UNIVERSITI PUTRA MALAYSIA

ELECTROCHEMICAL DNA BIOSENSOR BASED ON IRON OXIDE/NANOCELLULOSE CRYSTALLINE FOR DETECTION OF Mycobacterium tuberculosis

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By

CHE ENGKU NORAMALINA BT CHE ENGKU CHIK

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

December 2017

DEDICATION

This research work is dedicated to my lovely husband Mohd Hazim Abd Aziz, my late father, Che Engku Chik Che Engku Embong, my lovely mother, and my siblings, and my family in law.

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

ELECTROCHEMICAL DNA BIOSENSOR BASED ON IRON OXIDE/NANOCELLULOSE CRYSTALLINE FOR DETECTION OF *Mycobacterium tuberculosis*

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Chairman : Helmi Wasoh @ Mohamad Isa,PhD
Faculty : Biotechnology and Biomolecular Science

In this study, electrochemical DNA biosensor based on carboxyl functionalized iron oxide/nanocellulose crystalline surface modified with cetyl trymethylammonium bromide (COOH-Fe_3O_4/NCC/CTAB) for detection of *Mycobacterium tuberculosis* (MTB) was developed. The modification was made by drop coating a carboxyl COOH-Fe_3O_4 nanocomposite with NCC/CTAB on surface of screen printed carbon electrode (SPCE). The modified SPCE was electrochemically characterized using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) in the present of $Fe(CN)_6^{3/4-}$ as electrolyte. The electrochemical characterization confirmed the high rate of electron transfer, was due to increase of electroactive surface area and excellent conductivity of modified SPCE. The optimization of SPCE modified in term of COOH-Fe_3O_4 concentration, volume/volume ratio of COOH-Fe_3O_4: NCC/CTAB and scan rate was achieved by 0.5 mg/ml, 1:1 ratio and 100 mV/s; respectively. The present of functional groups were confirmed by physical characterization using fourier transform infrared spectroscopy (FTIR), the surface morphology was confirmed by field emission scanning electron microscopy (FE-SEM), and the elemental composition of modified SPCE was confirmed by energy dispersive X-ray spectroscopy (EDX). The deposition of modifier materials on the surface of SPCE was properly characterized through physical characterization. Their electrochemical activities and sensing capability towards detection of *Mycobacterium tuberculosis* (MTB) was investigated using differential pulse voltammetry (DPV). Ruthenium complex bipyridyl $Ru(bpy)_3^{2+}$ was used as redox indicator in monitoring hybridization events of the DNA. A 5'-NH_2- end of DNA probe was used to form a covalent bond of amide bond immobilized with carboxyl group via ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide EDC/NHS as coupling mechanism. The selectivity of the fabricated DNA sensor was confirmed by the different signal produced by $Ru(bpy)_3^{2+}$ in differentiate ssDNA

probe, dsDNA target complementary, non-complementary and the mismatch DNA sequences. The optimum condition were obtained using the following criteria such as Tris EDTA as supporting electrolyte pH 8, deposition potential (0.3V), deposition time (5 sec) probe DNA concentration, immobilization time (60 minutes), EDC/NHSS immersion, ionic strength (20 mM), hybridization time (35 minutes at 42ºC) and $Ru(bpy)_3^{2+}$ concentration (25 µM). Performance of the biosensor was investigated using different concentration of DNA target in the range of 1.0×10^{-12} to 1.0×10^{-6} M and the limit of detection was calculated to be at 7.96×10^{-13} M. The calibration curve showed a linear relationship with equation, $y=0.6936 \log (x) + 11.47$ and correlation coefficient of $R^2=0.9896$. DNA of MTB was extracted from clinical sample and underwent PCR procedure for real sample analysis. The developed biosensor was demonstrated the difference between positive and negative MTB sample, mycobacterium other than tuberculosis (MOTT) and respiratory-related bacterial samples, *Staphyloccocus aureus* and confirmed that this fabricated electrochemical DNA sensor have good selectivity and effectively detected the sequence of DNA from MTB using PCR product analysis. The studied of stability and reproducibility showed a satisfactory result with loss only 5.87% activity after 7 days and below 50% after 5 weeks and finally the reproducibility value of 3.87% (n=8). The repeatability showed quite high with value of 29.30% indicating to a good SPCE to be used for TB detection if compared to previous study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

DNA BIOSENSOR ELEKTROKIMIA BERASASKAN BESI BEROKSIDA BERFUNGSI KARBOKSIL/NANOCELULOSA KRISTAL UNTUK PENGESANAN *Mycobacterium tuberculosis*

Oleh

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Dalam kajian ini, elektrokimia biosensor DNA berdasarkan besi oksida difungsikan dengan kumpulan karboksil/nanocelulosa kristal diubahsuai dengan siltrimetilammonium bromida (COOH-Fe_3O_4/NCC/CTAB) untuk mengesan *Mycobacterium tuberculosis* (MTB) telah dibangunkan. Pengubahsuaian itu dibuat melalui salut titis COOH-Fe_3O_4 dan campuran NCC/CTAB pada permukaan paparan bercetak elektrod karbon (SPCE). SPCE yang telah diubahsuai dicirikan menggunakan kitaran voltametri (CV) dan spektroskopi impedans elektrokimia (EIS) dengan kehadiran $Fe(CN)_6^{3-/4-}$ sebagai elektrolit. Pencirian elektrokimia mengesahkan kadar pemindahan elektron yang tinggi, peningkatan kawasan permukaan elektroaktif dan kekonduksian SPCE yang diubahsuai. Pengoptimuman SPCE yang diubah suai dari segi kepekatan COOH-Fe_3O_4, nisbah isipadu/isipadu COOH-Fe_3O_4:NCC/CTAB dan kadar imbasan dicapai oleh 0.5 mg/ml, nisbah 1:1 dan 100 mV/s. Pencirian fizikal telah dikaji menggunakan 'fourier transform infrared spectroscopy' (FTIR) mengesahkan semua kehadiran kumpulan berfungsi, bidang imbasan pancaran elektron mikroskop (FE-SEM) mengesahkan morfologi permukaan, dan serakan tenaga X-ray spektroskopi (EDX) mengesahkan komposisi unsur SPCE yang diubahsuai. Pemendapan bahan pengubah di permukaan SPCE telah disahkan melalui pencirian fizikal. Kegiatan elektrokimia dan keupayaan mengesan MTB diselidiki menggunakan 'differential pulse voltammetry' (DPV). Ruthenium kompleks bipyridyl $Ru(bpy)_3^{2+}$ digunakan sebagai penunjuk redoks dalam memantau peristiwa hibridisasi DNA. DNA yang telah diubahsuai dengan 5'-NH_2- telah digunakan untuk membentuk satu ikatan kovalen amida dengan kumpulan karboksil melalui ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) sebagai mekanisme gandingan. Pemilihan sensor DNA yang dihasilkan telah disahkan oleh isyarat yang berbeza yang dihasilkan oleh $Ru(bpy)_3^{2+}$ dalam membezakan prob ssDNA, sasaran dsDNA pelengkap, DNA tidak

melengkap dan DNA dengan turutan yang salah. Keadaan optimum yang dicapai adalah ; Tris EDTA dengan pH 8 sebagai elektrolit sokongan, pemendapan potensi dan masa di 0.3 V dan 5 saat masing-masing, 10 µM kepekatan prob DNA dengan 60 minit DNA untuk immobilisasi, 10 minit rendaman dalam EDC/NHS, 20 mM kekuatan ionik, masa hibridisasi selama 35 minit pada suhu 42 ºC dan kepekatan dan 25 µM dari $Ru(bpy)_3^{2+}$. Prestasi biosensor disiasat menggunakan kepekatan DNA yang berbeza dalam julat 1.0×10^{-12} hingga 1.0×10^{-6} M dan batas pengesanan dikira pada 7.96×10^{-13} M. Lengkok penentukuran menunjukkan hubungan linear dengan $y = 0.6936 \log(x) + 11.47$ dan pekali korelasi $R^2 = 0.9896$. DNA MTB telah diekstrak daripada sampel klinikal dan menjalani prosedur PCR untuk analisis sampel sebenar. Biosensor yang dihasilkan telah menunjukkan perbezaan antara sampel MTB positif dan negatif, mikobakteria selain tuberkulosis (MOTT) dan sampel bakteria yang berkaitan dengan pernafasan, *Staphyloccocus aureus* mengesahkan bahawa fabrikasi DNA sensor elektrokimia ini mempunyai pemilihan yang baik dan berkesan mengesan produk PCR MTB. Kajian kestabilan menunjukkan hasil yang memuaskan dengan kehilangan aktiviti cuma sebanyak 5.87% selepas 7 hari dan kehilangan aktiviti di bawah 50% selepas 5 minggu. Manakala kebolehulangan pada elektrod yang berbeza nmempunyai nilai 3.87% (n=8). Kebolehulangannya pada elektrod yang sama menunjukkan nilai yang agak tinggi dengan 29.30% menunjukkan prestasi SPCE yang baik sebagai pengesan MTB jika dibandingkan dengan kajian terdahulu.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Fe_3O_4	Iron Oxide
COOH	Carboxyl
MPA	Mercaptopropanoic acid
NCC	Nanocellulose Crystalline
CTAB	cetyl trymethylammonium bromide
MTB	*Mycobacterium Tuberculosis*
TB	Tuberculosis
MOTT	Mycobacterium other than tuberculosis
SPCE	Screen printed carbon electrode
EIS	Electrochemical impedance spectroscopy
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
FTIR	Fourier transform infrared spectroscopy
FE-SEM	Field emission scanning electron microscopy
EDX	Energy dispersive X-ray spectroscopy
EDC/NHS	Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide
ssDNA	Single stranded deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
CE	Counter electrode
WE	Working electrode
RE	Reference electrode
g	Gram

mg	Milligram
M	Molar
µM	Micromolar
A	Ampere
µA	Microampere
µl	Microliter
^{o}C	Degree celcius
V	Volt

CHAPTER 1

INTRODUCTION

1.1 Background

Mycobacterium tuberculosis (MTB) is an infectious agent, which is responsible for causing Tuberculosis (TB). According to the World Health Organization (WHO), 10.4 million TB cases with 1.4 million death cases reported globally in 2015 (WHO, 2016). An effective diagnosis is considered crucial for TB control, both for the treating of the infected individual as well as for the intervention of the public to decrease further spreading (Ryu, 2015).

Nowadays, biosensor technology for improving global health issues is in great demand especially in the diagnosis of critical diseases. Biosensor has been introduced to be studied extensively for their role in detecting different analytes in clinical analysis and medical diagnosis. The first biosensor was invented by Clark and Lyons in 1962 using the concept of 'soluble' enzyme electrode which is considered as the marker for biosensor technology advancement (Tehrani & Bavarian, 2016).

In the past two decades, the interest towards DNA biosensors is increased due to its ability to detect specific nucleic acid hybridization. Conventionally, the nucleic acid hybridization was applied in various methods, such as southern blotting and polymerase chain reaction (PCR). By taking advantages of different organism's genomic sequence, it leads the ways for numbers of research opportunities especially in medical diagnosis to detect different diseases especially the diseases originated from bacteria.

Electrochemical DNA biosensor method was introduced to overcome the deficiencies of conventional method such as culturing and smear spectroscopy which is laborious, time-consuming and often susceptible to errors. Interestingly, this DNA biosensor works by immobilizing single stranded DNA (ssDNA) onto electrochemical transducer to be recognized by target complementary ssDNA (Rahman et al., 2015). The ability of biosensor system to detect various concentration of analyte shows their level of sensitivity, however, in the diagnosis of critical diseases more attention has to be given as several procedures are lacking and need improvements.

Additional knowledge of different nanomaterial's capability is very crucial in searching the best nanomaterial that can improve performance of biosensor. In this work, to improve the electrical conductivity of the biosensor system, a negatively charged functionalized COOH-Fe_3O_4, and positively charged NCC/CTAB were

introduced as sensor platform. The used of nanocomposites materials based on carboxyl functionalized iron oxide/nanocellulose crystalline/cetyl trimethylammonium bromide (COOH-Fe_3O_4/NCC/CTAB) were considered as one of additional knowledge to contribute to the promising electrochemical DNA biosensor development. In this project the overall hybridization event for the detection of *Mycobacterium tuberculosis* (MTB) was monitored using ruthenium bipyridyl complex as redox indicator.

1.2 Problem statement

Mantoux tuberculin skin test (TST) is the first evaluation method for TB detection. Good result for this test requires 48 to 72 hours incubation period. Besides, the test needs to be done by a trained health worker to reduce error. Chest radiograph also one of the method for TB evaluation to detect chest abnormalities from the lesion that appears in the lungs. Unfortunately, the present of lesion might be mistakenly diagnosed for other lung diseases, and therefore it cannot be used definitely to diagnose TB.

For better MTB confirmation, acid fast bacilli staining and microbial culture technique has been considered as the 'gold standard' method (Cross et al., 2015) for detection (Kritski et al., 2015). Although, acid fast bacilli staining is quick and simple, confirmation using microbial culture technique is still required as it shows low and variable sensitivity. However, a culture technique requires a lot of time as it involves cell culture of a slow growing mycobacterium (Pullan et al., 2016). Currently, many methods have been introduced to detect the TB disease rapidly by developing low time consumption and sensitive diagnosis including a molecular diagnosis method (Tiwari et al., 2017). Nonetheless, despite all advantages, the complex instrumentation and the cost for its reagent are still considered as the main obstacles for these methods.

Recently, as the sensing technology was improved, various studied based on biosensor in clinical diagnosis had been reported includes electrochemical, piezoelectric, and optical biosensor. Electrochemical method has attracted more attention (Arduini et al., 2016; Justino et al., 2016; Yang et al., 2015) due to its characteristics of simple, low-cost, rapid response, and offer ease of operation (Liu et al., 2014). The detection in variety of analytes such as enzymes, proteins and DNA was also observed to be more possible.

The performance of biosensor was improved through application of screen printed carbon electrode (SPCE) which offer capability for surface modification (Kurbanoglu et al., 2017) plus additional advantages such as low cost, disposable, mass production, and portable (Mat Zaid et al., 2016). Another strategy was adopted through modification of SPCE's surface to improve the analytical performance. By introducing nanomaterial on the surface of SPCE, the biocompatibility, additional binding sites and signal intensities are hoping to be subsequently improved as well as

the sensitivity and specificity of MTB detection. Therefore, interest in the electrochemical DNA biosensor has been considered as one of the efforts to improve the detection of TB (Zaffino et al., 2015).

1.3 The objectives of this study

The aim of this study is to develop a simple electrochemical DNA biosensor for the detection of specific DNA sequence related to MTB assisted by ruthenium complex. The sensor incorporates the use of carboxyl functionalized iron oxide nanoparticles (COOH-Fe_3O_4) with nanocellulose crystalline surface which was modified by cetyl trimethylammonium bromide (NCC/CTAB) for performance enhancement. It is very interesting to explore the potential of this nanoparticles as it has not been reported in any previous TB detection.

The objectives set to achieve these goals include:

i. To develop electrochemical DNA sensor based on carboxyl functionalized iron oxide/nanocellulose crystalline/cetyl trimethylammonium bromide (COOH-Fe_3O_4/NCC/CTAB) modified screen printed carbon electrode (SPCE).
ii. To evaluate the sensing capability of the specific gene sequence of DNA in the detection of *Mycobacterium tuberculosis* (MTB) through the interaction between ruthenium complexes $Ru(bpy)_3^{2+}$ with DNA molecules.
iii. To apply and validate the fabricated biosensor on the analysis of TB real sample.

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