UNIVERSITI PUTRA MALAYSIA

ELECTROCHEMICAL ENHANCEMENT USING IRON OXIDE-GOLD NANOCOMPOSITE FOR DETECTION OF TUBERCULOSIS BASED ON rGO-APTES MODIFIED SCREEN PRINTED ELECTRODE

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FS 2019 79

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By

SYAZANA AMEERA BINTI SYED AMRI

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

April 2018

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

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Chairman: Professor Nor Azah Yusof, PhD
Faculty: Science

A new electrochemical immunosensor based on Fe_3O_4-Au nanocomposite (NC) as signal enhancement was proposed for the detection of M*ycobacterium Tuberculosis* ESAT-6-like protein EsxB. In this study, Fe_3O_4-Au NC was synthesis via co-precipitation method and size of NC was viewed under high resolution transmission electron microscopy (HRTEM) with average diameter of 1.629-1.915 nm. Meanwhile, X-ray diffraction (XRD) analysis confirmed that Fe_3O_4-Au NC exhibited all possible peaks of Fe_3O_4 and new peaks at 38.37° (111) and 44.63° (200) are belongs to crystal plane of metallic gold. Additionally, UV-Vis absorption study shows peak at 545 nm was appeared indicating that Au shell was successfully coated on Fe_3O_4 NC. Characterization by FTIR demonstrated that the peak at 1670 cm^{-1} assigned to C=O group and peak at 3280 cm^{-1} indicated O-H group. The peak at 710 cm^{-1} represented C-S stretching. The IR spectra confirms successful functionalization of the Fe_3O_4-Au NC with ME-MDDA linker. In this study, rGO-APTES have been used to modify screen printed electrode (SPE) to serve as immobilization matrix for immunoassay interaction. The result from RAMAN spectroscopy, Field Emission Scanning Electron Microscope (FE-SEM) and Energy Dispersive X-ray Spectroscopy (EDX) confirmed rGO-APTES modified SPE successfully reduced. Additionally, the morphology of rGO-APTES modified SPE also revealed smoother surface and ready for immobilization of capture antibody (Ab). The effective surface area value of rGO-APTES modified SPE also has increase about 20 times larger compared to bare SPE of based on cyclic voltammetry (CV) studies. Moreover, the ratio of Ipc/Ipa = 0.9867 showed electron transfer process is towards reversible kinetics. The rGO-APTES modified SPE was further explored as immunoassays matrix for Ab_1 tagged with Fe_3O_4-Au NC (Ab_1- Fe_3O_4-Au) and further interact with M*ycobacterium Tuberculosis* ESAT-6-like protein EsxB were monitored by differential pulse voltammetry (DPV) technique using 0.1 M PBS as electrolyte. As a result, the decreased of peak current of Ab from 3.996 µA to 3.863 µA occurs after immobilization on rGO-APTES modified SPE confirmed that electron transfer resistance increased. At optimal condition, the DPV was studied in different

concentration of M*ycobacterium Tuberculosis* ESAT-6-like protein EsxB. The DPV current of immunosensor electrode increased as the M*ycobacterium Tuberculosis* ESAT-6-like protein EsxB increased. In this study, the lowest TB concentration that could be determined statistical by resulting immunosensor was calculated as 1.32 ng/mL. The Relative Standard Deviation (RSD) of the measurements for five electrodes was 6.4% suggesting the precision and reproducibility of the immunosensor was quite good.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENINGKATAN ELEKTROKIMIA MENGGUNAKAN KOMPOSIT NANO FERUM OKSIDA-EMAS BERDASARKAN ELEKTROD DIMODIFIKASI OLEH rGO-APTES UNTUK MENGESAN TUBERKULOSIS

Oleh

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Sensor elektrokimia imun baru berdasarkan komposit nano (NC) Fe_3O_4-Au sebagai isyarat peningkatan terhadap pengesanan EsxB protein *Mycobacterium tuberculosis* ESAT-6. Dalam kajian ini, Sintesis NC Fe_3O_4-Au dilakukan melalui kaedah pemendakan dan saiz NC Fe_3O_4-Au telah dilihat di bawah mikroskop elektron penghantaran resolusi tinggi (HRTEM) dengan diameter purata 1.629-1.915 nm. Manakala, pencirian difraksi sinar X (XRD) bagi NC Fe_3O_4-Au menunjukan semua kemungkinan puncak Fe_3O_4 dan puncak baru di 38.37 ° (111), 44.63 ° (200) adalah milik kristal logam emas. Sebagai tambahan, pencirian oleh UV-Vis mempamerkan puncak pada 545 nm muncul menunjukkan bahawa Au berjaya disalut pada NC Fe_3O_4. Pencirian oleh FTIR menunjukkan bahawa puncak pada 1670 adalah merujuk kepada kewujudan C=OH cm^{-1} dan puncak 3280 cm^{-1} merujuk kepada kehadiran kumpulan O-H Manakala puncak 710 cm^{-1} menunjukkan kehadiran kumpulan C-S. Pencirian oleh IR membuktikan bahawa fungsian pautan ME-MDDA terhadap NC Fe_3O_4-Au berjaya dihasilkan. Dalam kajian ini juga, rGO-APTES telah digunakan sebagai platfom pemegun hasil modifikasi di atas electrod skrin bercetak (SPE) untuk immbolisasi interaksi assay imun. Hasil dari analisis RAMAN, FESEM dan EDX mengesahkan elektrod dimodifikasi oleh rGO-APTES mengalami penurunan dan mendedahkan permukaan yang lebih licin dan bersedia untuk memegunkan antibodi tangkapan (Ab). Nilai kawasan permukaan berkesan bagi elektrod dimodifikasi oleh rGO-APTES juga telah bertambah kepada 20 kali lebih besar berbanding dengan elektod yang tidak dimodifikasikan berdasarkan pencirian CV. Manakala, nisbah I_{pc}/I_{pa} = 0.9867 menunjukkan proses pemindahan elektron adalah ke arah kinetik berbalik. Elektrod dimodifikasi oleh rGO-APTES kemudian diuji untuk memegun Ab, dan interaksi antara Ab dengan EsxB protein *Mycobacterium tuberculosis.* dipantau oleh teknik DPV menggunakan elektrolit 0.1 M PBS. Hasilnya, pengurangan arus puncak Ab dari 3.996 µA hingga 3.863 µA berlaku selepas pemegunan ke atas elektrod dimodifikasi oleh rGO-APTES menunjukan bahawa rintangan pemindahan electron telah meningkat.

Pada keadaan yang optimum, EsxB protein *Mycobacterium tuberculosis* ESAT dikaji pada kepekatan yang berbeza menerusi pencirian DPV. Arus DPV menunjukkan peningkatan apabila kepekatan EsxB protein *Mycobacterium tuberculosis* ESAT-6 meningkat. Dalam kajian ini, kepekatan EsxB protein *Mycobacterium tuberculosis* ESAT-6 yang paling rendah yang dapat ditentukan oleh statistik imunosensor yang dihasilkan adalah 1.32 ng / mL. Penyimpangan Standard Relatif (RSD) pengukuran untuk lima elektrod ialah 6.4% menunjukkan ketepatan dan kebolehulangan semula imunosensor adalah baik.

ACKNOWLEDGEMENTS

I sincerely thank everyone who has supported and contributed to this research. I would like to especially thank Professor Dr . Nor Azah Yusof, my supervisor, for her guidance on this research. Throughout my master study, her support and encouragement has been absolutely crucial to growth as a master research student. She has given me a lot of helpful suggestion. I am also very grateful for having an exceptional advisory committee and wish to thank Dr Jaafar Abdullah. I have learnt a lot from their advices on my research. I would like to thank Dr Hanun for her support on the Tuberculosis detection research. I would also like to thank Kementerian Pelajaran Malaysia (KPM) under MyBrain14 program for providing scholarship for my study.

I would also like to extend my sincerest gratitude to my family and best friends, Ashraf Azri, Zul Adhlan, Norasidah and Aishah for all their support and encouragement during my Master pursuit, in particular my parents**, Syed Amri Bin Abdul Manan** and **Rozaida Binti Musa** who constant source of support. I would have never made it this far alone. Everything I have accomplished has been a combination of the support from all those in my life.

This thesis was submitted to the Senate of 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

TB	Tuberculosis
POC	Point of Care
SPE	Screen Printed Electrode
NPs	Nanoparticles
NC	Nanocomposite
DPV	Differential Pulse Voltammetry
Ab	Capture Antibody
Ab_1	Primary Antibody
rGO	Reduced Graphene Oxide
GO	Graphene Oxide
WHO	World Health Organization
TST	Tuberculin Skin Test
IGRAs	Interferon Gamma (IFN-Γ) Release Assays
PCR	Polymerase Chain Reaction
NAAT	Nucleic Acid Amplification Tests
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
ELISA	Enzyme Linked Immunosorbent Assay
PQC	Piezoelectric Quartz Crystal
PNA	Peptide Nucleic Acid
CV	Cyclic Voltammetry
APTES	Aminopropyl-Triethoxysilane
DNA	Deoxyribonucleic Acid
FESEM	Field-Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
EDX	Energy Dispersive X-Ray
HR-TEM	High Resolution Transmission Electron Microscopy
UV-Vis	Ultraviolet Visible
LOD	Limit of Detection
LOQ	Limit of Quantification

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Tuberculosis (TB) is a disease caused by bacteria known as *Mycobacterium tuberculosis* that frequently attack the lungs and eventually damage other parts of body. TB spread through the air when individual with TB disease coughs, sneezes or talks. TB is infectious, but it is not easy to catch. The chances of catching TB from someone we live or work with are much higher than from a stranger. Regardless of high possibility to get infected, most people with active TB who undergo appropriate treatment for at least two weeks were no longer contagious.

In world population, about one-third of population is believed to have latent TB. This means people had actually been infected by TB bacteria but are not yet ill with the disease and cannot transmit the disease. People infected with TB bacteria have a 10% lifetime risk of falling ill with TB (McIntosh, 2017). However, persons with compromised immune systems, such as people living with HIV, malnutrition or diabetes, or people who use tobacco, tend to have higher risk of falling ill. A person with active TB disease may show some symptoms such as fever, cough, night sweats and weight loss which may look usual for many months. This will result in delays in seeking care and transmission of the bacteria to others might occur. People with active TB can infect 10-15 other people through close contact over the course of a year.

About 45% of HIV-negative people with TB on average and nearly all HIV-positive people with TB will die without proper treatment. In 2014, 9.6 million cases were reported globally, of these, 1.5 million died from the disease and 5.7 million were newly diagnosed (Global *Tuberculosis* Control Report, 2014). In 2016, World Health Organization (WHO) reported there were seven countries that are prevalence to TB disease. India led with the highest percent of morbidity, followed by Indonesia, China, Philippines and Pakistan, Nigeria and South Africa. India, China and Indonesia alone accounted for 45% of global cases in 2016. These reports are summarize in bar chart and showed in Figure 1.1.

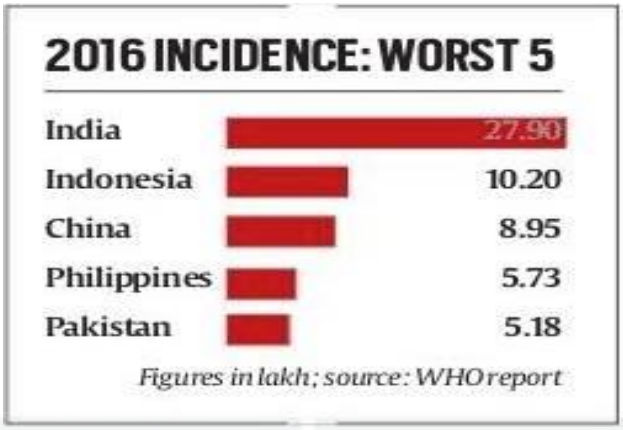

Figure 1.1: WHO Report in 2016 Showed the Worst 5 Countries that Prevalence to TB Disease. Resource from http://who.int/features/qa/79/en/.

TB is a treatable and curable disease. The majority of TB cases can be cured when medicines are provided and consumed properly. Early diagnosis is crucial to prevent TB bacteria from spreading and the treatment of infected individuals. Therefore, it is necessary to develop fast and precise diagnostic methods with higher sensitivity and specificity than existing assays.

1.2 Problem Statement

The world wide effort to stop TB has resulted in a cure and led to an increase in survival rate. However, the emergence of the human immune deficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) prevented global TB eradication, and TB remains a global hazard that requires attention. Furthermore, in the late 1990s, a new form of TB appeared known as drug-resistant TB (DR-TB). Through the first decade of the 21st century, the emergence of multidrug-resistant TB (MDR-TB) and extensively drug- resistant TB (XDR-TB) still obstruct control of TB. Most recently, rare but very dangerous forms of TB, extremely drug-resistant TB (XXDR-TB), super XDR-TB, and totally drug-resistant TB (TDR-TB) have emerged, especially in India and China (Cheon *et al.*, 2016). These drug-resistant TB strains arise owing to improper use of antibiotics in chemotherapy in patients with drug-susceptible TB, poor adherence to anti-TB drugs, and insufficient monitoring of drug resistance (Maitre *et al.*, 2017). In 2014, WHO estimated 9.6 million people suffered from TB and 1.5 million people with TB-related deaths internationally, and estimated 480,000 people worldwide developed MDR-TB and 190,000 people died of MDR-TB (World Health Organization, 2015a). TB is curable in 85% of cases with appropriate treatment, but a global epidemic may occur if MDR and XDR-TB cannot be diagnosed and treated in a timely manner, particularly in people co-infected with HIV.

Thus, the rapid diagnosis and treatment of infectors is crucial for the effective control of TB. Traditional microbial culture-based tests are the most common methodologies currently used. The methods involve are cell culture, cell counts and cell enrichment, however the methods are time consuming and laborious especially for slow growing bacteria like *Mycobacterium tuberculosis.* To date, various methods and techniques have been developed for rapid detection of M. tuberculosis, such as polymerase chain reaction (PCR), enzyme linked immunoassays (ELISA), TB rapid cultivation detection techniques, MB/Bact system and BactecMGIT 960 system promise more rapid and sensitive compare to traditional microbial cukture test, yet they cannot provide the detection results in real-time and required complex instrumentation and highly qualified technical staff.

Consequently, a rapid, sensitive and simple diagnostic test applicable in the field is highly needed. Making such a simple method available in the field, at the primary point of care (POC) could help diagnose of TB at early stage and may prevent more death from occur.

Recently, many studies have been engaged in developing biosensor to detect the TB antigen. Biosensor allows the detection of TB in complex sample matrices such as saliva, urine and serum. Biosensor offer high specificity by using biological sensing elements which can distinguish the targets from other microorganisms and the response time is rapid. It also provides continuous data with minimal quantity of the samples and it can detect the analytes on-line due to the sensor and the signal transducer in series.

Electrochemical immunosensor is a method of detection for TB that combine the high specificity of traditional immunochemical methods with low detection limits of modern electrochemical system (Gonzalez *et al*., 2005). For the design of an electrochemical immunosensor, the critical steps are the choice of the basis electrode and the immobilisation of the immunoreagent onto the electrode surface. Currently, several immunosensor devices have been developed on screen-printed electrodes (SPE). The miniature size of SPE and their ease of handling and also disposable has made the SPE ideal for immunosensor detection.

1.3 Nanomaterial Labels in Electrochemical Immunosensor

Electrochemical immunosensor have recently considerable because it offers high sensitivity, low cost and miniature. For immunoassay, the immunological materials are immobilized on SPE. Antibodies or antigen tagged with nanomaterials can retain their bioactivity and interact with their counterparts. The amount or concentration of analytes can be determined based on the electrochemical detection of those nano materials. The extensive signal enhancement associated with the use of nanomaterial amplifying tags and with the formation of nanomaterial–antibody–antigen assemblies increase sensitivity of immunosensor (Liu and Lin, 2007).

Magnetic nanoparticles (NPs) are the strong candidates for biological tags. Iron oxides (Fe_3O_4) are very promising as their biocompatibility has already been proven (Kekutia *et al.*, 2014) and its magnetic properties could separate non biological from biological tags with Fe_3O_4NPs which will be useful for separation purposes.

This research describes the development of electrochemical immunosensors based on iron oxide gold nanocomposite (Fe_3O_4-Au NC) as signals enhancement for the detection of *Mycobacterium tuberculosis*. Chapter 2 presents a review of the literature relevant to this research including current detection methods for TB and NPs-based biosensors. Chapter 3 describes the development of an electrochemical immunosensor for *Mycobacterium tuberculosis* ESAT-6-like protein $EsxB^2$ detection using Fe_3O_4-Au NC for target labelling and signal generation. Chapter 4 describes immunosensor based on Fe_3O_4-Au NC for *Mycobacterium tuberculosis* ESAT-6-like protein $EsxB^2$ detection using differential pulse voltammetry (DPV). Some discussion and the conclusion of this research are addressed in Chapter 5, as well as recommendations for future work.

1.4 Objective

The overall goal of this research is to develop a sensitive and rapid detection method using Fe_3O_4-Au nanocomposite as signal tags based on rGO-APTES modified SPE for *Mycobacterium tuberculosis* ESAT-6-like protein $EsxB^2$ detection. The immunosensor is based on primary antibody (Ab_1) conjugated Fe_3O_4-Au NC as target separation and target DPV for signal acquisition. According to the overall goal, the detailed objectives are:

1. To prepare rGO-APTES modified SPE via electrochemical reduction method and to characterize the resulting electrode.

2. To evaluate the capability of rGO-APTES modified SPE for interaction study between antibody and antigen in TB detection.

3. To characterize the sensing capability of immunoassays system for *Mycobacterium tuberculosis* ESAT-6-like protein $EsxB^2$ detection using Fe_3O_4-Au NC as signal enhancement.

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2. Workshop on advance Materials and Nanotechnology, UPM (4^{th} -5^{th} November 2015). (Participant).
3. Training at MIMOS Berhad (June 2015). (Participant).
4. Final Progress Seminar, Faculty of Science, UPM (6^{th} June 2016). (Participant).
5. 29th Malaysian Analytical Chemistry Symposium (SKAM 29), Penang (15^{th}-17^{th}\ August 2016). (Poster Presenter).
6. Workshop on in depth study of cyclic voltammetry (23^{rd} November 2016). (Participant).
7. Putra Conference: Sensor in Medical Diagnostic (4^{th} January 2017). (Participant).

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