
**UNIVERSITI PUTRA MALAYSIA**

# *DEVELOPMENT OF A NANOMATERIAL-BASED ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF AFLATOXIN B1 IN PEANUTS*

**FARAH ASILAH BINTI AZRI**

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# DEVELOPMENT OF A NANOMATERIAL-BASED ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF AFLATOXIN $B_1$ IN PEANUTS


**By**

**FARAH ASILAH BINTI AZRI**


**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science**

**March 2016**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**DEVELOPMENT OF A NANOMATERIAL-BASED ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTION OF AFLATOXIN $B_1$ IN PEANUTS**

By

**FARAH ASILAH BINTI AZRI**

**March 2016**

**Chairman : Professor Fatimah Abu Bakar, PhD**
**Faculty : Food Science and Technology**

Aflatoxin $B_1$ is epidemiologically implicated as carcinogen in humans due to contamination in several agricultural commodities either pre- or post-harvest under favourable conditions of temperature and humidity. Development of rapid and sensitive method for detecting aflatoxin $B_1$ is important for food safety and quality to minimise the exposure of this toxin towards consumers. In this study, the use of multi-walled carbon nanotube (MWCNT) with chitosan (CS) was investigated as a modifier on the screen-printed carbon electrode (SPCE). The modification of electrode was studied as a platform to fabricate the immunoassay for further electrochemical reaction to occur. MWCNT was first successfully functionalised via strong acid treatment ($H_2SO_4$:$HNO_3$) by adding carboxylic acid (-COOH) groups and increased the MWCNT's surface area. The characterisation was done by FTIR and the additional group was confirmed by the presence of peak at 1715 $cm^{-1}$ and shorter nanotube can be seen through FESEM. Based on the electrochemical analysis, the modification of SPCE with the functionalised MWCNT greatly enhanced the current response up to 7 times. The modification of SPCE was optimised through various parameters, which includes pH, scan rate, MWCNT ratio, drop coating volume and drying condition. Ten microliters of MWCNT:CS (with mixing ratio, 5:1) for electrode coating and oven dried for 10 minutes at 80$^0$C were optimal for electrode casting. Furthermore, the electrochemical scan was done under 0.1 $Vs^{-1}$ of scan rate and PBS pH7 was used throughout this study. An indirect competitive ELISA was optimised prior to transferring onto the modified electrode. Through checkerboard study, the concentration of $AFB_1$-BSA at 0.25 µg/mL and anti-$AFB_1$ of primary antibody at 1/5000 (v/v) gave high absorbance with low $IC_{50}$ value of 0.0183 ng/mL. Futhermore, 8% skimmed milk produced the lowest background reading and was used as blocking agent. By using multi-level factorial design, an interaction plot between pH, incubation time and incubation temperature was obtained. It can be deduced that pH 7 with 0.5 hour incubation time at 25$^0$C was optimal for the competition between primary antibody and free antigen to compete for binding site. From the result, the working range of $AFB_1$ was between 0.001 to 10 ng/mL ($R^2$=0.9875) and the LOD was found at 0.15 pg/mL. A stable fabrication of $AFB_1$-BSA onto the modified SPCE was obtained by activating the carboxylic acid groups using coupling agent, NHS and EDC. $AFB_1$ was quantified indirectly based on the



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activity of HRP enzyme that caused $TMB_{(ox)}$ to be reduced at potential between 0.2 to 0.3 V, where the peak was detected in electrochemical system. A non-linear calibration curve was plotted with $R^2$ of 0.9873 and $IC_{50}$ of 0.0015 ng/mL. The linear working range was between 0.0001 to 10 ng/mL with LOD of 0.03 pg/mL. Furthermore, the reproducibility and repeatability of this immunosensor were 4.78% and 2.71%, respectively. The test using spiked peanut sample gave good recovery of 80-127%. As a conclusion, a sensitive immunosensor with MWCNT/CS base electrode was successfully developed for detection of $AFB_1$.

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**PEMBANGUNAN SENSOR IMUNO ELEKTROKIMIA BERASASKAN BAHAN NANO UNTUK PENGESANAN AFLATOKSIN $B_1$ DALAM KACANG TANAH**

Oleh

**FARAH ASILAH BINTI AZRI**

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Aflatoksin $B_1$ dikaitkan sebagai karsinogen kepada manusia yang diakibatkan oleh kontaminasi pelbagai hasil pertanian sama ada sebelum atau selepas penuaian di bawah suhu dan kelembapan yang sesuai. Pembangunan kaedah pengesanan aflatoksin $B_1$ yang cepat dan sensitif adalah penting untuk pengawalan kualiti dan keselamatan makanan demi mengurangkan pendedahan toksin ini terhadap para pengguna. Dalam penyelidikan ini, penggunaan tiub nano karbon pelbagai dinding (MWCNT) dan kitosan (CS) telah dikaji sebagai pengubah suai elektrod skrin bercetak karbon (SPCE). Pengubah suaian elektrod ini dikaji sebagai tapak yang sesuai untuk fabrikasi asai imuno, di mana tindak balas elektrokimia akan berlaku. MWCNT telah berjaya difungsikan dengan menggunakan rawatan asid kuat ($H_2SO_4$:$HNO_3$) untuk menambahkan kumpulan asid karbosilik (-COOH) dan meningkatkan luas permukaan. Pencirian telah dilakukan dengan FTIR dan kehadiran puncak pada 1715 $cm^{-1}$ membuktikan penambahan kumpulan. Selain itu, tiub nano yang lebih pendek dapat dilihat melalui FESEM. Berdasarkan analisis electrokimia, pengubahsuaian elektrod dengan menggunakan MWCNT telah meningkatkan gerak balas arus sebanyak 7 kali ganda. Pengubahsuaian SPCE dioptimumkan melalui pelbagai parameter termasuk pH, kadar imbas, nisbah MWCNT:CS, isipadu penyalutan dan cara pengeringan. Sepuluh microliter larutan MWCNT:CS (dengan nisbah 5:1) lalu dikeringkan di dalam ketuhar pada suhu 80$^0$C selama 10 minit adalah optima untuk pengubahsuaian elektrod. Selain itu, imbasan elektrokimia telah dilakukan pada kadar imbas 0.1 $Vs^{-1}$ dan PBS pH 7 digunakan sepanjang kajian ini. Pengoptimaan ELISA dilakukan sebelum memindahkan asai ini ke atas elektrod yang telah diubah suai. Melalui petak segi empat, 0.25 µg/mL $AFB_1$-BSA dan 1/5000 (v/v) anti-$AFB_1$ memberikan nilai keserapan yang tinggi dengan nilai $IC_{50}$ yang rendah iaitu 0.0183 ng/mL. Tambahan pula, 8% susu siringan memberikan bacaan latar belakang yang paling rendah lalu digunakan sebagai agen penghalang. Dengan menggunakan pelbagai arak reka bentuk faktorial, plot saling tindak di antara tiga faktor iaitu pH, masa inkubasi dan suhu inkubasi telah dibina. Oleh itu, pH 7 dengan 0.5 jam masa inkubasi pada suhu 25$^0$C adalah keadaan yang optimal untuk persaingan di antara antibodi primer dan antigen bebas demi tapak penambatan. Berdasarkan hasil kajian, julat kerja bagi $AFB_1$ adalah di antara 0.001 to



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10 ng/mL ($R^2$=0.9875) dengan LOD adalah pada 0.15 pg/mL. Fabrikasi $AFB_1$-BSA yang stabil telah dilakukan di atas SPCE yang telah diubah suai dengan mengaktifkan kumpulan asid karboksilik dengan menggunakan agen pengganding, NHS dan EDC. $AFB_1$ dikesan secara tidak terus berdasarkan aktiviti enzim HRP yang menyebabkan $TMB_{(ox)}$ diturunkan pada keupayaan di antara 0.2 hingga 0.3 V, di mana puncak dikesan di dalam sistem elektrokimia,. Lengkung tidak linear telah dibina dengan nilai $R^2$=0.9873 dan $IC_{50}$ of 0.0015 ng/mL. Julat kerja yang linear adalah di antara 0.0001 hingga 10 ng/mL dengan LOD pada 0.03 pg/mL. Selain itu, kebolehasilan semula dan kebolehulangan semula bagi sensor imuno ini adalah pada 4.78% dan 2.71%, masing-masing. Ujian dengan menggunakan sampel kacang yang dipakukan menunjukkan perolehan yang baik dengan nilai 80-127%. Sebagai kesimpulan, sensor imuno yang sensitif dengan elektrod berasaskan MWCNT/CS telah berjaya dihasilkan untuk pengesanan $AFB_1$.

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I certify that a Thesis Examination Committee has met on 11 March 2016 to conduct the final examination of Farah Asilah binti Azri on her thesis entitled "Development of a Nanomaterial-Based Electrochemical Immunosensor for Detection of Aflatoxin $B_1$ in Peanuts" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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## TABLE OF CONTENTS



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## LIST OF TABLES



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## LIST OF FIGURES





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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| AF | Aflatoxin |
| $AFB_1$ | Aflatoxin $B_1$ |
| $AFB_1$-BSA | Aflatoxin conjugated with BSA |
| $AFB_1$-HRP | Aflatoxin conjugated with HRP |
| Ag | Antigen |
| Ag/AgCl | Silver/silver chloride |
| Anti-IgG-HRP | Anti-antibody IgG labeled with HRP |
| B | Background |
| BSA | Bovine Serum ALbumin |
| COOH | Carboxyl terminal |
| CS | Chitosan |
| CV | Cyclic voltammetry |
| CV% | Coefficient of variation |
| DPV | Differential pulse voltammetry |
| EDC | N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| CI-ELISA | Competitive Indirect Enzyme-Linked Immunosorbent Assay |
| f-MWCNT | Functionalised multi-walled carbon nanotube |
| FTIR | Fourier transform infrared spectroscopy |
| FE-SEM | Field Emission Scanning Electron Microscope |
| GPES | General Purpose Electrochemical Software |
| $H_2O_2$ | Hydrogen peroxide |



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| | |
|---|---|
| $H_2SO_4$ | Sulphuric acid |
| HRP | Horseradish peroxidase |
| $IC_{50}$ | Adequate sensitivity |
| $K_3[Fe(CN)]_6$ | Potassium Ferricyanide |
| LOD | Limit of detection |
| MWCNT | Multi-walled carbon nanotube |
| NHS | N-hydroxysuccinimide |
| Ox | Oxidised |
| PBS | Phosphate-buffered saline |
| PBS-T | 0.05% tween-20 (v/v) to the PBS |
| ppb | Part per billion |
| ppt | Part per trillion |
| Red | Reduced |
| RSD | Relative standard deviation |
| S | Signal |
| S/B | Signal/Background |
| SPCE | Screen-printed carbon electrode |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| $TMB_{(ox)}$ | Oxidised TMB |
| $TMB_{(red)}$ | Reduced TMB |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Aflatoxin is a secondary metabolites derived from the *Aspergillus* genus which are highly toxic, carcinogenic, teratogenic and mutagenic compounds (Afsah-Hejri *et al*., 2013). Fungi can be found widely in various food and feedstuffs namely peanuts, corn, rice, wheat, cottonseed and spices (Leong *et al*., 2010). Malaysia is a tropical country with high temperature and high relative humidity. Therefore, presence of mycotoxins can be expected. Arzandeh *et al.* (2010) reported that 75% (out of 84 samples) of raw peanut kernels, which were collected randomly from Malaysian supermarket was confirmed to contain aflatoxin $B_1$. Moreover, 10.71% of samples were exceeded the maximum tolerable limit of 15 ng/g set by Codex *(*Arzandeh *et al.*, 2010). Futhermore, Ali *et al.* (2005) also reported a high occurrence of aflatoxin $B_1$ (70%) in traditional herbal medicines from Malaysia and Indonesia.

Mycotoxin contamination of foodstuffs is a serious matter that causes a major health threat for human and animals, as well as affecting the economy, significantly. Aflatoxin exposure to human can be by either directly or indirectly consumption of the contaminated food or inhalation of $AFB_1$-contaminated dust (Afsah-Hejri *et al*., 2013). Due to the proven risk related with aflatoxins, the current maximum level for aflatoxins set by the European Commission is 2 ng/g for $AFB_1$ in corn, groundnuts, nuts, dried fruit and cereals (Piermarini *et al*., 2007). Hence, it is very important to protect humans and animals by restricting their exposure to aflatoxins.

Due to the significant health risks related with the occurrence of aflatoxins in food, and also to gratify the severe legal requirement, it is vital to have efficient techniques for the detection (Ammida *et al*., 2006). Immunoassay or enzyme-linked immunosorbent assay (ELISA) can be considered as a rapid method that offers simplicity, sensitivity and selectivity compared to other traditional techniques which required extensive clean up procedure, labour consuming and costly (such as liquid chromatography). ELISA is a calorimetric technique that can be used in huge scale screenings and chromogenic substrates offer long lasting stability of a coloured product after the reaction is complete (Kadir, 2010). Kolosova *et al*. (2006) have reported a direct competitive ELISA based on monoclonal antibody for detection of aflatoxin $B_1$ in grain samples. Hence, in this study, the incorporation of ELISA into electrochemical biosensing technique was investigated to enhance the sensitivity.

Biosensor is a device that can determine the analyte based on the incorporation of bioactive materials with physiochemical transducing element (Mosiello and Lamberti, 2011). The biological material can be classified as affinity or biocatalytic such as antibodies, DNA, receptor protein, enzymes, tissues, whole cells or organs interrelating with specific analyte. The interaction can then be converted by the transducer into a quantifiable electrical signal. In this study, an antibody-based



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biosensor, also known as immunosensor, was developed by integrating the optimised ELISA system onto the electrochemical sensing component. Holford *et al.* (2012) have reviewed the recent trends in antibody-based sensors in the past decades. They concluded that one major challenge for many of these immunosensors is limited use in the laboratory surroundings. Therefore, many researches were conducted by using technology of screen-printed electrodes, which can be manufactured reasonably cheap and can be advanced for commercial purposes. This technology can be applied into a portable sensor that can be used on site.

Many researches have been done over decades on the detection of aflatoxin $B_1$. Various techniques, types of electrodes, modifications, detector components as well as the type of sample have been explored. Nanomaterials including nanotube, nanofilm, nanoparticle and nanofiber display unique physical and chemical features which can help to enhance the sensitivity of the developed sensor (Sonawane *et al.*, 2014). Although substantial progress has been made in exploring carbon nanotube based as electrodes and biosensor, there is no report on commercialised device or for aflatoxin $B_1$ detection (Veetil and Ye, 2007). Therefore, this study was undertaken to discover the potential of multi-walled carbon nanotube and chitosan as the modifier on the sensing components and incorporating an indirect competitive ELISA under optimal conditions. Hypothesis that can be deduced for this study is that the implementation of nanomaterials and ELISA system in the development of electrochemical immunosensor can increase the sensitivity as well as improve the current conductivity. The importance of this study is to develop a sensitive immunosensor for rapid detection of aflatoxin $B_1$. Moreover, the finding may have significant contribution towards food safety and quality, subsequently emphasize the citizen on the risk of consumption.

## 1.2 Aim and objectives

The aim of this study is to develop a rapid and sensitive electrochemical immunosensor for detection of aflatoxin $B_1$ ($AFB_1$) in food samples, which is peanuts. The use of multi-walled carbon nanotube (MWCNT) and chitosan (CS) were implemented as nanomaterials to modify the electrode and subsequently enhance the performance of the sensor. In order to achieve this aim, there were three objectives that needed to be focused on including:

1. To modify the screen-printed carbon electrode with multi-walled carbon nanotubes and chitosan.
2. To optimise the competitive indirect enzyme-linked immunosorbent assay (ELISA).
3. To develop and characterise the immunosensor for aflatoxin $B_1$ based on indirect competitive ELISA.

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