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TYROSINASE AND LACCASE BIOSENSORS BASED ON GRAPHENE-GOLD-CHITOSAN NANOCOMPOSITE-MODIFIED SCREEN-PRINTED CARBON ELECTRODE FOR PHENOLIC COMPOUNDS DETECTION

FUZI MOHAMED FARTAS

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By

FUZI MOHAMED FARTAS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

September 2018

DEDICATION

This thesis is dedicated to my beloved parents and the entire Muslim Ummah.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Jaafar Bin Abdullah, PhD
Faculty : Science

Phenolic compounds are among the most pollutants present in ground and surface water. These compounds consider potentially harmful to human health and showing adverse effects on the environment. Bisphenol A (BPA) is one of phenolic compounds which is widely used as a raw material for common products, including food containers, an inner liner for food cans, beverage cans, water bottles and baby bottles. The hazard effect exposure to BPA released from these materials is increasing due to its endocrine-disrupting potential can cause several health diseases such as cancers, developmental problems and heart disease. The conventional methods used for detecting these pollutants suffered some limitations such as expensive instrumentation, time-consuming and not suitable for in-situ monitoring.

In this study, electrochemical biosensors were fabricated based on immobilization of tyrosinase and laccase onto graphene-decorated gold nanoparticle/chitosan (Tyr/Gr-Au-Chit and Lacc/Gr-Au-Chit) nanocomposite-modified screen-printed carbon electrode (SPCE) for the detection of phenolic compounds. The gold nanoparticles (AuNPs) decorated graphene nanosheets (Gr) in combination with chitosan was deposited onto SPCE via drop casting technique. The tyrosinase and laccase enzyme was immobilized onto the modified electrode via physical adsorption. The prepared nanocomposite was characterized by using Fourier Transform Infrared Spectroscopy (FTIR), Raman spectroscopic, Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM) and Energy dispersive X-ray (EDX). The FTIR spectra for Tyr/Gr-Au-Chit and Lacc/Gr-Au-Chit show new peaks at 1639 cm^{-1} and 1543 cm^{-1} for amide I and amide II are due to –C=O stretching, C–N stretching, and –N–H bending of vibration bands of amide groups of both enzymes which proved the successful immobilization of the enzymes

onto the Gr-Au-Chit/SPCE nanocomposite matrix. The increased in the intensity ratio of Raman spectra (ID/IG = 0.58) for Gr-Au/Chit compared to Gr-Au substrate (ID/IG = 0.34) demonstrated that the Gr-Au/Chit nanocomposite was successfully prepared. Electrochemical characterization of the modified electrodes revealed that the nanocomposite have increased its electro-active surface area and conductivity. The electroactive surface area (A) of bare SPCE and Gr-Au-Chit/SPCE was calculated to be 0.091 cm^2 and 0.488 cm^2, respectively. While the electron transfer resistance (Rct) at the bare SPCE and Gr-Au-Chit/SPCE was found to be (36 KΩ) and (89 Ω), respectively.

The modified SPCEs showed a remarkable activity which attributed to the excellent conductivity, large surface area, electrocatalytic activity and good synergetic effect of the bio-nanocomposite films towards phenol and bisphenol A. The fabricated Tyr/Gr-Au-Chit biosensor was applied for the voltammetric detection of phenol in water sample by differential pulse voltammetry (DPV). Under the optimum conditions, the Tyr/Gr-Au-Chit based biosensor displays a linear calibration curve in the phenol concentration range of 0.05 to 15.00 μM with sensitivity of 0.62 μA/μM and limit of detection (LOD) of 0.02 μM.

While differential pulse voltammetric technique was employed for second fabricated biosensor (Lacc/Gr-Au-Chit) for bisphenol A detection. Under the optimum conditions, the Lacc/Gr-Au-Chit based biosensor gave linear calibration curve in the bisphenol A concentration range of 0.05 to 12.00 μM with sensitivity of 0.27 μA/μM and detection limit of 0.02 μM. The proposed methods showed good selectivity of target analytes even in the presence of some foreign ions. The fabricated biosensor (Tyr/Gr-Au-Chit) was successfully applied for the determination of phenol in lake water sample while Lacc/Gr-Au-Chit was successfully applied for the determination of bisphenol A in different types of commercial plastic products with satisfactory recovery results from 96.40% to 101.16% and 98.31% to 102.92%, which comparable to HPLC technique. These findings suggests that the developed biosensors have a promising potential for the detection of phenol and bisphenol A in water sample and plastic samples for environmental monitoring and industrial quality control.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

BIOSENSOR TIROSINASE DAN LAKKASE BERASASKAN GRAFEN-EMAS-KITOSAN KOMPOSIT NANO MODIFIKASI ELEKROD KARBON BERCETAK UNTUK PENGESANAN SEBATIAN FENOLIK

Oleh

FUZI MOHAMED FARTAS

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Sebatian fenolik adalah antara bahan pencemar yang paling penting yang terdapat di dalam tanah dan air permukaan. Sebatian ini dianggap berupaya membahayakan kesihatan manusia dan menunjukkan kesan buruk kepada alam sekitar. Bisfenol A (BPA) adalah salah satu sebatian fenolik yang digunakan secara meluas sebagai bahan mentah untuk kebanyakan produk, termasuk bekas makanan, pelapik dalam untuk tin makanan, tin minuman, botol air dan botol bayi. Kesan bahaya pendedahan terhadap BPA yang dikeluarkan dari bahan-bahan ini semakin meningkat kerana potensi ganguan endokrin dan boleh menyebabkan beberapa penyakit kesihatan seperti kanser, masalah perkembangan dan penyakit jantung. Kaedah konvensional yang digunakan untuk mengesan bahan pencemar ini mengalami beberapa kelemahan seperti peralatan mahal, memakan masa dan tidak sesuai untuk pemantauan segera.

Dalam kajian ini, biosensor elektrokimia dibangunkan berdasarkan pemegunan tirosinase dan lakkase diatas komposit nano grafen dihiasi nanopartikel emas /chitosan (Tyr/Gr-Au-Chit) dan (Lacc/Gr -Au-Chit) yang diubahsuai elektrod karbon bercetak skrin (SPCE) yang ditandakan sebagai untuk pengesanan sebatian fenolik. Nanopartikel emas (AuNPs) dihiasi kepingan nano grafen (Gr) dalam kombinasi dengan kitosan dienapkan diatas SPCE menggunakan teknik titisan acuan. Enzim tirosinase dan lakkase telah dipegunkan diatas elektrod terubahsuai melalui penjerapan fizikal. Komposit nano yang disediakan dicirikan menggunakan spektroskopi inframerah transformasi fourier (FTIR), spektroskopi Raman, Mikroskopi Transmisi Elektron (TEM), pembelauan sinar-X(XRD), Mikroskop Elektron pengimbasan pelepasan medan (FESEM) dan penyebaran tenaga sinar-X (EDX), masing-masing. Spektrum FTIR untuk Tyr/Gr-Au-Chit dan Lacc/Gr-Au-Chit menunjukkan puncak baharu pada 1639 cm^{-1} dan 1543 cm^{-1} untuk amida I dan amida

II adalah disebabkan oleh regangan -C = O, C-N regangan, dan bengkokan -N-H bagi getaran kumpulan amida kedua-dua enzim yang membuktikan enzim berjaya dipegunkan diatas matriks komposit nano Gr-Au-Chit/SPCE. Peningkatan dalam nisbah intensiti spektra Raman (ID/IG = 0.58) untuk Gr-Au/Chit berbanding dengan Gr-Au substrat (ID/IG = 0.34) menunjukkan bahawa komposit nano Gr-Au/Chit telah berjaya disediakan. Pencirian elektrokimia elektrod yang diubahsuai mendedahkan bahawa luas permukaan elektroaktif dan kekonduksian komposit nano telah meningkatkan. Luas permukaan elektroaktif (A) SPCE pengosong dan Gr-Au-Chit/SPCE masing-masing adalah 0.091 cm2 dan 0.488 cm^2. Sementara rintangan pemindahan elektron (Rct) pada SPCE pengosong dan Gr-Au-Chit/SPCE masing-masing didapati adalah (36 KΩ) dan (89 Ω).

SPCE yang diubahsuai menunjukkan aktiviti yang luar biasa yang dikaitkan dengan kekonduksian yang sangat baik, luas permukaan yang besar, aktiviti elektrokatalik dan kesan sinergik yang baik dari filem bio-komposit nano terhadap fenol dan bisfenol A. Biosensor yang telah difabrikasi digunakan untuk pengesanan voltammetrik fenol dalam sampel air melalui kaedah voltammetri denyutan perbezaan (DPV). Pada keadaan optimum, biosensor Tyr/Gr-Au-Chit memaparkan lengkungan kalibrasi linear dalam julat kepekatan fenol di antara 0.05 hingga 15.00 μM dengan kepekaan 0.62 μA/μM dan had pengesanan (LOD) 0.016 μM.

Manakala, teknik voltammetri denyutan perbezaan telah digunakan untuk biosensor kedua (Lacc/Gr-Au-Chit) bagi pengesanan bisfenol A. Pada keadaan optimum, biosensor Lacc/Gr-Au-Chit memberikan lengkung kalibrasi linear dalam julat kepekatan bisfenol A di antara 0.05 hingga 12.00 μM dengan kepekaan 0.27 μA/μM dan had pengesanan 0.023 μM. Kaedah yang dicadangkan menunjukkan pemilihan yang baik bagi sasaran analitik walaupun dengan kehadiran ion-ion asing. Biosensor yang difabrikasi (Tyr/Gr-Au-Chit) berjaya digunakan untuk penentuan fenol dalam sampel air tasik manakala biosensor Lacc/Gr-Au-Chit berjaya digunakan bagi penentuan Bisfenol A dalam pelbagai jenis produk plastik komersial dengan keputusan perolehan semula yang memuaskan dari 96.40% hingga 101.16% dan 98.31% hingga 102.92%, yang mana setanding dengan kaedah kromatografi cecair prestasi tinggi (HPLC). Penemuan ini mencadangkan bahawa biosensor yang dibangunkan mempunyai keupayaan yang menyakinkan untuk pengesanan fenol dan bisfenol A dalam sampel air dan plastik bagi pemantauan alam sekitar dan kawalan kualiti industri.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AuNPs	Gold Nanoparticles
BPA	Bisphenol A
CE	Counter Electrode
CME	Chemically Modified Electrode
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
EC	European Commission
EDCs	Endocrine Disrupting Chemicals
EDX	Energy Dispersive X-Ray
EIS	Electrochemical Impedance Spectroscopy
FESEM	Field Emission Scanned Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
Lacc	Laccase
LC-MS	Liquid Chromatography-Mass Spectrometry
LDR	Linear Dynamic Range
LOD	Limit of Detection
LSV	Linear Sweep Voltammetry
Km	Apparent Michaelis Menten Constant
PBS	Phosphate Buffer Saline
PH	Logarithm of Proton Concentration
RE	Reference Electrode
SPCE	Screen-Printed Carbon Electrode
TEM	Transmission Electron Microscopy
Tyr	Tyrosinase
USEPA	United States Environmental Protection Agency
UV-Vis	Ultraviolet Visible
WE	Working Electrode
XRD	X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Water is one of the most important natural resources for all living beings in the world. With the rapid development of science and technology, many industries such as pharmaceutical, petrochemical, plastics, mining, textile and dye are grown around the world. Each of these industries requires a large quantity of water for daily activities and the water released from these industries are contaminated with pollutants such as pharmaceutical residues, dyes and endocrine disrupting chemicals (EDCs) which contributed to pollution in the ecosystem. In addition, the rapid growth of the population and the intensification of agricultural and industrial activities have led to a dramatic increase in the number of pollutants released into the environment. These pollutants raise a major environmental and public health concern.

Phenolic compounds are one of the pollutants that present a growing environmental problem and have affected several components of the environment, including terrestrial and aquatic biota. Phenolic compounds are one of the most dangerous water pollutants, extremely destructive to nature and harmful to human health. Phenols are easily adsorbed into human's skin, regardless of the type of exposure. In addition, these compounds have a high toxicity on the human health when they are present above certain concentration limits.

The discovery of endocrine disruptor chemicals (EDCs) in the wastewater lately is being alarming. The sources of EDCs are mainly from anthropogenic chemicals which have the ability to damage endocrine systems (Ghazali & Johari, 2015). Phenolic compounds such as phenol, bisphenol A, chlorophenols and alkylphenols have been classified as one of the endocrine disrupting chemicals (EDCs) and regarded as priority pollutants by both the United States Environmental protection agency (USEPA) and European Commission (EC) (Guan et al., 2016; Wang et al., 2014; Zolgharnein, et al., 2013). In addition, several studies have reported that various phenolic compounds such as 2,4-dichlorophenol, 2-nitrophenol ,4-nitrophenol, phenol, bisphenol A were detected in water samples (Guan et al., 2016; Kong, et al., 2009; Santhi et al., 2012; Veerasingam et al., 2013; Wei et al., 2015; Zhang et al., 2011) and bisphenol A was also detected in polycarbonate drinking bottle (plastic products) (Cosio et al., 2017; Li et al., 2016; Wang et al., 2015; Zhao et al., 2017).

In 1991, a spillage of phenolic compounds in the Linggi River, Negeri Sembilan was occurred and the concentration detected was in the range of 0.8 to 53.6 µg/L. The main sources of the detected phenols were identified as effluent from sawmills, rubber factories, motor and battery shops, engineering workshops and agricultural activities

(Abdullah and Nainggolan, 1991). It was also found that the levels of phenols in the Linggi River was exceeded from the recommended Malaysian water standard of 2.0 µg/L for raw water and 1.0 µg/L for the treated water (Malaysian Ministry of health 1987). In Malaysia, the maximum permissible limit of phenolic compounds in surface water for drinking is in the range of 1-2 µg/L, while the acceptable limit in wastewater and industrial effluents is in the range of 1-5 mg/L.

Therefore, monitoring of the water pollution and to comply with the strict environmental regulation, researchers have focused on the development of effective analytical methods for the determination of phenolic compounds in several fields, such as environmental monitoring, food quality control and medical diagnosis. Several analytical methods were used for the detection of phenolic compounds including high-performance liquid chromatography (HPLC) (Zhong et al., 2016)), liquid chromatography-mass spectrometry (LC-MS) (Regueiro & Wenzl, 2015), gas chromatography-mass spectrometry (GC-MS) (Pastor-Belda, et al., 2017) and capillary electrophoresis ((Hernández-Chávez & Guemes Vera, 2015). Although, these methods have reliable sensitivity and low detection limit, but they have some limitation such as high maintenance cost, time-consuming, complex instrumentation and require highly qualified personnel. Therefore, the development of simple, highly sensitive, selective, accurate method and portable for the detection of phenolic compounds is extremely important for monitoring and controlling the release of contaminants which have harmful effects on human health.

Biosensor is the best candidate to overcome this situation. Currently, several studies on biosensor have been reported for the detection of phenolic compounds which includes an electrochemical biosensor (Caetano et al., 2018), optical biosensor (Gupta & wood, 2017) and colorimetric (Arciuli et al., 2013). The mentioned biosensors have obtained an excellent performance. An electrochemical biosensor is considered as the best choice for in situ detection of phenolic compounds (Gul et al., 2017; Mayorga et al., 2013). While offering simplicity in the operation and sample-handling, the electrochemical biosensor also provides highly sensitive and specific measurements for a broad range of analytes (Putzbach & Ronkainen 2013; Wang et al., 2014). In addition to that, the sample size required for performing electrochemical biosensors is small, ranging from several microliters to hundreds of nanoliters, which includes simple pretreatment of samples (Wei et al., 2010).

Furthermore, the latest technology has introduced commercially available screen-printed carbon electrode (SPCE) which offer inherent advantages including low cost, simple fabrication, miniaturization, disposability and portability (Jin et al., 2017). Moreover, screen-printed technology for production of electrochemical biosensors have been widely used in various fields, including environmental, pharmaceutical, clinical, food quality control (Trojanowicz, 2016). On the other hand, the most important characteristic of electrochemical biosensors is their potential to be easily transformed from the laboratory instrument to the point of care (POC) devices for environmental monitoring and healthcare testing.

With noticeable achievements in nanotechnology and nanoscience, the amplification of electrochemical signals based on nanomaterials has a great potential to be used for improvement sensitivity and selectivity of electrochemical sensors and biosensors (Zhu et al., 2015). Graphene nanosheets are one of the advance nanomaterials has been extensively explored as an electrode modifier in electrochemical biosensor provided a biocompatible matrix to improve the performance of electrochemical biosensor (Bollella et al., 2017). More recently, the applications have been extensively explored in the modification of the working electrode by combining graphene materials with nanoparticles for the formation of nanocomposite which may contribute to the best performance with excellent stability and improved sensitivity (Kumar et al., 2015).

Over the past few years, gold nanoparticles (AuNPs) have attracted much attention for sensing application due to the unique properties including excellent biocompatibility, high surface activity, high conductivity and good stability (Zhang et al., 2016). Thus, the combination of graphene and gold nanoparticles provide a promising platform for biomolecule immobilization without losing their activity. Nowadays this method is well established for the construction of biosensor device. Additionally, it is expected that the designed biosensor based on the graphene/gold nanocomposite display excellent analytical performance for the detection of phenolic compounds with high sensitivity, wide linearity range and lower detection limit.

1.2 Problem statement

Industrial effluents that contain toxic compounds are discharged into water bodies would cause serious environmental problems. Phenolic compounds are one of the common pollutants in many industrial wastewaters. In addition, most phenolic compounds are highly toxic chemicals, which causes hazard to human health and the environment. Bisphenol A (BPA) is one of the most commonly used compounds which have attracted great concerns nowadays because it can disrupt the normal regulatory function of endocrine systems. Several adverse effects such as infertility, cancer and suppression of the immune system could be induced by these endocrine disrupting compounds (EDCs) (Ismail et al., 2018). The alteration of the endocrine system is due to the ability of EDCs to block and copy the normal effect of hormones, interrupt the synthesis and metabolism of hormones and alter the levels of hormone receptors (Ismail et al., 2018).

Human exposure to phenolic compounds causing various diseases including prostate and breast cancer. It was also found to be associated with obesity, diabetes and heart disease. Despite the diseases associated with BPA, it is still used in the manufacture of commonly used goods, for example, plastic bottles, polyethylene bags, thermal papers, optical and dental purposes (Yang et al., 2014). To avoid the harmful effects of phenolic compounds, an efficient tool for environmental monitoring system is urgent required.

Commonly, the most frequently used methods for the detection of phenolic compounds are gas chromatography and liquid chromatography. These methods have the advantage of high accuracy and sensitivity, however they suffered of some limitations such as expensive instrumentation, involve complicated procedure, time-consuming, require pre-treatment and extraction steps and large size which unsuitable for on-site analysis. These limitations could be overcome by using electrochemical technique due to its exceptional properties, for example, low cost, simple, selective, sensitive and possible for portability which makes them suitable for on-site application.

Therefore, in this study, the utilization of tyrosinase and laccase in the fabrication of electrochemical biosensors can improve the selectivity and sensitivity towards the detection of phenol and bisphenol A that cannot be achieved with the traditional technique.

1.3 Scope of study

The conventional analytical techniques for the detection of phenolic compounds are mainly based on high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrum (GC-MS). However, these techniques involve a tedious extraction procedure, time-consuming and require skilled analysts to obtain the optimal result. Therefore, enzyme-based electrochemical biosensor was suggested as an alternative method for the detection of phenolic compounds that offers considerable advantages, such as rapid analytes detection, simple or no sample pre-treatment, low-cost and portable equipment. In addition, the suggested method also offers quantifiable measurement, sensitivity and selectivity compared to the chromatographic technique

1.4 Objectives of the study

The goal of this study is to develop a simple and sensitive electrochemical biosensor for the detection of phenolic compounds in aqueous samples. This can be achieved by immobilizing enzymes (tyrosinase or laccase) on graphene-decorated gold nanoparticles/chitosan composite modified screen printed carbon electrode as a sensing material for enhancement sensitivity of the detection. To achieve the aim of this research the following specific objectives are outlined:

i. To prepare and characterize nanocomposite based on graphene decorated gold nanoparticles/chitosan deposited onto screen printed carbon electrode.
ii. To immobilize tyrosinase or laccase onto screen printed carbon electrode modified with graphene-decorated gold nanoparticles/chitosan for the detection of phenolic compounds.
iii. To evaluate the performance of the developed biosensors for the detection of phenolic compounds and real sample analysis.
iv. To validate the proposed method with high performance liquid chromatography technique for the detection of phenolic compounds in real samples.

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LIST OF PUBLICATIONS

Fartas, Fuzi M.; Abdullah, Jaafar; Yusof, Nor A.; Sulaiman, Yusran; Saiman, Mohd I. 2017. "Biosensor Based on Tyrosinase Immobilized on Graphene-Decorated Gold Nanoparticle/Chitosan for Phenolic Detection in Aqueous." Sensors *17, no. 5: 1132*.

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Laccase Biosensor Based on Graphene-Gold Nanocomposite Film for Bisphenol A Detection. Fundamental Science Congress (FSC 2017). University Putra Malaysia, 21–22 November 2017.

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