analysed without requiring elaborate instrumentation.

6.6 Sample analysis

Overall, this work has demonstrated that competitive immunoassays can readily be developed for detection of mycotoxins found in food products. Although both AFB_1 and Fms could be detected in spiked peanut and corn, sensitivity was a serious issue for both: FDA advisory levels for both are in the ppb ($\mu g\ L^{-1}$), rather than ppm ($mg\ L^{-1}$) range. From the performance of the immunosensor developed it can be considered that the sensor is able to detect both mycotoxins in real samples. The principal intention of the immunosensors is to minimize the matrix interference and inhibition effects of the immunoassays of real samples. The first evaluation was carried out of the matrix effect in immunoassays, and the second was the study of the extraction efficiency of AFB_1 and Fms from contaminated (or spiked) peanut and corn samples to verify the accuracy of the sensors, respectively. For this purpose, both non contaminated and unknown samples were used. Peanut and corn samples were selected because those are the commodities that are most susceptible to high aflatoxin and fumonisin contamination, respectively, and they are greatly consumed in my country (Malaysia). Thus, some selected samples were brought from Malaysia and some from the United Kingdom.

The efficiency of the detection of mycotoxins in food matrix is depending on the effectiveness of the sample preparation. Sample preparation is normally a major concern, and may require optimisation for each food matrix being tested. There are a number of commercially available rapid sample preparation and/or solvent clean-up kits that can be used to accomplish the same purpose (Anderson *et al*., 2010; Krska *et*

al., 2005). The higher percent of recovery found was shown with the high level performance of the extraction method.

In this study, two different techniques for sample preparation have been developed: a simple extraction method, as described in the ELISA kit method, and a clean-up method using the SPE column developed. Normally, a simple extraction method is for evaluating the specificity of the antibody to the antigen in a matrix sample and as a rapid method of detection. This is suitable for screening methods to monitor direct detection of analytes in samples, while the clean-up technique is for reducing or removing interference in the matrix samples due to the increased sensitivity of the assay. For both systems, the peanut and corn samples were extracted in an aqueous-organic solvent (methanol-water) to liberate AFB_1 and Fms from the sample before diluting using buffer (for immunoassay) or re-dissolved with another organic solvent (for HPLC). The 70% methanol-water solution is the one most commonly used to extract aflatoxin from peanuts according to the official AOAC method. While, using 70% aqueous methanol to extract fumonisin from corn sample also provided best overall performance of extraction (80 to 100 % of recovery test) (Kulisek & Hazebroek, 2000; Sewram *et al*., 2003). The selection of a solvent for extraction and re-dissolution exerts a great influence on the recovery of mycotoxins. Thus, the solvents employed have to be taken into account regarding the individual properties of toxins and solvents, especially their polarities. Many complex sample mixtures cannot be accurately analyzed without prior purification. The SPE C18 cartridges used in this method represent a rigorous but relatively simple purification method for cleanup of these samples. The cartridges can be eluted in the reverse-phase format using very polar solvents such as methanol, acetonitrile, and water. Using these methods in sequence, the entire aflatoxin B_1 and Fms from the sample extracted can be separated for analysis.

With the SPGE and MEA performing well in a pure buffer solution, a further examination was carried out to assess the performance in a peanut and corn sample. The calibration curve of AFB_1 and Fms in a non contaminated peanut and corn sample was established which found a similar limit of detection (LOD). Then, these calibration curves were applied for calculating the amount of those toxins in the spiked samples or unknown samples.

Table 6.2 shows the performance of the sensors using these two methods, based on the average of % recovery and %CV. Then the result of the peanut and corn analysis using the gold immunosensor (SPGE and MEA) was compared with the HPLC analysis. In order to achieve more comparable results to the HPLC and to validate these as well, the peanut and corn samples needed to be pre-treated using the same method before analysis. The data was also compared to the ELISA method thus providing a simple and rapid method. The reliable data between 70 to 110 % was observed from both extraction methods at all detection methods. The data from developed sensors were significantly similar to the HPLC and ELISA.

This shows that the developed methods (SPGE and MEA) were demonstrating a high performance of detection.

Table 6.2: The performance of the electrochemical gold immunosensor using SPGE and MEAs for the detection of AFB_1 and Fms in spiked sample extracts (peanut and corn), respectively. The data presented is based on the average of recovery and CV of 4,40,100 and 250 µg Kg^{-1} (AFB_1) and 50, 250, 500 and 2500 µg Kg^{-1} (Fms).

Analytes	Detection method	Extraction Methods			
		Without clean-up		Clean-up procedure	
		%R	%CV	%R	%CV
Aflatoxin B_1					
	ELISA	82.5	6.9	-	-
	SPGE/Nano	82.7	8.5	103.1	8.7
	MEA	85.7	8.3	-	-
	HPLC	-	-	110	6.7
Fumonisins					
	ELISA	80.9	7	-	-
	SPGE	73.5	6.1	98.6	6.2
	MEA	87.3	5.3	-	-
	HPLC	-	-	91.8	5.2

%R: Percent of recovery, %CV: Percent of Coefficient variance, SPGE: Screen-printed gold Electrode, MEA: Microelectrode array, HPLC: High performance Liquid Chromatography.

From the Table, high recovery test was observed from either of the samples after the clean-up process compared to without clean-up. This is likely to have been due to the use of the SPE C18 column as a pre- treatment for the sample analysed. The pre-treated method showed greater removal of interference of co-extracted compounds in peanut and corn samples. In the case of simple extraction method (without clean-up), the lower recovery test was observed compared to clean-up method. This phenomenon shows that the high amount of substances in the matrix samples was inhibited the analysis. However, the recovery result was still reliable for the detection of the toxins in matrix samples. We would expect here that the strong captured antibody specific to analytes were presenting a high performance of SPGE and MEA methods. In this case, the major advantage of the proposed gold immunosensor is that pre-treatment is also not necessary.

6. 7 Final Conclusions

The first stage of the biosensor development was discussed in Chapter 1 about the potential of the electrochemical immunosensor technique demonstrating the capability of AFB_1 and Fumonisin detection. From the literature obtained, immunoassays in sensor application methods potentially have advantages over the other procedures because of their specificity, sensitivity and applicability of the immunosensor to the buffer and real samples. Advanced technology in microsensor array and the functionalization of nano-particles utilising current instrumentation with new modelling technology, high ability and more stable receptors and the miniaturization of microelectrode means that the focus will be on developing sensitive, simple, rapid, environmentally-safe and inexpensive methods.

The investigation of immunosensor development first involved the characterisation of the bio-molecule recognition (antibody-antigen) elements using enzyme immunoassays (ELISA). The reagents were purchased and investigated. The use of reagents in a spectrophotometric immunoassay study based on indirect and direct format for AFB_1 detection has been developed and optimised. The establishing of optimal parameters coated in microtitre plate for indirect assay were 1 µg mL^{-1} of BSA-AFB_1, 10 µg mL^{-1} of monoclonal antibody and 1 µg mL^{-1} of anti-antibody

labelled HRP. For the direct immunoassay it was 10 µg mL^{-1} of anti-antibody IgG coated, 20 µg mL^{-1} of monoclonal antibody bonding and 1: 10 dilution of AFB_1 labelled HRP concentration. PVA blocking is a suitable solution used in preventing non specific binding of antibody and enzyme conjugate to the surface. The use of a monoclonal antibody and using a horseradish peroxidase labelled antibody or antigen in both systems provided ranges of detection which were 0.1 to 10 µg L^{-1}. An indirect and direct competitive ELISA were shown to perform satisfactorily for the analysis of AFB_1.

The feasibility of determination of AFB_1 concentration by SPGE based competitive immunoassay has been demonstrated in this work. The ELISA method with the optima concentration and condition were then transferred to an in house screen-printed electrode (SPGE Ercon) and commercial printed one (SPGE Dupont). Optimal concentrations of 5 mM TMB and 0.075% H_2O_2 for enzyme activity (HRP) substrate was performed for immunosensor measurement on both electrodes at an applied -100 mV versus Ag/AgCl. Physical adsorption of antibodies to SPGE Ercon and DuPont in an indirect and direct immunosensor format showed a detection limit range of 0.01 to 10 µg L^{-1}. This work shows that probably the use of the indirect competitive method provides a better immunosensor using SPGE-DuPont. SPGE–DuPont presents a higher current response, with a wide current range and still produces a low background reading**.** The development of an electrochemical immunosensor (SPGE-Dupont) using a DTDPA modified surface has been shown to be a useful device. The SAMs thiol modification using a 3,3-dithiodipropionic acid method allowed the current to penetrate through the monolayer and undergo an electron transfer with the surface. The improvements of the orientation binding and lower non specific binding was seen by employing a SAMs thiol monolayer on the gold working surface rather than passive adsorption immobilised. The developed immunosensor for AFB_1 result was 0.01 to 10 µg L^{-1} (LOD = 5 ngL^{-1}) applying a gold electrode modified with DTDPA. The application of immuno gold nano-particles labelled HRP on the SAM thiol gold electrode surface enhanced the sensitivity of the assay and also proposed fewer incubation steps. The sensitivity of the assay was increased 5 times (LOD = 1 ng L^{-1}). The calibration curve displayed a dynamic range, covering a lower concentration of interest and also covering detection of about 1-20 µg L^{-1} of AFB_1 (level permitted).

In this study, we have developed a direct competitive ELISA and immunosensor for fumonisin detection in buffer solution and matrix corn sample. There was better orientation of the antibody specific for the fumonisin by precoating the immobilisation antibody before it was coated with a monoclonal antibody on the ELISA plate. The establishment of the optimal parameters coated in the microwell plate were 20 µg mL^{-1} of ant-IgG, 1:50 dilution of monoclonal antibody and 1:5 dilution of Fumonisins-HRP. After optimisation of the reagents using the ELISA method, the direct competitive assay exhibited a linearity range between 100 to 1000 µg L^{-1}. The electrochemical direct competitive immunosensor for fumonisin detection was also investigated using SPGE-DuPont. The sensor was pre-coated with 10 µg mL^{-1} of anti-IgG before being coated with 1:50 dilution of the monoclonal antibody and the addition of 1:5 dilution of Fms-HRP and free Fms in the sample competing for the binding sites on the surface. Highly specific binding on the covalent binding assay has been achieved by pre-incubation free Fms for 30 min before competing with Fms-HRP on antibody sites. The immunosensor technique in this work exhibited a linearity range between 1-500 µg L^{-1}, more sensitive than the ELISA method (100–1000 µg L^{-1}). The use of the monoclonal antibody against fumonisins (MAbFms) specific had high cross reactivity to FmB_1 and FmB_2, which means the immunosensor was able to detect both fumonisins.

For better sensitivity, the use of a microelectrode array as an alternative transducer for the electrochemical immunosensor was investigated. The reusable microelectrode after cleaning using a piranha solution and O_2 plasma provided sensitive detection in the sensor application. The surface was modified with an aminosilane anchor and PDITC cross linker to provide covalent bonding capture antibody immobilisation. The competitive immunoassay of AFB_1 and Fms solution on the modified MEA was then performed by transferring the chemistry from successfully on the SPGE. The sensor devices were reproducible and very sensitive as they can detect 0.001 µg L^{-1} (1 ng L^{-1}) and above of AFB_1 in buffer and peanut matrix. This developed microsensor was 5 times better than SPGE. In the case of Fms detection, the low level can be detectable at 1 µg L^{-1} and above in buffer and corn extract.

By using a standard curve of AFB_1 and Fms in non contaminated matrix peanut and corn samples, the detection of AFB_1 and Fms in the peanut and corn samples

respectively was investigated using the proposed methods. The performance of the proposed sensor was applied using the rapid extraction method and a pre-treated clean-up process after extraction. The gold immunosensor method (immuno gold SPGE) for AFB_1 detection has shown gold results achieving an average of 82.7% and 103.1% for rapid and pre-treated clean-up, respectively, while the MEAs in the rapid extraction method obtained 85.7% of average recovery of the spiked peanut sample. In the case of Fms detection, the rapid extraction and clean-up process method presented an average recovery of 73.5% and 98.6% using the SPGE immunosensor developed, while the rapid extraction method obtained 87.3% of average recovery of the spiked corn sample using the microsensor. The reproducibility of both proposed sensors has also shown a good performance with a low coefficient of variation (%CV) between 6.1 to 8.7% (less than 10%). The reliability of both format immunosensors for the analysis of AFB_1 and Fms in spiked or real samples was verified by comparison of the data with the fully validated confirmatory ELISA kit and HPLC results. These methods (SPGE and MEA) are highly sensitive to specific antibodies and provide an analytical system capable of detecting very low levels of toxin. This high sensitivity is also due to the powerful catalytic ability of the enzyme conjugate selected. For all the immunoassay tests using ELISA, SPGE and microelectrode array are compatible with the requirements of EU legislation in food and feed; that is, a range between 1- 20 and 2000 - 5000 µg L^{-1} for AFB_1 and Fms, respectively.

6.8 Future work

The overall results achieved from the immunosensors developed (SPGE, gold nano-particle/SPGE and MEA) in this work were very encouraging and met the required detection limits needed for aflatoxin B_1 (AFB_1) and fumonisins (Fms) determination in peanuts and corn samples. The sensors have been successfully designed to be cost effective, easy to use and sensitive. However, there are several suggestions to improve the developed immunosensors for future applications;

- The development of electrochemical immunosensors using screen-printed gold electrode and the application of gold nano-particle on SPGE for AFB_1 analysis still needs to be further investigated. The target for achieving high sensitivity

immunosensor has been succesfully compared to other method. However, for rapid detection, optimisation and stability of the gold nano-particle immunosensor further investigation is needed to characterise the system. The study should optimize the incubation time in every steps of the immunoassay immobilisation in order to reduce total analysis time required for the sensors. Different immobilisation techniques need to be investigated in order to reduced the immobilisation steps used. Even though the stability of the immuno gold nano-particle conjugate antibody/HRP is good, this also needs to be further optimised and investigated for long time storage stability. This is in order to confirm the performance of the immunosensors and establish sensitivity of the devices with lower levels of AFB_1.

- The development of electrochemical immunosensor for Fms detection using SPGE has been successfully constructed in this work. However, to increase the sensitivity, stability and reduce time of analysis (rapid detection) further investigation using different kind of immobilisation techniques and immuno reagents optimisation, re-optimising time of incubation should also be investigated. Furthermore, the application of nano-particle should also be used in this study in order to increase the sensitivity of the assay and reduce immobilisation steps.

- In case of AFB_1 and Fms detection using MEA, the immunosensor developed has been very sensitive, robust, easy to use, reusable and require less time of analysis. For further investigation, we suggesting that analysis need to be improved by improving the system using MEA multi-analyte based sensor. This is in order to produce a useful device for simultaneous detection of several mycotoxins in food samples.

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APPENDICES

Appendix 1

List of Publications

M.K.A. Kadir and I.E. Tothill (2007). Development of affinity sensor for aflatoxin B_1 detection. Young researcher's meeting "Contamination in the environment", Central Science Laboratory, York, 24 September 2007. (Poster presentation)

M.K.A. Kadir and I.E. Tothill (2008). Immunosensor for aflatoxin B_1 detection using gold working electrode. Cranfield Multi-Strand Conference: Creating Wealth Through Research and Innovation (CMC 2008), 6-7 May 2008, Business Development Centre, Cranfield University, Bedfordshire, MK43 0AL (Oral presentation)

M.K.A. Kadir and I.E. Tothill (2008). Immunosensor for aflatoxin B_1 detection using gold working electrode. The Tenth World Congress on Biosensors May 14 – 16, 2008, Shanghai, China. (Poster presentation).

M.K.A. Kadir and I.E. Tothill (2008). Immunosensor for aflatoxin B_1 detection using gold working electrode. Cranfield Health Postgraduate Conference, 18 September, 2008. (Poster presentation).

M.K.A. Kadir and I.E. Tothill (2008). Microsensor array for mycotoxin detection. The World Mycotoxin Forum – the fifth conference. Conference 17-18 November 2008. Netherland. (Poster Presentation).

M.K.A. Kadir and I.E. Tothill (2009). The Application of Nano-particles in the Development of an Electrochemical Immunosensor for Aflatoxin B_1. 12^{th} Conference in Genomics and Proteomics of Human Patogens, "Target Molecules and Biomarkers in the Characterisation of Microbes in Disease and the Environment" 25-26^{th} June 2009, Health Proctection Agency, Centre for infection, London**.** (Poster presentation)

M.K.A. Kadir and I.E. Tothill (2009). The Application of Nano-particles in the Development of an Electrochemical Immunosensor for Aflatoxin B_1. 9^{th} Workshop on (Bio) sensors and Bioanalytical microtechniques in environmental and clinical analysis, June 14-17^{th} 2009, Montreal, Canada (Poster presentation).

M.K.A. Kadir and I.E. Tothill (2009). Development of electrochemical immuno gold nano-particle sensor for aflatoxin B_1 detection. ISM conference 2009, 9-11 September, Tulln, Austria. (poster presentation)

M.K.A. Kadir. (2009). Microsensor array for mycotoxin analysis. Cranfield Health, Postgraduate Conference, 16th September 2009, Cranfield Health, Vincent Building, Cranfield University, MK43 0AL (Oral presentation).

M.K.A, Kadir and I.E. Tothill. (2010). Development of an electrochemical immunosensor for fumonisins detection in foods. *Toxins*, **2**, 382-398.

M.K.A. Kadir and **I.E. Tothill** (2010). Immunochip microsensor array for mycotoxin detection. Abstract accepted for 11th World Congress on Biosensors, May 26 – 29, 2010, Glasgow, UK. (Oral presentation by Tothill)

Submitted Papers:

Mohamad Kamal Abdul Kadir, Damien W. M. Arrigan and Ibtisam E. Tothill. Immunochip microsensor array for aflatoxin B_1 detection in peanut.

Mohamad Kamal A Kadir and Ibtisam E. Tothill. Immuno gold nano sensor for aflatoxin B_1 detection in peanut.

Appendix 2

DEVELOPMENT OF AFFINITY SENSOR FOR AFLATOXIN B_1 DETECTION

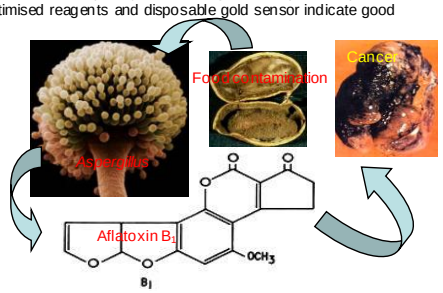
Mohamad Kamal Abdul Kadir and Ibtisam E.Tothill
Cranfield Health, Cranfield University, Silsoe, Bedfordshire, MK45 4DT, UK

Abstract

This paper reports on the development of an affinity sensor based on gold working electrode combined with indirect competitive enzyme-linked immunosorbent assay (ELISA). Immunoassay parameters such as AFB_1-BSA conjugate, monoclonal antibody (anti-AFB_1 antibody), anti-antibody labelled with the enzyme HRP, buffer, blocking reagent and incubation time (coating, blocking, competition and enzyme reaction steps) were evaluated and optimised. An ELISA microtitre plate assay was used in a preliminary phase of development, prior to transferring the assay to the screen printed gold electrode (SPGEs). The electrochemical system was then developed by immobilizing the biological component directly (passive absorption) on the surface of SPGE. Preliminary result using optimised reagents and disposable gold sensor indicate good sensitivity toward the toxin.

Introduction

Aflatoxin contamination (produced by members of *Aspergillus)* in foods is of global concern due to their potential toxicity, carcinogenicity and also their immunosuppressive ability for the mammalian system. Therefore, concern has been expressed regarding the effect of this toxin on human health since Aflatoxin B_1 (AFB_1) is known to be the most predominant and most toxic. Hence, Aflatoxin B_1 sensing techniques must be able to detect this compound with high sensitivity, selectivity and accuracy. Current analysis is generally based on the use of conventional methods such as HPLC which can be difficult to perform on site and also expensive. Therefore, a need exits for an alternative methods which are rapid and portable that exhibit the sensitivity and low detection capability of conventional methods.



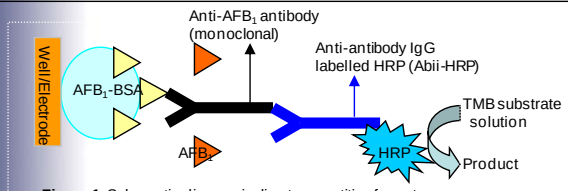


Figure 1, Schematic diagram indirect competitive format.

Material & Methods

Indirect competitive immunoassay were developed, initially by ELISA and subsequently transferred to an electrochemical immunosensor format using screen printed gold electrode.

Figure 1, show the assay format implemented to develop the ELISA microtitre plat assay.

ELISA

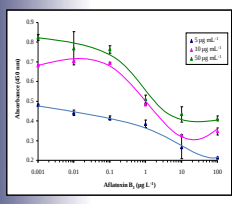


Figure 2. Calibration curve of AFB_1 with different concentration of monoclonal antibody (Mab).

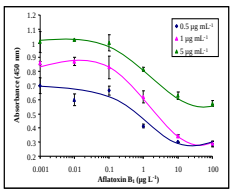


Figure 3. Calibration curve of AFB_1 with different concentration of anti-antibody IgG labelled HRP (Abii-HRP).

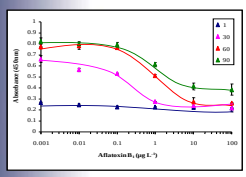


Figure 4. Effect of incubation times at 37ºC on competition step for AFB_1 detection.

Optimisation experiments show that the best competitive condition were obtained using 5 µg mL^{-1} Abii-HRP and 50 µg mL^{-1} Mab. Best incubation time for specific binding (antibody – antigen) is 90 minutes at 37ºC. The range of detection was observed from 0.01 to 10 µg L^{-1}.

Electrochemical Immunosensor - SPGE

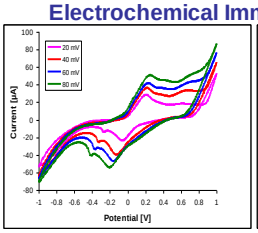


Figure 5. Cyclic voltammetry of 5 mM potassium ferrocyanide at different scan rate using the SPGE.

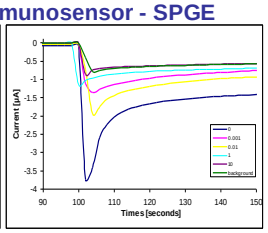


Figure 6. Chronoamperometric measurement. Change in current of different concentration AFB_1 using TMB/H_2O_2 substrate.

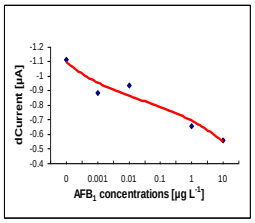


Figure 7. Calibration curve of AFB_1 using electrochemical detection on the surface of the gold sensor.

Figure 5, indicate that the redox reaction at the screen printed gold electrode is a quasireversible. The concentration of AFB_1 was determined based on different current response using TMB/H_2O_2 substrate by chronoamperometry. The calibration curve shows detection range is lower than ELISA assay. That is indicate good sensitivity toward AFB_1.

Conclusions

Preliminary data indicate that the electrochemical immunosensor using screen printed gold electrode as well as ELISA based on indirect competitive format were acceptable for analyses AFB_1 at level required by the regulation (1 to 20 ppb).

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Appendix 3

IMMUNOSENSOR FOR AFLATOXIN B_1 USING GOLD WORKING ELECTRODE

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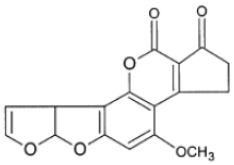
Introduction

Aflatoxins contamination in foods and animal feeds is of global concern due to their potential toxicity, carcinogenicity and also their immunosuppressive ability for the mammalian system. Aflatoxins are a group of highly toxic secondary metabolites produced by the fungi *A. flavus* and *A. parasiticus*. The major occurring aflatoxins are aflatoxin B_1 (AFB_1), B_2 (AFB_2) (produced by *A. flavus* and *A. parasiticus*) G_1 (AFG_1) and G_2 (AFG_2) (produced by *A. parasiticus*) plus two additional metabolic products, M_1 (AFM_1) and M_2 (AFM_2). Therefore, concern has been expressed for the effect of this group of toxins on human health since AFB_1 (Figure 1) is known to be the most predominant and most toxic.



Aflatoxin B_1

Figure 1: Chemical structure of AFB_1 (Hussein and Brasel, 2001)

To control and manage the level of aflatoxins, many countries worldwide have set permitted exposure limits for food and feed intended for direct human consumption or for use as ingredient in animal foodstuffs, which vary from 1- 20 $\mu g\ kg^{-1}$. The maximum acceptable level for AFB_1 in food is set at 2 $\mu g\ kg^{-1}$ (ppb) in 29 countries, which include EU countries (van Egmond and Jonker, 2004).

Therefore, current research focuses on the development of AFB_1 sensing techniques based on immunosensor systems. This developed technique must be able to detect the toxin at low levels (ppb or ppt range) as well as to be rapid and simple to use (Logrieco et al., 2005). The adopted approach is based on an electrochemical sensor platform fabricated using screen printed gold electrode combined with a specific competitive immunoassay methods using an antibody immobilised on the gold sensor

Appendix 4

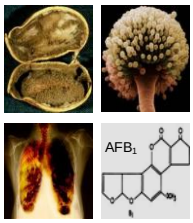
IMMUNOSENSOR FOR AFLATOXIN B_1 USING GOLD WORKING ELECTRODE

M. A. Kadir and I.E. Tothill*

Cranfield Health, Cranfield University, Silsoe, Bedfordshire, MK45 4DT, UK

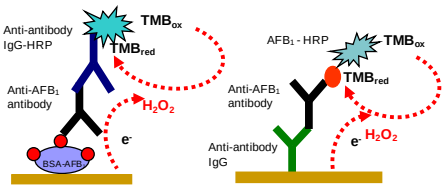
Introduction

Aflatoxins contamination in foods and animal feeds is of global concern due to their potential toxicity, carcinogenicity and also their immunosuppressive ability for the mammalian system. Aflatoxins are a group of highly toxic secondary metabolites produced by the fungi *A. flavus* and *A. parasiticus*. The major occurring aflatoxins are aflatoxin B_1 (AFB_1), B_2 (AFB_2) (produced by *A. flavus* and *A. parasiticus*) G_1 (AFG_1) and G_2 (AFG_2) (produced by *A. parasiticus*) plus two additional metabolic products, M_1 (AFM_1) and M_2 (AFM_2). Therefore, concern has been expressed for the effect of this group of toxins on human health since AFB_1 (Figure 1) is known to be the most predominant and most toxic. To control and manage the level of aflatoxins, many countries worldwide have set permitted exposure limits for food and feed intended for direct human consumption or for use as ingredient in animal foodstuffs, which vary from 1- 20 µg kg^{-1}. The maximum acceptable level for AFB_1 in food is set at 2 µg kg^{-1} (ppb) in 29 countries, which include EU countries. Therefore there is an essential need for sensitive and rapid detection methods for the detection of this toxin.



The project focuses on the development of an electrochemical sensor based on gold working electrode fabricated using screen printing combined with a specific competitive immunoassay methods with an immobilised antibody on the gold sensor surface.

Electrochemical Immunosensor (Figure 2)



An electrochemical immunosensor system based on gold working electrode has been developed using indirect and direct competitive format for AFB_1 detection. For the indirect immunoassay, free AFB_1 in the sample compete with the immobilised BSA-AFB_1 for the anti-AFB_1 monoclonal antibody added with the sample. Signal detection is carried out using labelled secondary antibody (anti-mouse IgG-HRP) (Figure 2 a). In case of direct format assay, the AFB_1-HRP and free AFB_1 in the sample compete for the binding sites of the anti-AFB_1 monoclonal antibody immobilised through an anti-mouse IgG adsorbed on the gold sensor (Figure 2 b).

Signal generation was through the use of TMB/H_2O_2 as the enzyme substrate system and the current recorded at -200 mV. The current is proportional to the amount of HRP conjugate bound on gold electrode surface.

ELISA

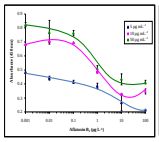


Figure 3. Calibration curve of AFB_1 with different concentration of monoclonal antibody (Mab).

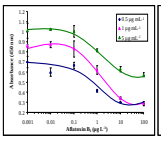


Figure 4. Calibration curve of AFB_1 with different concentration of anti-antibody IgG - HRP

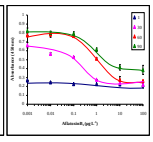


Figure 5. Effect of incubation times at 37ºC on competition step for AFB_1 detection.

Optimisation experiments show that the best competitive (indirect format) condition were obtained using 5 µg mL^{-1} anti-IgG-HRP and 50 µg mL^{-1} Mab. Best incubation time for specific binding (antibody – antigen) is 90 minutes at 37ºC.

Immunosensor

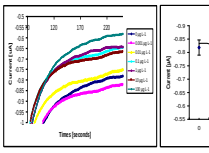


Figure 7 :The calibration curve for indirect competitive immunoassay for AFB_1 on SPGE by chronoamperometry detection.

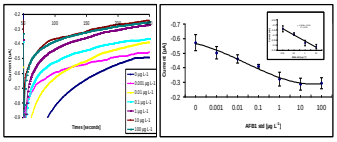


Figure 8 :The calibration curve for direct competitive immunoassay for AFB_1 on SPGE by chronoamperometry detection.

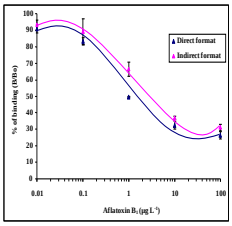


Figure 6: Standard curves for AFB_1 standard solution by indirect and direct competitive ELISA. These were then transferred to the electrochemical immunosensor using SPGE. Optimum concentration of reagents and also conditions were applied in these tests. The absorbance decreases with the increasing of AFB_1 concentration. Linear range covers for both format from 0.1 to 10 µg L^{-1}

For both assay formats the readings decreased from low to high concentration of AFB_1 and declined steadily after 10 µg L^{-1}.

The linear range for indirect assay was ~ 0.01 to 1 µg L^{-1} and using linear regression the R^2 value is 0.917 (Figure 7).

In the direct assay a pre-coating of anti- IgG antibody was used which gives better orientation of the anti-AFB_1 antibody binding sites (Figure 8). The linear range was ~ 0.01 to 10 µg L^{-1} with R^2 value of 0.97.

Both assays gave a linear range able to detect 1 to 20 µg L^{-1} of AFB_1concentration.

Conclusions

Data indicate that the electrochemical immunosensor using screen printed gold electrode based on indirect and direct competitive ELISA format were suitable for AFB_1 analysis at the level required by the regulation (1 to 20 ppb).

Acknowledgment

The authors would like to thank the Malaysian Agricultural Research & Development Institute (MARDI) for funding this research degree and DuPont UK Limited (the Microcircuit Materials) for collaborative work in providing the gold screen printed sensors.

Appendix 5

MICROSENSOR ARRAYS FOR MYCOTOXIN DETECTION

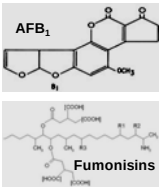
M. A. Kadir[1], D.W.M. Arrigan[2] and I.E. Tothill[1]*

[1]Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK
[2]Tyndall National Institute, Lee Maltings, University College, Cork, Ireland.

Introduction

Mycotoxins are toxic fungal metabolites that occur in food and feed products due to fungal contamination. The toxins are chemically and structurally diverse and stable and hence pose a threat to human and animal health. Concerns regarding their high toxicity resulted in the U.S. Food and Drug Administration (FDA) and the European Union (EU) strict regulatory limits on the permissible levels in food. Significant emphases have also been placed in devising sensitive and rapid diagnostics methods for risk assessment and management. Hence, the need for rapid and sensitive methods that is able to analyse a range of these toxins at the required detection limits.

The project focuses on the development of affinity based microsystems for the detection of Aflatoxin B_1 and Fumonisins. Electrochemical microsensor arrays were designed and fabricated by photolithography and characterized using electrochemical approaches for both analysis.



Electrochemical Microsensor

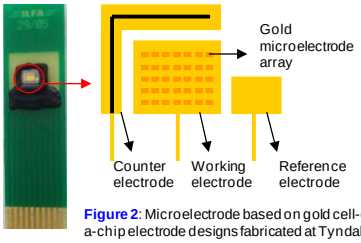


Figure 2: Microelectrode based on gold cell-on-a-chip electrode designs fabricated at Tyndall.

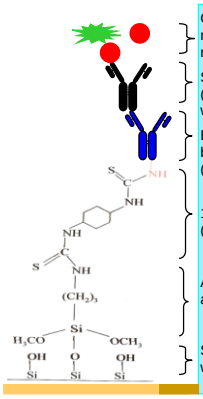
An electrochemical immunosensor system based on gold cell-on-a-chip microelectrode (including on chip reference and counter electrodes) were designed and fabricated by photolithography at Tyndall National Institute (Figure 2).

The microelectrode consisted of a gold microelectrode array working electrode (35 microsquare electrode with 20 um x 20um, 200um spacing) with a reference (0.4 mm x 0.4 mm) and counter (100 um L-shape) electrode.

In this study, surface modification (working electrode) based on an amino silane anchor (3-aminopropyltrimethoxysilane and PDITC (1,4-phenylene diisothiyanate) cross-linker for covalent binding of direct competitive ELISA format for AFB_1 and fumonisins detection were performed.

Signal generation was through the use of TMB/H_2O_2 as the enzyme substrate system and the current recorded at 150 mV. The current is proportional to the amount of HRP conjugate bound on electrode surface.

Immunoassay development



- Competition binding within free mycotoxins and conjugated mycotoxins with HRP
- Second coating with 1º antibody (monoclonal antibody) after block with 1% PVA
- Binding with 2º antibody before adding NH_4OH (deactivated reactive sites)
- 1,4-phenylene diisothiyanate (PDITC) cross-linker
- Amino silane anchor (3-aminopropyltrimethoxysilane)
- Silicon surface treated with oxygen plasma

Figure 3: Schematic of surface modification based on an amino silane anchor and PDITC cross-linker and binding with ELISA

Surface modification with amine group on protein to improve covalent binding and increase of biomolecule recognition amount on surface.

Immunosensor

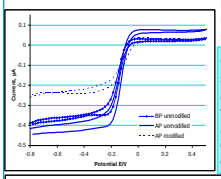


Figure 4: Cyclic voltammetry of modified and unmodified surface before and after oxygen plasma using 5 mM ferrocyanide in 0.1KCl at 40 mVs^{-1}.

CV redox peaks potential was reduced after surface modification, showed that attachment of amino silane achor and PDITC cross linker are taking place on gold surface. However, the adsorption of the molecules still permitted the diffusion of electrons which is showed current can be detectable.

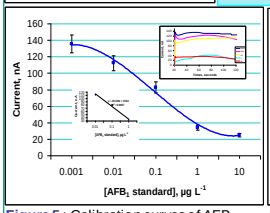


Figure 5 : Calibration curves of AFB_1 solution using microelectrode by chronoamperometry detection.

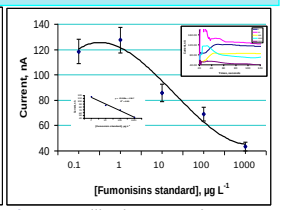


Figure 6 : Calibration curves of Fumonisins solution using microelectrode by chronoamperometry detection.

For both detection, the readings decreased from low to high concentration of AFB_1 and Fumonisins declined steadily after 1 and 1000 µg L^{-1}, respectively. Greater response with improve sensor signal (displaying high in range between each concentration). The linear range from 0.001 to 1 µg L^{-1} with R^2 value, 0.991 for AFB_1 and from 1 to 1000 µg L^{-1} with R^2 value, 0.99 for fumonisins. That is indicate good sensitivity toward mycotoxins.

Conclusions

Data indicate that the electrochemical immunosensor using modified microelectrode binding with direct competitive ELISA format were suitable for AFB_1 and fumonisins analysis at the level required by the legislation (1 to 20 and 2000 to 4000 ppb, respectively).

Acknowledgment

The authors would like to thank the Malaysian Agricultural Research & Development Institute (MARDI) for funding this research degree and Tyndall National Institute for collaborative work in providing the microelectrode sensors.

Appendix 6

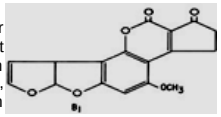
The Application of Nanoparticles in the Development of an Electrochemical Immunosensor for Aflatoxin B_1

M. A. Kadir[1] and I.E. Tothill[1]*
[1]Cranfield University, Cranfield Health, Cranfield, Bedfordshire, MK43 0AL, UK

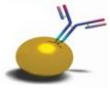
Introduction

Mycotoxins contamination in foods is of global concern due to their potential toxicity, carcinogenicity and their immunosuppressive effect on mammalian systems. This has led to concern being expressed for the detrimental effect Aflatoxin B_1 (AFB_1) can have on human health. AFB_1 is known to be the most predominant and toxic mycotoxin produced by fungi. Aflatoxin B_1 sensing techniques must therefore be able to detect this compound with high sensitivity, selectivity and accuracy. Current analysis is generally based on the use of conventional methods such as High Performance liquid Chromatography (HPLC), which are laboratory based, difficult to perform on- site and expensive. Hence, a need exits for an alternative method that is rapid, portable and exhibit the sensitivity and low detection capability of conventional methods.

This research focuses on the development of an immuno-gold nanoparticle enzyme complex and its use as the detection reagent in combination with a gold screen-printed working electrode for aflatoxin B_1 detection.



Structure of AFB_1



Immuno-gold nanoparticle

Electrochemical Immunosensor System

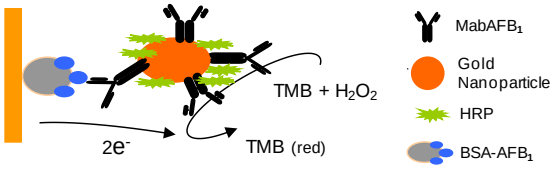


Figure 1: Schematic diagram of the detection system used for AFB_1 analysis. The assay is based on an indirect ELISA format on the SPGE surface.

The BSA-AFB_1 conjugate was immobilised on the surface of the screen-printed gold working electrode (SPGE) using passive adsorption. The competition between free AFB_1 in the sample and the immobilised AFB_1 was carried out in the presence of gold nanoparticle conjugated to monoclonal anti-AFB_1 antibody and HRP (Figure 1).

Electrochemical signal generation was through the use of TMB/H_2O_2 as the enzyme substrate system. The current produced was amplified using immuno-gold nanoparticle-HRP conjugate at -100 mV vs. onboard Ag-AgCl pseudo-reference electrode. The current is inversely proportional to the amount of toxin in the sample.

Results

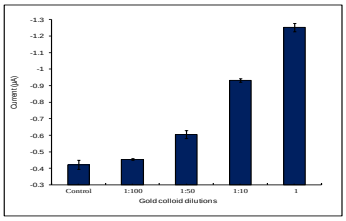


Figure 2: The current signal achieved using different concentrations of gold nanoparticles conjugate applied in an indirect non competitive assay.

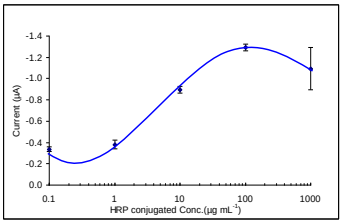


Figure 3: The current signal of different HRP concentrations conjugated to gold nanoparticles in indirect non competitive assay

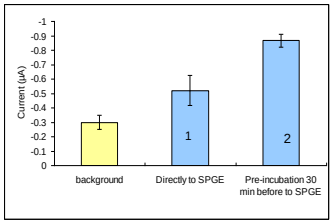


Figure 4: Current response of indirect competitive assay; 1) no pre-incubation; 2) with 30 minute incubation with the sample.

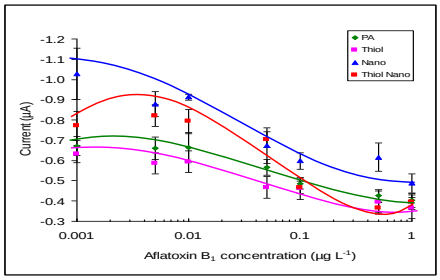


Figure 5: Comparison of Calibration curve of AFB_1 by indirect competitive format using with and without immuno-gold nanoparticle.

Figure 2 and 3 show the optimisation results of the immuno-gold nanoparticles conjugated HRP used in developing the sensor assay on the SPGE. The high current response was obtained from undiluted gold colloid with optimal HRP concentrations conjugated to immuno-gold nanoparticle was found to be at 100 µg mL^{-1}.

Figure 4, show the results achieved when using a sample pre-incubation step with the immuno-gold nanoparticle conjugated HRP (30 minute) before applying to the sensor surface. A one fold increase in the signal/background ratio was achieved when compared to sample analysis without a pre-incubation step.

Figure 5, show that the assay sensitivity increased by using immuno gold nanoparticle achieving a detection limit of (<0.005 µg L^{-1}) compared to assay without gold nanoparticle (>0.005 µg L^{-1}). The results also showed that antibody immobilisation on the sensor surface by physical adsorption showed higher signal when compared to antibody immobilisation by SAM thiol monolayer.

Conclusion

Data indicate that the electrochemical immunosensor sensitivity increased when using immuno-gold nanoparticle conjugated HRP applied to an indirect competitive assay. This sensor is suitable for AFB_1 analysis at levels below the required EU legislative limit (1 to 20 µg L^{-1}).

Acknowledgment

The authors would like to thank the Malaysian Agricultural Research & Development Institute (MARDI) for funding this research degree and DuPont Microcircuit Materials (UK) for collaborative work on the SPGE.

Appendix 7

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Article

Development of an Electrochemical Immunosensor forFumonisins Detection in Foods

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Abstract: An electrochemical affinity sensor for the determination of fumonisins mycotoxins (Fms) using monoclonal antibody modified screen-printed gold electrode with carbon counter and silver-silver chloride pseudo-reference electrode is reported in this work. A direct competitive enzyme-linked immunosorbent assay (ELISA) was initially developed, exhibiting a detection limit of 100 μg·L^{-1} for fumonisins. This was then transferred to the surface of a bare gold screen-printed electrode (SPGE) and detection was performed by chronoamperometry, monitoring the reaction of 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB) and hydrogen peroxide (H_2O_2) catalysed by HRP at −100 mV potential *vs.* onboard Ag-AgCl pseudo-reference electrode. The immunosensor exhibited detection limit of 5 μg·L^{-1} fumonisins with a dynamic range from 1 μg·L^{-1}–1000 μg·L^{-1}. The sensor also performed well in extracted corn samples.

Keywords: fumonisins; mycotoxins; screen-printed gold electrode; electrochemical Immunosensor

1. Introduction

In recent years, fumonisins which are mycotoxins produced by a variety of fungi of the *Fusarium* genus have become one of the most widely researched areas of mycotoxins contamination in food. These toxins were first isolated from a culture of *F. verticillioides* by Gelderblom and co-workers [1].

Fumonisins are considered as natural contaminants of cereal grains worldwide and are mostly found in corn and corn product [2–5]. The major compounds of fumonisins are B_1 (FB_1) and B_2 (FB_2), with more than eleven structurally related compounds has been discovered to date. However, fumonisin B_1 is the most abundant and toxic of this family of mycotoxins. Fumonisins have been linked with induce equine leukoencephalomalacia in horses and other equids [6], pulmonary edema in swine and pigs [7] and esophageal cancer in humans [8,9]. Also according to Wang *et al*., [10], fumonisins have been linked with various diseases associated with liver and kidney toxicity and carcinogenicity and immunosuppression. Therefore, the US Environmental Protection Agency (EPA) classified fumonisins as category 2B carcinogens [11]. The U.S. Food and Drug Administration (FDA) have recommended a maximum level of fumonisins (2–4 mg L^{-1}) on the hazard of animal studies for the protection of human consumption. As evidence mounts worldwide implicating fumonisins in human and animal diseases, increasing efforts to develop sensitive, selective, rapid and versatile procedures for detecting fumonisin levels in foods have emerged.

Normally, a program of monitoring and surveillance of fumonisins level is therefore necessary to ensure safe exposure, especially in the food and feed supply. Therefore, any analytical method used must be able to detect fumonisins at least down to the specified regulatory level. Fumonisins detection techniques are typically based on chromatographic separation, quantification and identification. This includes methods such as thin-layer chromatographic (TLC) [12], liquid chromatography (LC), liquid chromatography - mass spectrometry (LC-MS) and post-hydrolysis gas chromatography (GC) and. GC-MS. All of the methods above require sample extraction and clean-up steps before the analysis [13,14]. However, some of these methods are very sophisticated, expensive, time consuming and are generally unsuitable for rapid, on site or wide–scale monitoring programs. Also many chromatography methods used for mycotoxins require a form of chemical derivatization of the sample before detection is possible (with the exception of using mass spectrometry).

The growing number of mycotoxin to be controlled worldwide requires that rapid and reliable methods must become available for risk assessment and management of foods and feeds and to comply with the legislation. Because of the necessity, a number of fumonisin antibodies have been developed to allow for a rapid analysis by immunoassay techniques. Therefore, immunoassay methods for fumonisins analysis using polyclonal and monoclonal antibodies have been developed in the past two decades because of their, simplicity and selectivity [11]. Hence, on- site immunochemical techniques such as dipstick [15] immunochromatography [16], immunofiltartion [17], enzyme–linked immunosorbant assays (ELISA) and immunosensor techniques [18,19] are gaining interest for mycotoxin detection.

Nowadays, immunosensor techniques with different sensing receptors and transducers are considered as a major development in screening methods for use in fumonisins determination. The possible advantages of immunosesnor over conventional ELISA methods are; an increase in sensitivity and decrease in low detection limit; cost effective simple to use, decrease amount of expensive reagent and portable devices with digital signal outcome [20]. In case of fumonisins detection, many immunosensors have been reported using optical sensing principle such as surface plasmone resonance [18] and fiber optic [21–23]. The lower detection limit of FB_1 obtained were 50 $\mu g \cdot L^{-1}$ [21] and 10 $\mu g \cdot L^{-1}$ [23] using SPR and fiber optic immunosensors respectively. However, there are no reported literatures on the use of screen- printed electrodes (SPE) for fumonisins detection.

In this paper, we report on the development of an electrochemical immunosensor for fumonisins detection using a screen- printed gold electrode (SPGE). The sensor is as an attractive alternative to immunoassay techniques and also to the common use of carbon electrode in biosensors developments. Electrochemical immunosesnors have been proven to be very sensitive analytical tools obtaining low detection limits and offer reduced instrumentation costs compared to their optical counterpart [24]. The developed sensor is based on a competitive reaction between free fumonisins in the sample and a fumonisin- horseradish peroxidase conjugate, for an immobilised monoclonal anti- fumonisins antibody. Chronoamperometry was used as the electrochemical detection method for the signal generated by the use of TMB/H_2O_2 to ascertain the concentration of HRP on the sensor and consequently the concentration of fumonisins in the sample. The immunosensor was optimised regarding the immobilization of the ELISA reagents on the surface of the gold electrode. The optimum conditions were then employed to monitor the concentration of fumonisins in buffer solutions and then in extracted samples. The electrochemical detection involves chronoamperometry and the uses a TMB/H_2O_2 substrate catalysed by horseradish peroxidase has been widely used with screen- printed immunosensors [25–28].

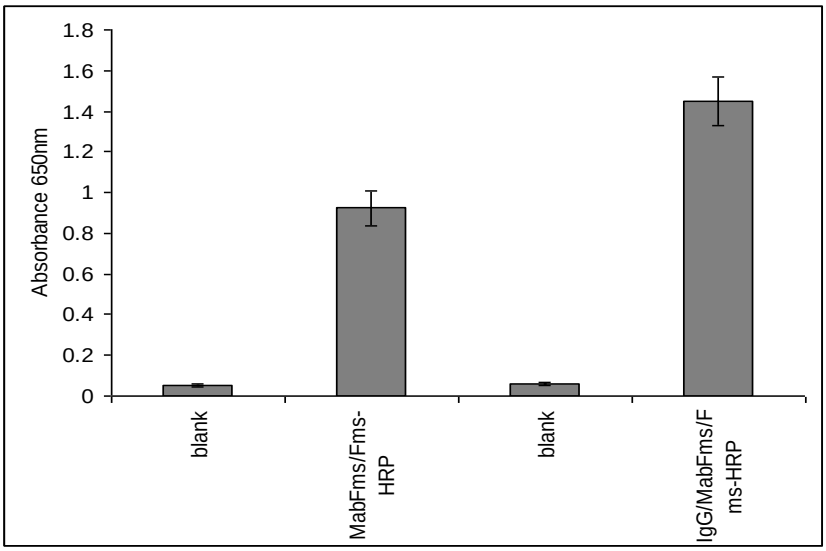
2. Results and Discussion

2.1. Optimization of the ELISA method

A spectrophotometric competitive enzyme-linked immunosorbent assay (ELISA) for fumonisins detection was first developed and optimized before moving the assay to the electrochemical transducer. The tests were performed in a microwell plate based on direct method. A checkerboard titration method was used to optimize the reagents concentrations (coating antibody, monoclonal antibody and fumonisin- HRP conjugate) followed by optimization of the assay conditions (incubation times and temperatures). The use of anti-IgG to pre-coat solid faces surfaces before immobilising the anti-capture antibody has been reported to increase the detection limit of aflatoxin M_1 [29]. Therefore, to maximize the ELISA signal the use of pre-coated microtitre wells was investigated in this study. The results achieved (Figure 1), show that the use of a pre-coated wells produced a much greater signal than non-coated wells.

The increase in the signal indicates a better binding orientation of the antibodies in the assay and therefore it was applied in future tests and sensor development. The use of 1% PVA in PBS as a blocking solution in the assay produced the optimal results for non-specific binding when compared to other blocking agents (Bovine serum albumen or Gelatin) and therefore, it was used in further tests (data not shown). These results were similar to those reported by Warwick [30]. The optimal concentrations and conditions for the ELISA assay using the direct competitive format were found to be; pre-coat the wells with Anti-IgG antibody (20 $\mu g \cdot mL^{-1}$, overnight, 4 °C); block using 1% PVA in PBS (1 hour); immobilize the monoclonal anti fumonisins on the coated and blocked surface(1:50 dilution, 2 hours at 37 °C) and lastly the use of a fixed amount of Fms-HRP (1:10 dilution, 30 minutes, 37 °C).

Figure 1. Comparison of different immobilization protocols of the monoclonal anti-fumonisin in a direct non competitive format using either non-coated (immobilized monoclonal anti-fumonisins, 1:50 $\mu g \cdot mL^{-1}$, blocked with 1% PVA) or pre-coated wells(immobilized anti-antibody IgG, 20 $\mu g \cdot mL^{-1}$, blocked with 1% PVA then monoclonal anti-fumonisins, 1:50 $\mu g \cdot mL^{-1}$) before continue with Fms-HRP from Veratox kit (1:10). Error bar=SD, n=3.



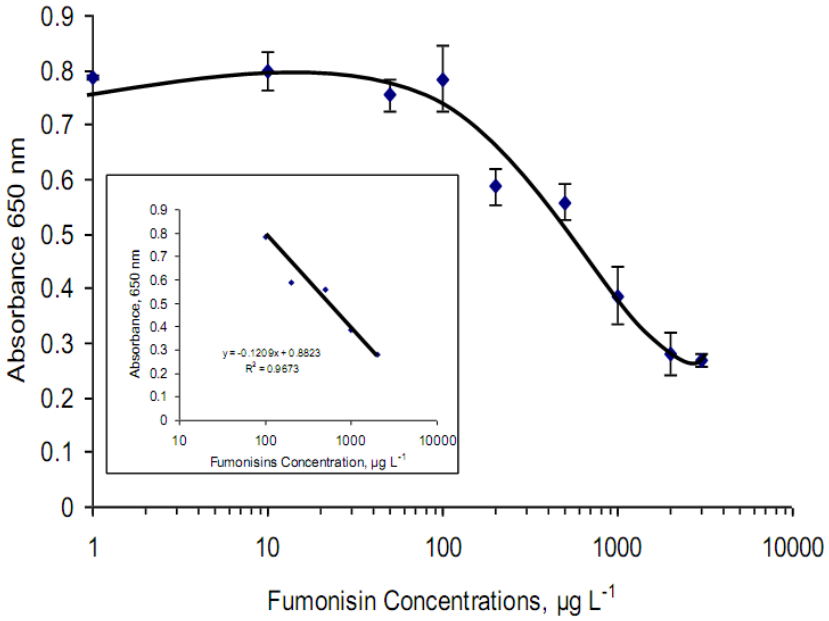
Note: IgG: anti-IgG antibody; MabFms: monoclonal antibody against fumonisins; Fms-HRP: fumonisin conjugated HRP.

Utilizing the derived optimal concentrations and conditions for the competitive assay, a calibration curve for fumonisins was then carried out with free (0–3000 $\mu g \cdot L^{-1}$) and HRP labelled fumonisins (Fms) in buffer, mixed and added to the wells where they compete for the anti-fumonisin active sites (Figure 2). The calibration curve was fitted using 'non-linear regression plot' [31]. A dynamic range from 100 to 2000 $\mu g \cdot L^{-1}$ (R^2 = 0.967) with an LOD of ~100 $\mu g \cdot L^{-1}$, CV = 9.3% was achieved using the linear section of the curve to calculate the data.

2.2. Optimization of the immunosensor

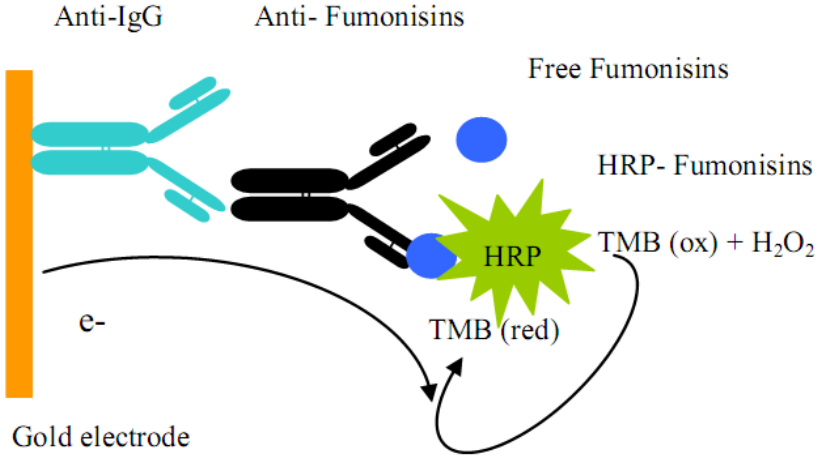
For the immunosensor development an electrochemical transducer was chosen for the detection system, since no previous literatures have been reported for fumonisin analysis using an electrochemical immunosensor. Recent literature have shown the use of electrochemical immunosensor for a range of mycotoxin detection such as aflatoxin B_1 [32], aflatoxin M_1 [26,29] and ochratoxin A [33] with good detection limits below the required European Union (EU) legislation.

Figure 2. Standard curves for the detection of fumonisin using a spectrophotometric ELISA assay. Signals were obtained using a pre-coated wells with anti-antibody IgG (20 $\mu g \cdot mL^{-1}$), blocked with 1% PVA then anti-fumonisin antibody (MabFms) (1:50 dilution) before continue with competition Fms-HRP from Veratox kit (1:5) and free Fms (0 to 3000 $\mu g \cdot L^{-1}$). Error bar = standard deviation, n=3.



Therefore, an electrochemical immunosensor system was developed in this work by transferring the ELISA assay to the gold screen-printed electrode surface, where the horseradish peroxidase (HRP) enzyme label activity is detected using chronoamperometry. TMB/ H_2O_2 were chosen as the mediator /substrate system. (See Schematic diagram below).

Scheme 1. Caption.

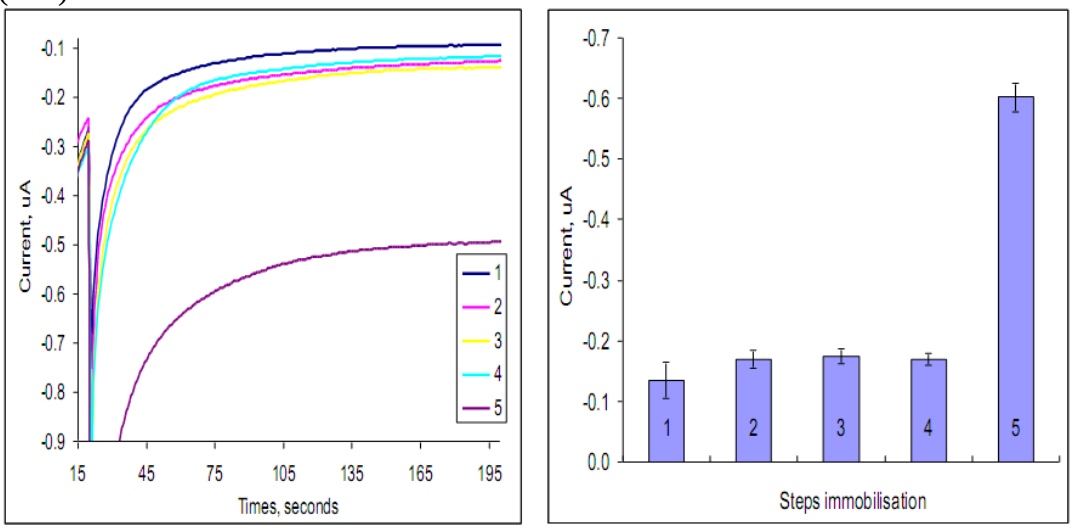


According to Parker & Tothill, [26] and Volpe *et al*., [34] the use of TMB as the mediator with H_2O_2 has greater electrochemical detection properties than other mediators system used with HRP enzyme. Therefore, in order to obtain the optimum applied potential, chronoamperometry detection for HRP using TMB/H_2O_2 substrate was investigated for the DuPont gold screen-printed electrode. Our previous work [25] has shown that the optimal potential using the same substrate /mediator system with an Ercon (Inc. USA) gold screen-printed electrode (R-464 (DPM-78)) was at −200 mV versus Ag/AgCl. Therefore, selecting the correct potential to use with the DuPont gold ink (BQ331 gold) on the screen-printed electrode was also investigated. The technique was evaluated by the ratio of the signal (S) current to background (B) at a constant step potential range from −600 to +600 mV. The results (data not shown) indicated that the maximum value of signal/ background ratio for the best potential

was at −100 mV. Therefore this potential was selected for the electrochemical detection system using the DuPont gold ink as the working electrode.

The effect of the different immobilization steps taking place for fumonisins detection on the electrochemical signal as a background and assay signal was also investigated. Figure 3, show the signals achieved from; (1) gold bare electrode; (2) pre-coating of anti-antibody IgG (anti-IgG); (3) anti-IgG, blocking with PVA and MabFms (anti-IgG/PVA/MabFms); (4) anti-IgG/PVA/Fms-HRP and (5) anti-IgG/PVA/MabFms/Fms-HRP. This is an important characterization step to evaluate the performance of different reagents adsorption as well as to study the effect of the adsorbed reagents on the electron transfer characteristics of the sensor. In this study TMB/H_2O_2 was also used as the enzyme substrate for the measurement of the enzyme activity on the electrode surface at a potential −100 mV. Figure 3, indicate that the reagents immobilization did increase the background signal slightly but had a minimal effect on the electrochemical characteristic of the sensor and was able to detect the signal achieved from the activity of the enzyme label at high sensitivity. According to Fu, [35] conducting this type of test is important to ensure that the enzyme label is still active and the electrode surface is not blocked due to the chemical modification of the sensor surface.

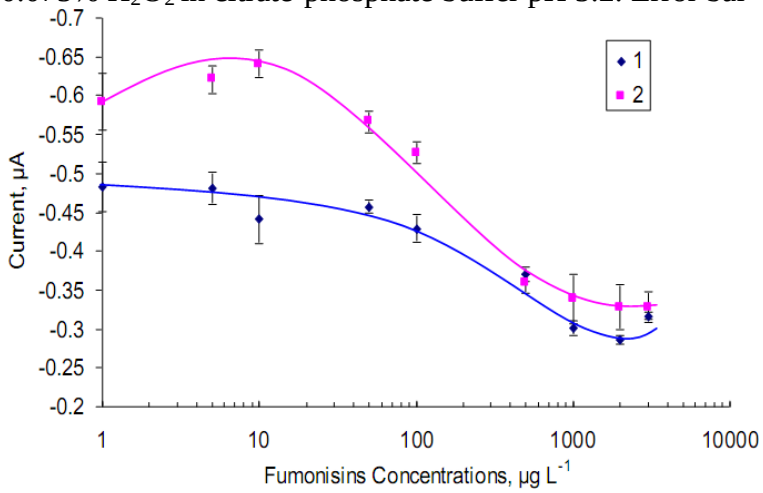
Figure 3. Chronoamperometric response of the different immobilisation steps of direct non competitive format using different gold electrode including 5 steps; (1) bare electrode,(2) Anti-IgG, (3) Anti-IgG/PVA/MabFms (4) Anti-IgG/PVA/Fms-HRP and (5) Anti-IgG/PVA/MabFms/Fms-HRP using TMB/H_2O_2 as the enzyme substrate (n=3).



A standard curve was then conducted on the screen-printed gold electrode similar to that used for the ELISA assay. The competition system was employed within free fumonisins concentrations at different range (0 to 3000 µg·L^{-1}) and Fms-HRP (1:10) through optimal condition and concentrations of reagents as described previously. The standard curves of competition binding of antigen by two different assay protocols were obtained as shown in Figure 4. The first protocol gave a working range of 50 to 2000 µg·L^{-1} and LOD at ~50 µg·L^{-1}, when free and labelled fumonisins were mixed together and incubated on the working electrode surface. The second protocol, a pre-incubation step was introduced (30 minutes) for the free fumonisin in the sample on the electrode surface before the addition of the HRP fumonisins conjugate. A working range of 10 to 1000 µg·L^{-1} and an LOD of ~ 10 µg·L^{-1} were achieved using this

second assay system. The current response is inversely proportion to the fumonisin concentrations. The second method of analysis showed an increase in the assay sensitivity with a lower LOD (CV = 9.6%). Therefore, this method was used in further assays. However, two point's needs to be considered here, the first is that this method adds an extra step to the assay protocol and the second is that by changing the incubation time and optimize it further the method without the pre-incubation step (1, Figure 4) may prove to be adequate for fumonisin analysis at the required legislative limit. The data shown in Figure 4 were carried out with two different sets of sensors. The pre-incubation tests were conducted using newly fabricated sensors, and therefore they show a higher current density. These data suggest that the reliability of the electrochemical immunosensor with the direct competitive ELISA format were suitable for fumonisins analysis at the level much lower than that required by the EU legislation (2000 to 4000 $\mu g \cdot L^{-1}$).

Figure 4. Standard curve of electrochemical competitive immunoassay on the screen-printed gold electrode for fumonisin detection: (1) the curve by direct mixing of free Fms and Fms-HRP on the gold working electrode surface; (2) the curve via a pre-incubation by adding the free Fms for 30 min before the Fms–HRP conjugate. Gold working electrodes were pre-coated with Anti-IgG antibody (20 $\mu g \cdot mL^{-1}$) and coated with MabFms (1:50 dilution) before competitive binding. The chronoamperometry, applied potential: −100mV; chromogen/substrate of HRP: substrate; 5mM TMB + 0.075% H_2O_2 in citrate-phosphate buffer pH 5.2. Error bar = standard deviation, n=3.



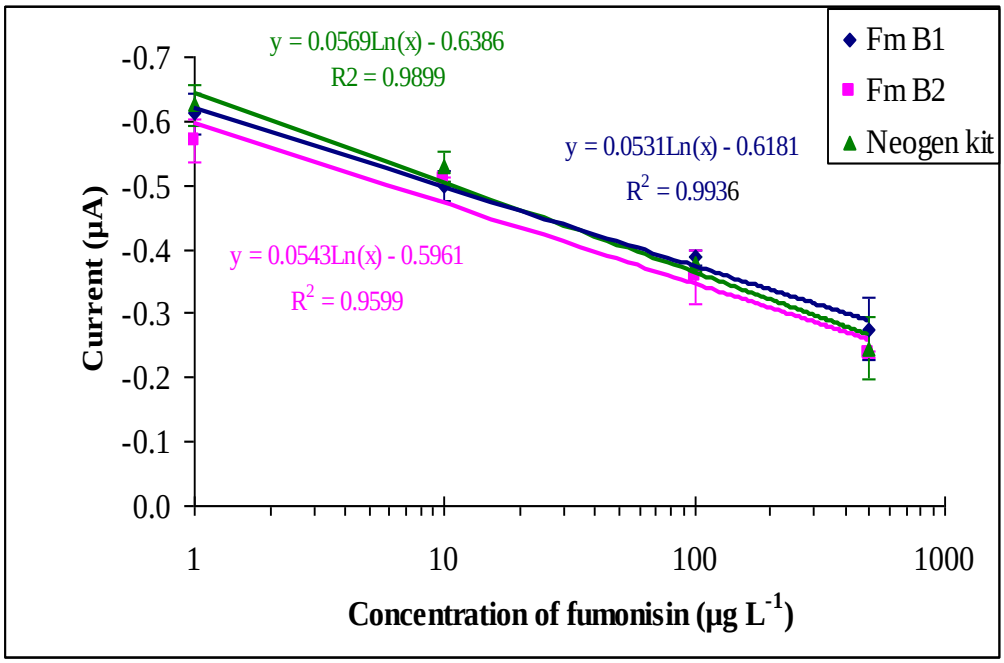
2.3. Cross-reactivity study

The specificity of the developed immunosensor for the detection of Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2) was investigated in this system. The purpose of the test is to determine the specificity of the sensor for these two structurally related toxins (Figure 5).

Figure 5, indicate that the monoclonal anti-fumonisins used in the development of the immunosensor had similar specificity for both toxins and showed similar cross-reactivity for the two structurally relater toxins. The reactivity of MabFms with different fumonisin analogs provides an insight as to the antibody ability to bind to fumonisins toxins. A standard fumonisin solution provided by the Neogen ELISA kit was also tested using the sensor and sensor response to this solution was also compared to response achieved using FB1 and FB2 toxins. Furthermore, the sensor was also tested for the detection of aflatoxin B_1 and Ochratoxin A, which are non structurally related toxins, and gave negative results which are below the 1% cross

reactivity (data not shown). This indicates the specificity and sensitivity of the developed sensor for fumonisins detection and analysis.

Figure 5. Cross reactivity of MAbFms against the standard solution of fumonisin provided in the Neogen Elisa kit, FmB_1 and FmB_2. Direct competitive of Fms immunosensor response on a modified gold working electrode by chronoamperometry at potential −100 mV and using a mixture of TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Screen printed gold electrode were coated with anti-IgG (10 µg·mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continue with Fms (1–1000) for 30 minutes pre-incubation times before adding of Fms-HRP (1:10). Error bar = standard deviation, n=3.

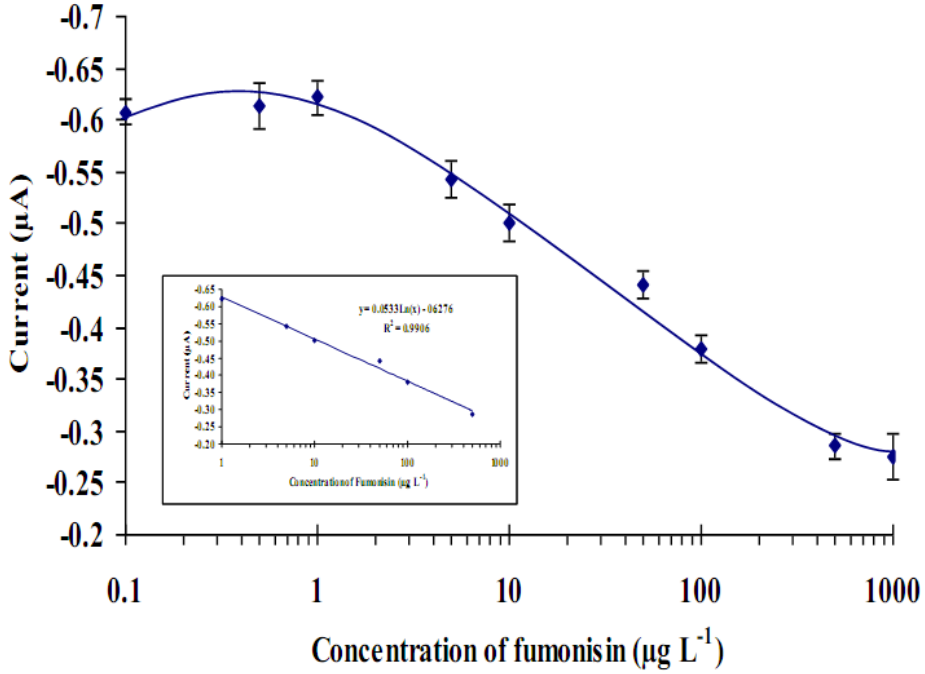


2.4. Optimized immunosensor calibration curve

The calibration curve for the immunosensor was conducted using all the optimized parameters for the competitive reaction on the gold sensor surface with a low range of fumonisins standard solution (0.01 to 1000 µg·L^{-1}) and a 30 minutes pre- incubation step (toxins solutions incubated on the monoclonal antibody immobilized on a thiol modified gold surface). The results are shown in Figure 6.

Figure (6), show that the decrease in current response was proportional to the fumonisin concentration in the range of 1 to 500 µg·L^{-1}. The linear regression equation is µA = 0.053 × $C_{[Fms]}$ – 0.63 with a limit of detection (LOD) of ~5 µg·L^{-1} (estimated to be 3× the standard deviation of blank-dose signal, n = 3, R^2 = 0.991). In this case, the LOD obtained in this final optimized assay system with the pre-incubation step was lower than reported previously. This sensor proved to be very sensitive and for low level of fumonicins detection in buffer solutions. Using this device and technique with highly contaminated food samples (above 500 µg·L^{-1}) may require the sample to be diluted to obtain the toxin concentration.

Figure 6. Calibration curve of direct competitive immunosensor for fumonisins analysis on thiol modified gold electrode surface. Measurements conducted by chronoamperometry at −100 mV, TMB (5 mM) and H_2O_2 (0.075%) as a substrate. Modified screen- printed gold electrode were coated with anti-IgG (10 μg·mL^{-1}), blocked with 1% PVA followed by MAbFms (1:50) then continue with Fms (0.01–1000) for 30 minutes pre-incubation time before adding the Fms-HRP (1:5). The curve was fitted by non linear regression, with the insert showing the liner section of the curve. Error bar = SD, n = 3.



2.5. Sensor response in extracted corn samples

To test the performance of the sensor in real sample matrix and conduct a recovery test for fumonisin, ground corn samples were spiked with various concentrations of mixed FB_1 and FB_2, The samples were first extracted using methanol/water mixture (70:30) and then either first used without any further treatment or the second were cleaned and concentrated using a clean-up procedure (C-18 SPE column). Samples were then dried and resuspended in PBS buffer and quantified using the developed electrochemical immunosensor assay. Other none spiked corn samples were also extracted and determined in order to evaluate possible matrix effect compared to fumonisins in buffer samples. For non spiked samples background signal, the current archived was found to be similar to the signal normally achieved in buffer (data not shown). This indicates that there is minimum matrix effect on the background electrochemical signal of the immunosensor by following the developed procedure. In spiked corn samples, the recovery of fumonisins was determined from the analyzed concentrations of fumonisins in the corn samples, compared to the expected values spiked to the sample. Using a calibration curve of fumonisin in a non spiked corn extract and C-18 cleaned samples, the obtained recovery results for spiked samples were calculated and these are shown in Table 1.

Table 1. A comparison results of corn samples spiked with mixed Fumonisins (FB_1 and FB_2), and using two different extraction methods, the samples were analysed using the electrochemical immunosensor.

Spiked Corn (µg kg^{-1})	Without clean-up			Clean-up using C18		
	Found (µg kg^{-1})	% R	SD	Found (µg kg^{-1})	% R	SD
Non Spiked	< 1		6.12	<1		4.08
50	25.05	60.1	9.43	50.85	101.7	7.6
250	144.65	69.86	5.54	190.1	76.04	8.49
500	410.9	82.18	5.96	560.3	112.06	4.61
2500	2055.8	82.23	3.61	2616.05	104.64	3.87

When the two different extraction procedures were compared in terms of recovery, different percentage recovery were found. The average of recovery result for the extraction method without a clean-up step (rapid extraction technique) was much lower than the average recovery with a clean-up and concentration step (using C-18 SPE) (Table 1). The lower concentration of spiked fumonisins (50 and 250 µg kg^{-1}) obtained less recovery compared to higher concentration (500 and 2500 µg kg^{-1}). This may suggest that substances in the samples containing low fumonisins inhibited the toxin signal response. By using a clean-up procedure (C-18 SPE column) which will help in removing these substances, the recovery of fumonisins have increase due to their removal from the sample extract. According to Muscarella *et al*. [36], and in agreement with regulation 401/2006/EC, that recovery in the range of 60–120% for fumonisins is expected for samples containing ~500 µg kg^{-1}. From the achieved results conducted in this study, the use of a clean-up procedure is recommended to improve fumonisins recovery especially at the low contamination level. Also conducting a standard curve for fumonisins using unspiked corn samples extracted and cleaned using C-18 gave very comparable results to fumonisin in buffer analysis (data not shown). The performance of the immunosensor and the results achieved from analysing the corn samples indicate its useful application for the analysis of fumonisins in corn samples. This indicates the developed SPGE immunosensor using electrochemical detection is rapid and reliable for corn samples analysis.

3. Experimental Section

3.1. Reagents and solutions

Mouse monoclonal anti-fumonisin antibody (MabFms) was purchased from Abcam Ltd. (Cambridge, UK), fumonisin- HRP conjugate and a chromogen/substrate solution was from Veratox kit from Neogen Corporation (UK). Affinity purified, anti-mouse IgG (H+L) from goat was obtained from Pierce Ltd. (UK). Fumonisins standards (Fumonisin B_1 and Fuminisin B_2), polyvinyl alcohol (PVA), 30% hydrogen proxide (H_2O_2), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (powder), Tween 20, potassium chloride (KCl) and all other buffer reagents were purchased from Sigma-Aldrich Co. Ltd (UK). TMB solution for ELISA tests was purchased from Insight Biotechnology (UK). Organic solvents (methanol and acetonitrile) were HPLC grade, purchased from Fluka (UK). Sodium carbonate buffer (CB), 100 mM, pH 9.6, was prepared by dissolving Na_2CO_3 (1.59 g) and $NaHCO_3$ (2.93 g) in in 1 L distilled-deionised water and the pH adjusted to 9.6. Phosphate buffered saline (PBS), 10 mM, pH 7.4 was prepared by dissolving 5 buffer tablet in 1 L distilled-deionised water. PBS-T solution was prepared by adding 0.05% tween-20 (v/v) to PBS buffer.

Citrate–phosphate buffer, 50 mM, pH5.5 was prepared by dissolving one buffer tablet in 100 ml of distilled-deionised water. TMB substrate solution was prepared by dissolving 1 mg of TMB in 150 µL of distilled- deionised water. The Fumonisins (Fms) standard stock solutions were prepared by dissolving 1 mg in 1 mL methanol (HPLC grade) and stored at −18 °C in tightly capped and dark bottle. When needed the stock solutions were diluted in PBS, 10 mM, pH 7.4 to the concentration range of 0–3,000 $\mu g \cdot L^{-1}$ and used for ELISA and immunosensor tests.

3.2. Fabrication of screen-printed gold electrodes

Screen-printed gold electrodes (SPGE) consisting of gold working electrode, carbon counter electrode and silver–silver chloride pseudo-reference electrode were fabricated using a procedure similar to that described in details by Noh and Tothill [37]. The electrodes used in this work were printed using the screen-printing facilities at DuPont Ltd. (Bristol, UK). The printing pastes used were 107255-135E carbon, BQ331 gold, 5874 Ag/AgCl and 5036 blue encapsulant, all inks were from DuPont Ltd.). The SPGE used in this work, consisting of a gold working electrode with a 5 mm diameter giving a 19.6 mm^2 planar area, printed on a graphite ink layer (dried at 120 °C, 30 mins). All electrodes were then tested using a multimeter before use. The sensors edge connector was purchased from Maplin Electronics Ltd. (Milton Keynes, UK).

3.3. Immunoassay developments

ELISA tests were first developed using a micro well polystyrene plates, MaxiSorp (Nunc Immuno), purchased from Fisher Scientific (Loughborough, UK). An incubator/shaker HT from Labsystem iEMS were used for temperature control incubation of every step of the immunoassay reaction. Spectrophotometric analysis of colour developed was performed using a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, UK).

3.3.1. Direct immunoassay test

A checkerboard ELISA procedures were carried out to evaluate and optimise the concentrations of the assay reagents used in the test. Anti-mouse IgG (H+L) was first used to pre-coat the ELISA plate at a concentration range from 0 to 50 µg mL^{-1}. Anti-Fms (primary) monoclonal antibodies were immobilised on the pre-coated plates using a concentration range of 1:25 to 1:5000 dilutions) and Fms-HRP conjugate using a concentration range of 1 to 1:50 dilutions. The tests were performed in a 96-well microplate according with a direct ELISA format based on the method described by Anna *et al*., [38].

3.3.2. Competitive assay

The competitive assay was carried out using the following procedure; the microtiter plate was pre-coated with anti-mouse IgG antibody (20 µg mL^{-1}, 50 µL/well) in 0.1 M carbonate buffer pH 9.6, for 18 hours (overnight) at 4 °C, followed by washing twice with 150µl/well phosphate buffered saline containing Tween 20 (PBS-T) and once with PBS alone. The plate was then blocked with 1% PVA (50 µL/well) and incubated for 1 hour at 37 °C. After washing as above, the plate was coated with anti-Fms monoclonal antibody (1:50 dilution, 50 µL/well) in PBS (v/v) for 2 hours incubation at 37 °C, followed by washing. The competition solution was prepared by mixing (50 µL/well) of free fumonisin B_1 (0–3000 µg L^{-1}) in PBS (v/v) with fixed dilution of a solution of fumonisin-HRP conjugate (1:5 dilution) in PBS (v/v). The competition reaction was allowed to proceed for 30 minutes at 37 °C and

then rinsed with PBS-T (twice), followed by PBS (once). Finally, the absorbance was measured by the addition of TMB substrate solution (50 µL/well) to each well and incubated at room temperature for 15 minutes before added a 25 µL of stop reagent (H_2SO_4) and then measuring at650 nm using the plate reader.

3.4 Electrochemical immunosensor

3.4.1.Electrochemical measurements

Electrochemical procedures were conducted using a computer controlled four channel Autolab electrochemical analyser multipotentiostat (Eco Chemie, Utrecht, The Netherlands) throughout which allows the simultaneous detection of four sensors. Data capture was through the supplied GPES version 4.9 software installed onto a PC. The screen-printed electrodes were connected to the Autolab, using an in house fabricated connector from a PCB edged IDC socket, aluminum instrument box, ribbon cable and 4 mm cable sockets. The individual components were purchased from Maplin Electronics (Milton Keynes, UK). For the C.V. scans a 100 µL drop of 5 mM potassium hexacyanoferrate (III) in 0.1 M KCl was placed onto the electrode and each electrode was disposed of after each scan. The scanning range was from −0.3 to +0.8 V at a rate of 50 mVs^{-1} relative to the on board Ag-AgCl reference electrode. For samples analysis each measurements was carried out in triplicate using a new strip in a non-deaerated and unstirred solution. For the selection of optimal constant potential for the enzymatic reaction (TMB-H_2O_2-HRP) system, choroamperometry was conducted with bare screen-printed gold electrode with buffer solution (50 mM citrate-phosphate buffer, pH 5.5, in 0.1 M KCl), substrate (5 mM TMB, 0.07% H_2O_2) with fumonisin- HRP conjugate in citrate-phosphate buffer –0.1 M KCl. Step amperometry was conducted at a range of potential from +600 mV to −600 mV within 600s.

3.4.2. Direct competitive assay

A 10 µL of anti-antibody (anti-mouse IgG (H+L)) primary layer (capture species) in CB, pH 9.6 were placed on the gold working electrodes then kept overnight at 4 °C. A 20 µL of 1 % PVA (w/v) in PBS was then used to block the electrode surface for 1 hour at 37 °C. Then, 10 µL of anti-Fms antibody capture was coated on the electrode for 2 hours at 37 °C. The electrodes were then ready for fumonisins analysis using the competitive assay. A standard curve for fumonisin was then constructed on the gold electrode surface by mixing (v/v) fumonosin-HRP conjugate with free fumonisin in buffer (0–3000 µg L^{-1} range) and placing a 10 µL of this mix on the electrode surface for the competitive assay (incubation for 30 minutes at 37 °C). The electrodes were washed between steps by rinsing using PBS-T (twice) followed by PBS (once). A substrate solution was then placed on the electrode surface and the signals measured using amperometric analysis as described above.

3.4.3. Calibration plot and interpretation of result

ELISA and immunosensor calibration curves were fitted by non-linear regression using the following four parameter logistic function [31]:

$$F(x) = (a - d)/[1+(x/c)^b]+ d$$

Where parameter *a* and *d* are the asymptotic maximum and minimum value of the calibration curves, respectively, *x* the concentration at the EC_{50} value, *c* the analyte concentration and *b* is the hill slope. EC_{50} is an effective concentration for 50% value.

The limit of detection (LOD) was defined as the concentration of toxin equivalent to three times the value of standard deviation (σ). This was calculated based on the following equation:

$LOD = x\,[a - d/\,(a - d) - 3\sigma]^{-1/b}$

Where σ is the standard deviation of the zero value.

3.5 Corn samples analysis

3.5.1. Extraction without clean-up

Samples preparation and extraction were conducted following the procedure as described by the Veratox ELISA kit (Neogen Corporation, UK). A corn sample was first ground and then mixed thoroughly before adding different concentrations of fumonisins standard (spiked). A 5 gram of ground corn sample was used, mixed with 25 mL of 70% methanol and 30% water and then shaken vigorously for 3 minutes. The extract was filtered through a Whatman #1 filter paper to remove the solid material and the filtrate was then collected as the sample for analysis without further preparation.

3.5.2. Clean up using C-18 solid phase extraction (SPE)

For this procedure, samples extraction was conducted similar to the procedure described above. Sample filtrates were collected and cleaned using C-18 SPE (Waters, Milford, MA) following the procedure accompanying the SPE columns. The C-18 SPE column was first conditioned by sequentially passing 5 ml methanol and 5 mL water through the column. A 4 mL of sample filtrate was then passed through the column, followed by 6.0 mL deionised water. The fumonisins was then eluted from the SPE column by rinseing with 2.0 mL of methanol:water (70:30). The sample eluted was dried under nitrogen stream, re-dissolved in 0.01 M PBS pH 7.4 before 10 µL of samples used for the ELISA or immunosensor analysis.

4. Conclusions

An electrochemical immunosesnor based on gold working electrode using screen-printed sensor was developed for the detection and analysis of fumonisins in food samples such as corn.A monoclonal antibody against fumonisins was applied in this work in order to enable the analysis of fumonisins toxins (FB_1 and FB_2). A spectrophotometric immunoassay test was first developed in order to optimise the assay conditions and concentrations before moving the test on the surface of the electrochemical sensor. The final developed SPGE immunosensor exhibited a working ranges from 1000 µg L^{-1} and the LOD was ~5 µg L^{-1}. This is well below the detection limit required for EU legislation of 2–4 mg L^{-1} of Fms (level required by official European Union). The sensor was also examined in extracted and cleaned up (using C-18 SPE columns) corn samples and showed very low matrix interference, high sensitivity and reproducibility. We have demonstrated that a competitive immunoassay for Fms, on an electrochemical screen-printed sensor device is capable of detecting fumonisins in corn samples and therefore has a great potential for on-site and rapid analysis.

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Appendix 8

Submitted Paper 1

IMMUNOCHIP MICROSENSOR ARRAY FOR AFLATOXIN B_1 DETECTION IN PEANUT

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Abstract

The development of a microelectrode array (MEA) immunosensor for the detection of aflatoxin B_1 is presented in this paper. Gold MEAs consisting of 35 microsquare electrodes with 20µm x 20µm dimensions and edge-to-edge spacing of 200µm, together with on-chip reference and counter electrodes were fabricated using standard photolithographic methods. The MEAs were characterised by cyclic voltametric and the behaviour of the on-chip electrodes were evaluated. The microsensor arrays were assessed for their applicability to the development of an immunosensor for the analysis of aflatoxin B_1 (AFB_1) directly in peanut samples. The microarray sensor surface was then silanized and used to immobilise the antibodies by cross-linking with 1,4-phenylene diisothiocyanate (PDITC). An indirect competitive enzyme linked immunosorbent assay (ELISA) assay was then developed for aflatoxin B_1 on the electrode sensor surface using horseradish peroxidise (HRP) as the enzyme label coupled with chronoamperometric detection with 3,3-5',5'-tetramethylbenzidine (TMB)/H_2O_2 substrate system. The performance of the assay and the microsensor was characterised in pure buffer before applying in peanut extract. The approached method was showed the limit of detection of 0.001 µg L^{-1} for AFB_1, which meets the required legislative limit of analyses recommended by the EU (1-20 µg L^{-1}). The gold immunosensor of MEA system shows high performance to detect AFB_1 in peanut matrix with average recovery achievement was 85.7%, which is the minimal interference found. The sensor also designed for reusable application, after cleaning using piranha solution and O_2 plasma, the sensitivity of the assay reduced after three times uses. This sensor system is highly sensitive and rapid for on-site mycotoxins analysis.

Keywords: aflatoxin B_1; mycotoxins; microelectrode array; electrochemical immunosensor;

Appendix 9

Submitted paper 2

ELECTROCHEMICAL IMMUNO GOLD NANO SENSOR FOR AFLATOXIN B_1 DETECTION

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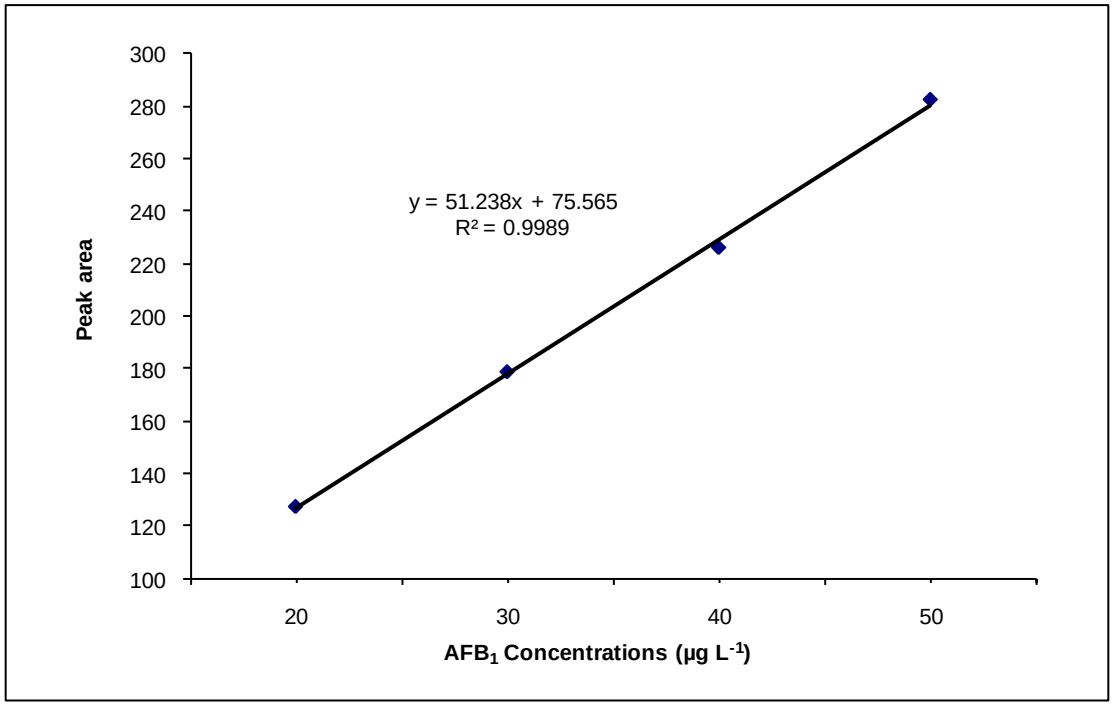
Abstract:

This paper reports on the development of an immuno-gold nanoparticle-enzyme complex and its use as the detection reagent in combination with a gold screen-printed working electrode for aflatoxin B_1 (AFB_1) detection based on electrochemical measurment. The detection reagent for electrochemical immunosensor was using combination of monoclonal anti-aflatoxin B_1 ($MAbAFB_1$) as an antibody sites and horseradish peroxidase (HRP) that conjugated to the nanoparticle. The competitive assay was conducted between the immobilized bovine serum albumine-AFB_1 (BSA-AFB_1) on the gold sensor surface and frees AFB_1 for the antibody sites in the immuno gold-nanoparticle enzyme system. Horseradish peroxidase was used as the enzyme label with chronoamperometric detection carried out by using 3,3,5'5'-tetramethylbenzidine dihydrochloride/hydrogen peroxide (TMB/H_2O_2) substrate system at -100 mV constant potential. The immuno gold nanoparticle sensor with the 3,3-dithiodipropionic acid (DTDPA) SAM thiol monolayer on the gold electrode showed great sensitivity with a detection limit (LOD) of 1 ng L^{-1} (ppt) achieved for AFB_1. The achieved detection range for AFB_1 was within the required legislative limit of analyse. This nanoparticle based electrochemical imunosensor show enhanced sensitivity for mycotoxins detection.

Keywords: aflatoxin B_1; immuno gold nano-particle; electrochemical immunosensor; nano-particle.

Appendix 10

Standard Curve of Aflatoxin B_1 (HPLC)



Standard Curve of total fumonisins (HPLC)