**UNIVERSITI PUTRA MALAYSIA**

# *DEVELOPMENT OF PNA ELECTROCHEMICAL BIOSENSOR BASED ON SCREEN-PRINTED ELECTRODE-MODIFIED AMINE-FUNCTIONALIZED GRAPHENE COMPOSITE FOR Mycobacterium tuberculosis DETECTION*

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**ITMA 2018 11**

# DEVELOPMENT OF PNA ELECTROCHEMICAL BIOSENSOR BASED ON SCREEN-PRINTED ELECTRODE-MODIFIED AMINE-FUNCTIONALIZED GRAPHENE COMPOSITE FOR *Mycobacterium tuberculosis* DETECTION

**By**

**MOHD HAZANI BIN MAT ZAID**

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

**October 2017**

Abstract of the thesis submitted to the senate of, Universiti Putra Malaysia, in fulfillment of the requirement for the Degree of Doctor of Philosophy

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**October 2017**

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Agarose gel electrophoresis is a fundamental and essential technique for analysis of PCR products related of mycobacterium tuberculosis. However this technique imposes some sort of limitation such as unquantifiable results and low specificity. Therefore, a novel PNA electrochemical biosensor based on modified screen printed carbon electrode (SPCE) has been developed for detection of *mycobacterium tuberculosis* which could overcome the limitation of agarose gel electrophoresis technique. In this study, the fabricated electrochemical biosensor SPCE has been modified with two different nanomaterial consist of amine functionalized reduced graphene oxide ($NH_2$-GO) composited with water soluble CdS Quantum dots (QDs) and $NH_2$-GO composited with Tempo-nanocellulose (TNCC) for enhance sensitivity detection of target ssDNA sequence related to *Mycobacterium tuberculosis*. The prepared composite materials have been characterized by Raman, FTIR, and TEM. Meanwhile, field emission microscope (FE-SEM) and energy dispersive X-ray spectroscopy (EDX) analysis had confirmed that both nanomaterials ($NH_2$-GO/QDs and $NH_2$-GO/TNCC) were deposited and uniformly distributed on the surface of SPCE. Furthermore, based on impedance spectroscopy (EIS) and cyclic voltammetry characterization (CV), the modified electrode has shown an enhancement of surface active area and better conductivity compared to the unmodified electrode. Subsequently, both fabricated electrode was further explored as electrochemical biosensor platform based on immobilized PNA probe and methylene blue (MB) was used as an electrochemical indicator to evaluate the performance of PNA electrochemical biosensor. At optimum condition, PBS buffer was choose as supporting buffer, PNA probe concentration of 10 µM, ratio of EDC/NHS of 4 mM:5 mM, MB immersion time of 45 min, MB concentration of 35 µM, pH MB of 7.5, and hybridization temperatures of 27 $^{O}$C for $NH_2$-GO/QDs/SPCE biosensor. Meanwhile, for biosensor based on $NH_2$-GO/TNCC/SPCE, The optimum detection can be reach by using borate saline buffer as supporting buffer, PNA probe concentration of 20 µM, ratio of EDC/NHS concentration of 6 mM:5 mM (v/v), MB immersion time of 30 min, MB concentration

of 45 µM, pH MB of 9 and hybridization temperature at 40 $^{O}C$ . Moreover, reproducibility study exhibited good result with the RSD value of 4.46% for SPCE/$NH_2$-GO/QDs and 5.96% for SPCE/$NH_2$-GO/NCC, respectively with sufficient selectivity to discriminate between complementary, non-complimentary and one base mismatch DNA. The reduction peak current of MB after hybridization was proportional to the concentration of target MTB DNA in the range from $1.0\times10^{-13}$ to $1.0\times10^{-6}$ mol/L with a detection limit of 8.948 $\times10^{-13}$ M for $NH_2$-GO/QDs/SPCE and 3.14 x $10^{-14}$ M for $NH_2$-GO/TNCC/SPCE, respectively. In addition, the stability study has also shown that both fabricated biosensor could be stored at 4$^{O}C$ for 4 weeks with minimum degradation. Meanwhile, for the detection of *Mycobacterium tuberculosis* from the real sample, DNA amplification step is necessary to amplify genomic MTB DNA to obtain a highly specific MTB DNA fragment sequence. Six difference samples were tested on both fabricated biosensors and demonstrate good result with real sample based on polymerase chain reaction (PCR) amplification product of *M. tuberculosis* DNA. Both fabricated biosensor also showed successful discrimination on the positive and negative sample with a limit of detection of 2.49 ng/µl ($NH_2$-GO/QDs/SPCE) and 1.52 ng/µl ($NH_2$-GO/TNCC/SPCE), respectively. Therefore, the combination of PCR amplification with electrochemical detection provides a sensitive method for specific sequence detection of *mycobacterium tuberculosis* (MTB).

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PEMBANGUNAN BIOSENSOR PNA ELEKTROKIMIA BERASASKAN ELEKTROD SKRIN BERCETAK KARBON DIUBAHSUAI BAHAN KOMPOSIT AMINA GRAFIN OKSIDA UNTUK PENGESANAN *Mycobacterium tuberculosis***

Oleh

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Elektroforesis gel agarose adalah teknik asas dan penting untuk analisis produk PCR berkaitan *mycobacterium tuberculosis*. Walau bagaimanapun teknik ini mempunyai beberapa jenis kelemahan seperti menghasilkan keputusan yang tak kuantitatif dan mempunyai kepekaan yang rendah. Oleh itu, satu biosensor elektrokimia PNA baharu berdasarkan elektrod karbon dicetak skrin yang diubahsuai (SPCE) telah dibangunkan untuk pengesanan *mycobacterium tuberculosi* yang mana boleh mengatasi kelemahan teknik elektroforesis gel agarose. Dalam kajian ini, SPCE telah diubahsuai dengan dua nanomaterial komposit yang berbeza mempunyai fungsian amina grafin oksida terturun ($NH_2$-GO) dikompositkan dengan bahan larut air CdS titik kuantum (QDs) dan $NH_2$-GO dikompositkan dengan nanoselulos kristal ($NH_2$-GO/NCC) bagi meningkatkan sensitif pengesanan jujukan DNA sasaran berkaitan *Mycobacterium tuberculosis*. Bahan komposit yang disediakan ini telah diciri menggunakan Raman, FTIR dan TEM. Manakala, mikroskop medan pelepasan (FESEM) dan spektroskopi penyebaran tenaga sinaran-x (EDX) telah mengesahkan bahawa kedua-dua bahan-nano iaitu $NH_2$-GO/QDs dan $NH_2$-GO/NCC telah didepositkan dan diselerakkan secara sekata diatas permukaan SPCE. Selain itu, berdasarkan pencirian spektroskopi impeden (EIS) dan voltametri berkitar (CV), elektrod diubahsuai telah menunjukkan peningkatan luas permukaan aktif dan kekonduksian yang lebih baik berbanding elektrod yang tidak diubahsuai. Selanjutnya, kedua-dua elektrod yang difabrikasikan telah diterokai sebagai tapak biosensor elektrokimia berasaskan prob PNA pegun dan metilena biru (MB) telah digunakan sebagai bahan penunjuk elektrokimia untuk menilai prestasi kedua-dua biosensor PNA elektrokimia. Pada keadaan optimum, pemilihan PBS sebagai penampan sokongan , kepekatan prob PNA 10 μM, kadar EDC/NHS 4 mM:5 mM, masa rendaman MB 45 minit, kepekatan MB 35 μM, pH MB 7.5, dan suhu hibridisasi pada 27 $^{O}$C telah digunakan untuk biosensor $NH_2$-GO/ QDs/SPCE. Sementara itu, untuk biosensor berdasarkan $NH_2$-GO/TNCC/SPCE menggunakan larutan penampan salin borate sebagai penampan sokongan, kepekatan probe PNA 20

μM, nisbah kepekatan EDC/NHS sebanyak 6mM: 5mM (v/v), masa rendaman MB30 minit, kepekatan MB 45 μM, pH MB pada 9 dan suhu hibridisasi 40 $^{O}$C untuk mendapatkan tahap pengesana optimimum. Tambahan lagi, kajian kebolehulangan juga menunjukkan hasil yang baik dengan nilai RSD iaitu 4.46% untuk $NH_2$-GO/QDs/SPCE dan 5.96% untuk $NH_2$-GO/NCC/SPCE, masing-masing dengan keselektifan memadai dapat membezakan diantara DNA pelengkap, DNA bukan-pelengkap dan DNA satu asas tidak sepadan. Penurunan puncak arus MB selepas penghibridan berkadar terus kepada kepekatan DNA MTB sasaran dalam julat $1.0\times10^{-13}$ to $1.0\times10^{-6}$ mol/L dengan had pengesanan (LOD) masing-masing iaitu 8.948 × $10\text{-}^{13}$ M untuk $NH_2$-GO/QDs/ SPCE dan 3.14 x $10\text{-}^{14}$ M untuk $NH_2$-GO/NCC/SPCE. Tambahan pula, kajian kestabilan juga telah menunjukkan bahawa kedua-dua biosensor yang difabrikasikan boleh disimpan pada 4°C selama 4 minggu (28 hari) dengan kemerosotan yang minimum. Sementara itu, untuk pengesanan *Mycobacterium tuberculosis* dari sampel sebenar, dapat dilihat bahawa peningkatan isyarat arus MB dicerap apabila penghibridan prob PNA dengan DNA genomik berlaku. Walaubagaimanapun, hasil yang ditunjukan tidak cukup jelas untuk membuktikan keupayaan kedua-dia biosensor yang telah difibrikasikan kerana isu pelekatan tidak spesifik dari DNA genomik yang panjang. Oleh itu, teknik tindakbalas rantai polimerase (PCR) telah digunakan untuk pengandaan jujukan spesifik sasaran untuk mendapatkan jujukan spesifik DNA MTB yang tinggi. Enam sampel berlainan telah diuji keatas kedua-dua biosensor yang difabrikasi dan menunjukkan hasil yang baik dengan sampel sebenar berasaskan penggandaan produk DNA M. tuberculosis melalui tindakbalas rantai polimerase (PCR). Kedua-dua biosensor juga berjaya membezakan diantara sampel positif dan negatif dengan had pengesanan (LOD) adalah 2.49 ng/µl ($NH_2$-GO/QDs/SPCE) and 1.52 ng/µl ($NH_2$-GO/TNCC/SPCE), masing-masing. Oleh itu, kombinasi penggandaan produk DNA M. tuberculosis mengunakan amplifikasi PCR dan pengesanan elektokimia telah meyediakan kaedah yang berkesan untuk pengesanan mycobacterium tuberculosis (MTB).

# ACKNOWLEDGEMENTS

Alhamdulillah, this is long journey for me. All the praises and gratitudes are due to Allah S.W.T. First and foremost I would like to thanks my supervisor Dr. Jaafar bin Abdullah for his great guidance, patience, constructive comment and support during all the stages of this Ph.D. study. I would also like to greatly appreciate for my co-supervisor Prof. Nor Azah Yusof and Dr. Helmi Wasoh for their limitless help during my research study.  Gratefully acknowledge to Dr. Rahizan Issa for her helps and advice during my Ph.D. program. Special thank goes to my close research partner Puzi Alttas, Fariza, and Amalina. I am also thankful to the people in the biosensor group in 103 lab and bacteriology unit staff under Institute of medical research (IMR). I am grateful and pray for your success in this life and the next to come. To the University Putra Malaysia (UPM) thank you so much for providing research facilities and Kementerian Pengajian Tinggi Malaysia (KPTM) for providing financial aids in Mybrain 15 (My Ph.D.)

Special thanks to my mother Nahyati binti Salleh and my father Mat Zaid bin Ismail for their love, support, and encouragement. Not forget to my brothers and lastly, I would acknowledge to Nur Hafiza Nasir who accompanied me throughout the entire process in pursuing my degree of doctor of philosophy. Thank you very much

I certify that a Thesis Examination Committee has met on 25 October 2017 to conduct the final examination of Mohd Hazani bin Mat Zaid on his thesis entitled "Development of PNA Electrochemical Biosensor Based on Screen-Printed Electrode-Modified Amine-Functionalized Graphene Composite for *Mycobacterium tuberculosis* Detection" 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 Doctor of Philosophy.

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| | |
|---|---|
| PNA | Peptide Nucleic Acid |
| DNA | Dioxyribonucleic Acid |
| GO | Graphene Oxide |
| RGO | Reduce Graphene Oxide |
| QDs | Quantum Dots |
| TNCC | TEMPO-Nanocrystalline cellulose |
| NCC | Nanocrystalline cellulose |
| MTB | *Mycobacterium tuberculosis* |
| SPCE | Screen Printed Carbon Electrode |
| MB | Methylene Blue |
| CV | Cyclic Voltammetry |
| EIS | Electrochemical Impedance Spectroscopy |
| DPV | Differential Pulse Voltammetry |
| PCR | Polymerase chain reaction |
| $E^{O}$ | Formal Reduction Potential |
| Ei | Initial Potential |
| $E_{pa}$ | Anodic Peak potential |
| $E_{pc}$ | Cathodic peak potential |
| $\Delta E_{p}$ | Peak potential separation |
| i | Current |
| $i_{pa}$ | Anodic Peak Current |
| $i_{pc}$ | Cathodic Peak Current |
| n | Number ofelectron |
| Q | Quantity of charge |

| | |
|---|---|
| POC | Point Of Care |
| EDC/NHS | 1-Ethyl-3-(3-dimethylaminopropyl (EDC)/ N-hydroxysuccinimide (NHS) |
| $T_m$ | Melting temperature |
| Thyb | Hybridization temperature |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of study

*Mycobacterium tuberculosis* (MTB) is an obligate pathogenic bacterial species in the family of *mycobacteriaceae* and a causative agent of most cases of tuberculosis. Tuberculosis bacteria can spread through the air, either by coughing, sneezing, talking, or spitting. Only a few of these bacteria can inflict a healthy person to be infected (WHO, 2015).

In the current situation in Malaysia, tuberculosis (TB) is fast rising as a non-communicable disease (NCD), with the death rate even higher than that of dengue where a total of 1,520 deaths from TB were recorded in 2012 compared to 34 deaths from dengue in the same year. Latest data provided by world health organization (WHO) shows that a total number of TB cases were recorded in Malaysia about 24220 TB cases with the Figures of mortality was 2400 people (WHO, 2015b). Traditionally, microbial culture-based tests were used as a diagnostic method to identify *mycobacterium* bacteria based on sputum sample from the patient. However, this approach is time consuming and laborious, especially for the slow-growing bacteria like MTB (Tortoli et al., 1997; Colebunders et al., 2000). As time evolved, alternative methods that have been developed for rapid detection of MTB include polymerase chain reaction (PCR) (Brisson et al., 1999, Wilson et al., 1994), Latex agglutination (Krambovitis et al., 1984), enzyme-linked immunosorbent assay (Chan et al., 2000), radiometric detection (Middlebrook et al., 1997), gen-probe amplified *M. tuberculosis* direct test (AMTDT) (Gamboa et al., 1997), TB rapid cultivation detection technique, such as MB/Bact system (Liu et al., 2001), BactecMGIT 960 system and flow cytometry (Qin et al., 2008). These methods are more sensitive and rapid than the traditional microbial culture-based method (Siddiqi et al., 2003). However, they cannot provide the detection results in real-time, and most of these methods require complex instrumentation and highly qualified personnel. Therefore, the development of portable, real-time, sensitive, rapid, and accurate methods detection is essential to prevent tuberculosis infection effectively.

The biosensor is the best candidate to overcome this situation. Currently, various reviews on biosensors have been reported for tuberculosis detection includes electrochemical sensors (Liu et al., 2014), piezoelectric biosensors (Kaewphinit et al., 2010), optical biosensors (Prabhakar et al., 2008) and magnetoelastic biosensors (Park et al., 2012). Although the reported biosensors have gained excellent performance. However, the sensitivity is a major issue that requires further improvement.

Electrochemical DNA biosensors recently have found significant applications for on-site detection especially for clinical and medical diagnostics (Zhou et al., 2011). While offering simplicity in operation and sample manipulation, the electrochemical biosensor also yields highly sensitive and specific measurements for a broad spectrum of biomolecules (Liepold et al., 2005). Moreover, sample size requirement for performing electrochemical biosensors is small, ranging from several microliters to hundreds of nanoliters, which includes the sample pretreatment reagents (Wei et al., 2010). Additionally, the latest technology has introduced commercially available screen printed electrode (SPE) which offer disposability, lower cost, and miniaturization (Dominguez et al., 2007). However, the most important feature of electrochemical biosensors is their potential to be easily transformed from a laboratory-based instrument to the point of care (POC) device.

Compared to their DNA counterparts, Peptide Nucleic Acid (PNA) recognition layers can offer significant advantages for sequence-specific DNA biosensors. As previously reported, PNA provides higher sensitivity and specificity including greater discrimination against single-base mismatches, faster hybridization at room temperatures, and minimal dependence on ionic strength, and promising for possible employment in the fabrication of biosensors for various applications (Wang et al., 1996). As a consequence, PNA has stimulated several studies to test new capabilities and opportunities afforded by the use of PNA as surface probes.

With exceptional achievements in nanotechnology and nanoscience, nanomaterial-based electrochemical signal amplifications offer huge potential of improving both sensitivity and selectivity for electrochemical sensors and biosensors (Zhu et al., 2015). Graphene oxide (GO) is one of the advance nanomaterials can be used as an electrode modifier in electrochemical biosensor provided a biocompatible matrix to improve the performance of electrochemical biosensor (Sharma et al., 2015). More recently, the applications of being explored widely on the surface functionalization of GO by combination with long chain aliphatic group, aromatic amines, amino acids, and polymers (Kuila et al., 2012). As a result, reduced GO is also called functionalized GO or chemically reduced GO provide abundant structural defects and functional groups offer advantageous for electrochemical applications (Tang et al., 2009).

In addition, the combination of GO or reduced graphene oxide (rGO) with others nanoparticles may contribute additional advantages such as to enhance the shelf-life of the chosen bio-recognition element (Artiles et al., 2011). Over the past few years, semiconductor quantum dots (QDs) have attracted increasing attention for sensing application due to its unique properties such as high surface activity, promote electron transfer and fixing the immobilization of biomolecules (Matsuno et al., 2006). The combination of reduced graphene oxide (rGO) and QDs will be given advantages in the development of electrochemical sensors, especially as immobilization matrix. Moreover, hydrophilicity, biocompatibility, high specific surface, good electrical conductivity and be able to form a good film on electrode surface also provide extra benefits for sensor febrication. Although these types of method are well established to be used as a detection mechanism, however, the nanocomposite material based on reduced graphene oxide composited quantum dots has not been reported yet for the *M. Tuberculosis* detection.
Nanocrystalline cellulose (NCC) is another nanomaterial with high surface area that can be easily functionalized and is biocompatible and ecofriendly. It has been used stand alone and in combination with other nanomaterials to improve biosensor

fabrication (Edward et al., 2013). Depend on preparation method, the obtained NCC currently has been studied due to its biocompatible, mechanical, functional, and biodegradable properties, which enable a wide dimension application in composite material (Lin et al., 2014). All these features offer the great potential of NCC in the electrochemical biosensor construction. Thus, combination NCC and GO will provide a new platform for the attachment of biomolecule without losing their bioactivity. Additionally, the designed biosensor based on the GO and NCC composite is expected to display excellent analytical performances for the detection of *mycobacterium tuberculosis* with high sensitivity, wide linearity range and lower detection limit.

## 1.2 Problem Statement

Early detection of tuberculosis remains crucial for its effective control as it is one of the leading causes of death worldwide. It is essential for global public health protection to detect, analyze, and quantify *Mycobacterium tuberculosis* (MTB) by sensitive and cost effective methods.

Current clinical diagnostic for the detection of mycobacteria relies on molecular biology diagnostic via polymerase chain reaction (PCR) combine with agarose electrophoresis technique for separation and purification of nucleic acids. This approach is proven to be highly sensitive and can easily be detected by simple visual observation of the test and the control tubes even at the lowest concentration of the targeted sequence (Alli et al., 2011). However, the main limitation when agarose gel electrophoresis is employed for identifying genes (DNA) of *mycobacteria tuberculosis* by size separation of the DNA fragment (Brody et al., 2004). Firstly, gel electrophoresis relies on the observation on band density of different spots on the gel and observation of this band density does not yield unquantifiable results and has low specificity, therefore cannot differentiate the size of amplified DNA between target DNA and non-complimentary target DNA. Secondly, this method has some degree of error since the bands formed in the gels tend to be fuzzy and spread apart and samples are usually run multiple times to get clean results. Thirdly, the analysis procedure of agarose gel electrophoresis is time consuming, tedious step, and require hazardous element such as ethidium bromide for gel staining to visualize DNA in agarose gel. Thus, electrochemical PNA biosensor seems to be the best candidate to replace agarose gel electrophoresis part in PCR diagnostics method because PNA electrochemical biosensor could display a high specificity of amplified nucleic acid between specific and nonspecific DNA target. Moreover, the level of concentration in amplified nucleic acid can be quantified by monitoring the current signal. However, electrochemical detection needs to undergo electrode surface modification process to enhance the sensitivity of the electrodes. For this purpose, utilization of electrode modifier material for amplification purposes is necessary for the bare electrode.

To date reduced graphene oxide (rGO) incorporate with quantum dots (QDs) and nanocrystalline cellulose (NCC) as a novel nanomaterial platform for PNA immobilization for *Mycobacterium tuberculosis* detection has not yet been explored. Thus, suitability of these composite materials for PNA immobilization and hybridization is still unknown. Additionally, understanding the nature of PNA as a hybridization probe on GO with QDs and GO with NCC probably produce another advantage that offers compatible microenvironment with DNA related *Mycobacterium tuberculosis.* Hence, the potential applications of both composite materials are beneficial in the designs for point of care device based biosensors. Additionally, the utilization of PNA instead of DNA as sensing probe in the fabrication of electrochemical biosensor can enhance selectivity and sensitivity of detection of *Mycobacterium tuberculosis* DNA that cannot be achievable with traditional oligonucleotides technique.

## 1.3 Objective of the study

The goal of this study is to develop a simple and sensitive PNA electrochemical biosensor as an alternative to the gel electrophoresis method for early detection of nucleic acid related *Mycobacterium tuberculosis*. This can be achived by incorporate reduced graphene oxide (rGO) with quantum dots (QDs) and reduced grapehene oxide (rGO) with nanocrystalline cellulose (NCC) as a sensing material for enhance the sensitivity of detection. The following specific objectives are designed to achieve the aim of this research;

1) To prepare and characterize amine functionalized graphene oxide/CdS quantum dots and amine functionalized graphene oxide/Tempo-nanocellulose composite as a sensing material.

2) To fabricate PNA electrochemical biosensor based on amine functionalized graphene oxide/CdS quantum dots and amine functionalized graphene oxide/TEMPO-nanocellulose composite composite for the detection of *mycobacterium tuberculosis.*

3) To evaluate the analytical performance of the fabricated electrochemical PNA biosensor from amplified real sample related to *Mycobacterium tuberculosis.*

## 1.4 Novelty and benefits of research

Presently, the application of electrochemical biosensor PNA probe as biorecognition element on the modified electrode based on reduced graphene oxide composited water soluble quantum dots (CdS) is the first being employed. Similarly, reduced graphene oxide composited nanocrystalline nanocelulose was first use with PNA probes for the detection of *Mycobacterium tuberculosis.* Additionally, both fabricated electrochemical PNA biosensor could be used as an alternative way to the gel electrophoresis in PCR detection method greatly reduce the cost of chemical and time detection.

## 1.5 Scope and limition of study

Agarose gel electrophoresis is common method for the detection of *M. tuberculosis* from clinical samples by PCR. However, this method relies on the band density (darkness) of the different spots on the gel which are analysis of sample is based on qualitative determination. Another limitation of this method are involve tedious preparation procedure, required longer time for analysis of sample (more than one hour) and the sample usually have to be run multiple times to obtain clean and accurate result. Therefore, PNA based electrochemical biosensor was suggested as an alternative method for the detection of *M. tuberculosis* which offers rapid detection time, simple pretreatment of sample, less expensive and possible for portable equipment. Moreover, the suggested method also offers quantifiable measurement, sensitivity and selectivity detection compared to agarose gel electrophoresis technique.

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