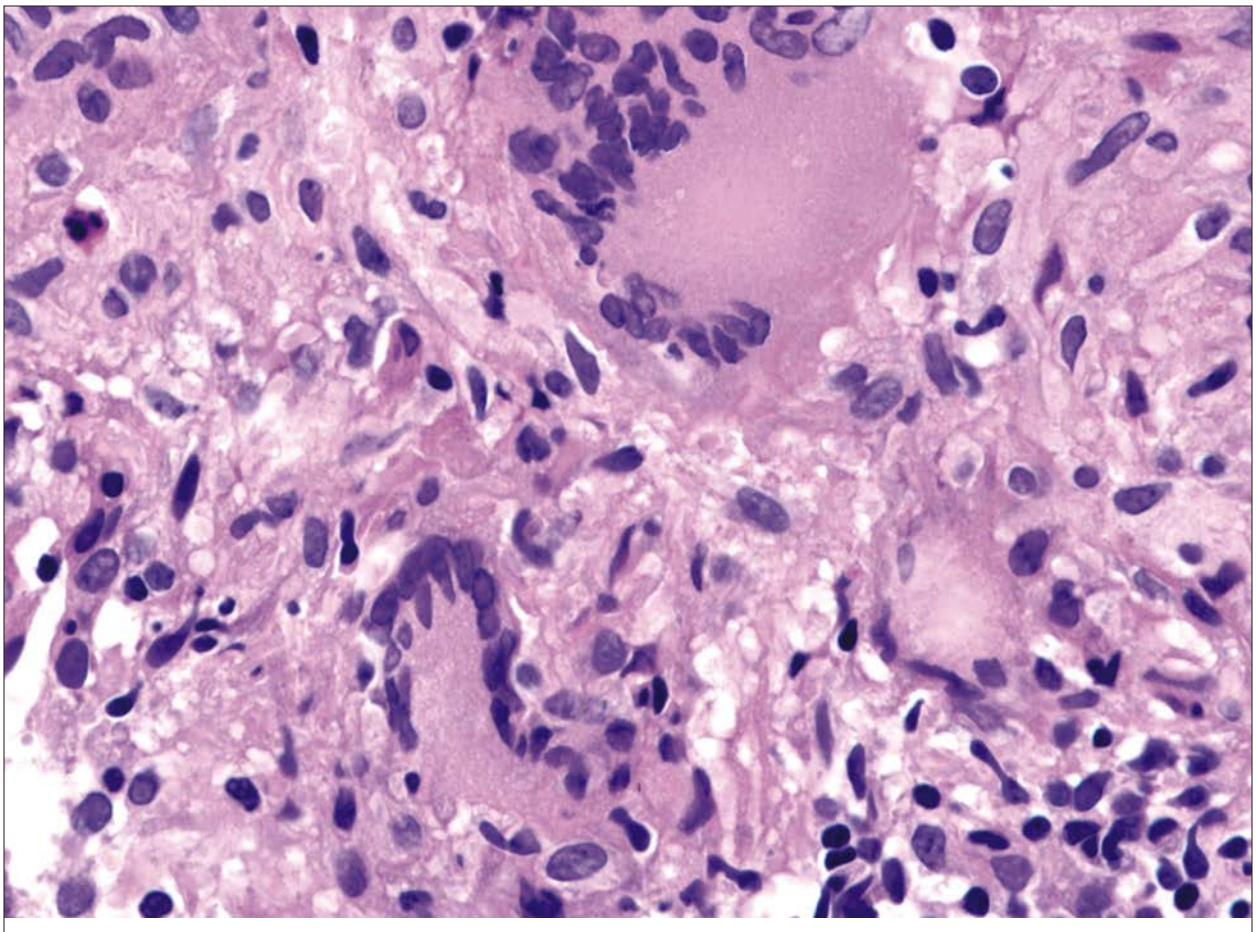

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Electrochemical Technique using Methylene Blue with Pencil Graphite Electrode for Optimum Detection of *Mycobacterium tuberculosis* DNA

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ABSTRACT

The analysis of nucleic acids recognition using genosensor provides a rapid, sensitive and inexpensive detection for infectious and genetic diseases, bacteria food contaminations, forensic and environmental research. A simple application of electrochemical biosensor for the alternative detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) using Pencil Graphite Electrode (PGE) and Methylene Blue (MB) as electroactive intercalators via non-covalent attachment was developed. Synthetic oligonucleotides of *M. tuberculosis* which consisted of M. Probe, M. Target, M. Non-complementary and M. Mutation were used for DNA hybridization detection on PGE. Differential Pulse Voltammetry (DPV) was performed using a PalmSens Electrochemical Portable Apparatus controlled by a Pocket PC. Various parameters affecting the response of the signals were explored and optimized including M. Probe concentration, immobilization time and hybridization time of immobilized M. Probe, concentration and accumulation time of MB, as well as the concentration of M. Target. The results obtained after measurement showed that the voltammetric signal of MB before hybridization was higher compared to after hybridization. This indicates that MB has high affinity towards guanine bases. Differences in the signals of MB for hybridization between M. Probes and Polymerase Chain Reaction (PCR) amplified products were observed. The Relative Standard Deviation (RSD) for TB PCR positive sample, TB PCR positive control, TB PCR negative sample, TB PCR negative control and TB PCR blank were 11.5, 2.9, 22.8, 13.8 and 20.3%, respectively which can be applied for the detection of *M. tuberculosis*.

Key words: DNA biosensor, pencil graphite electrode (PGE), non-covalent attachment, differential pulse voltammetry (DPV), methylene blue (MB)

INTRODUCTION

Mycobacterium tuberculosis is an infectious agent that causes tuberculosis (TB) (Nikalje and Mudassar, 2011). The bacteria are released when a person with TB

disease is coughing, sneezing, talking or even laughing (Talat *et al.*, 2002). According to the WHO (2010), tuberculosis can be defined as a disease of poverty which affecting young adults in their most productive years. Furthermore, majority of the TB death occurred in the developing world. In 2009, about 1.7 million people died from TB including 380,000 women and 380,000 people with HIV disease which are equal to 4,700 deaths a day. This infectious disease is among the three greatest causes of death among women mostly aged between 15 to 44. Meanwhile, as reported in the Health-Fact 2009 and Health-Fact 2008 by Ministry of Health of Malaysia (2008, 2009), the incidence rate recorded by WHO (2009) was 63.95 cases per 100,000 population compared to 63.10 cases per 100,000 population in 2008. The mortality rate for 2009 and 2008 were 5.59 and 5.49 cases per 100,000 populations.

Conventional methods had been conducted to detect TB including acid fast staining (Ziehl-Neelsen) and culturing on Lowenstein-Jensen media (El-Demellawy *et al.*, 2006). Now-a-days, the molecular assays are used such as Real Time RT-PCR and ELISpot as to replace the conventional method (Abdelwahab, 2009). As reported by Abdelwahab (2009) the conventional methods are not suitable to be conducted in the laboratories due to their insensitive nature. PCR is rapid, sensitive and specific molecular assay (El-Demellawy *et al.*, 2006) so it can be used to diagnose pulmonary and extra pulmonary tuberculosis. Furthermore, more than ten samples can be analyzed at the same time.

Yean *et al.* (2008) reported that various type of problems were faced in conducting the diagnosis of tuberculosis in the clinical samples. The utilization of toxically harmful agents such as UV light and ethidium bromide during conducting agorose gel electrophoresis might contribute to respiratory system disorder. When using specialized instrument such as real-time PCR, some expensive chemical reagents are required include of SYBR green dye, Taqman or molecular beacons. In addition, misinterpreted may also occurred caused either by cross-contamination, inhibitors existence or handling error of the clinical samples (El-Demellawy *et al.*, 2006). Furthermore, the usage of an expensive instrument such as real time RT-PCR requiring high ability and expertise in handling the instrument.

Genosensor is also known as an electrochemical hybridization biosensor and widely used in electrochemical analysis. Genosensor can be described as a small device that has the biological recognition properties which converts a biological response into an electrical signal (Suman and Kumar, 2008). According to Erdem *et al.* (2001) and Suman and Kumar (2008), electrochemical genosensor provides fast, simple and sensitive method for the detection of DNA sequences in human, viral and bacterial nucleic acids. Other advantages can be obtained from identification of nucleic acids which include of the detection of human diseases, bacterial food contaminations to the forensic and environmental research (Kuswandi and Sevilla, 2002; Suman and Kumar, 2008).

Methylene Blue (MB), Meldola Blue (MDB) and cobalt (II) bipyridine are commonly used as a hybridization indicator in the electrochemical studies to investigate and identified the DNA nucleotides sequences (Karadeniz *et al.*, 2006). Methylene Blue (MB) is an effective hybridization indicator had been widely used in various types of research especially in the detection of infectious diseases. MB has an aromatic heterocyclic structure which is belonged to the phenothiazine family (Kara *et al.*, 2002; Meric *et al.*, 2002b). The interaction between MB with DNA probe can be investigated and explored through electrochemical methods. MB has higher affinity towards guanine bases (Meric *et al.*, 2002a). As reported by Yan *et al.* (2001), MB could be the effective electroactive hybridization indicator compared to rubidium (II) complex with 2, 2'-bipyridine ligand

[$(Ru(bpy)_3)^{2+}$] according to the differences between the absorbance spectra and voltammetric signal. MB intercalated itself directly to the guanine bases of DNA and improves the DNA binding affinity by electrostatic interaction with negative charge of phosphate backbone (Kara *et al.*, 2002). Erdem *et al.* (2002) also supported that lower current signal was due to less binding of MB to dsDNA because of the inaccessibility of the guanine bases after hybridization.

This study described a fast, sensitive and simple application of genosensor in electrochemical analysis using Pencil Graphite Electrode (PGE) as a transducer and Methylene Blue (MB) for the detection of *M. tuberculosis*. The interaction of MB with DNA oligonucleotides on modified Pencil Graphite Electrode (PGE) surface were explored and measured via non-covalent attachment. The results were applied for the detection of amplified PCR products of *M. tuberculosis*.

MATERIALS AND METHODS

Apparatus: Three electrodes system were used consisted of an Ag/AgCl as reference electrode, platinum electrode as an auxiliary electrode and a disposable graphite electrode as working electrode. A mechanical pencil model Zebra TS-3 Mech Pencil made from Japan was used as graphite leads holder. The graphite lead was 0.5 mm Ultra-Polymer-Leads Mines from Tombo, Japan. A metallic wire was soldered to the metallic part as to provide the electrical contact with the lead (Fig. 1). Differential Pulse Voltammetry (DPV) measurements were carried out by using a PalmSens Electrochemical Portable Apparatus controlled by a Palmsens, PC (The Netherlands). The convective transport was provided by a magnetic stirrer.

Reagents: Methylene blue was purchased from Sigma. The DNA oligonucleotides of *M. tuberculosis* were purchased (as lyophilized powder) from Bio Basic Inc. (Torbay Road Markham Ontario, Canada) consisted of 20-mer sequence. The base sequences are shown below:

- M. Probe: 5'-CTC gTC CAg CgC CgC TTC gg-3'
- M. Target: 5'-CCg AAg Cgg CgC Tgg ACg Ag-3'

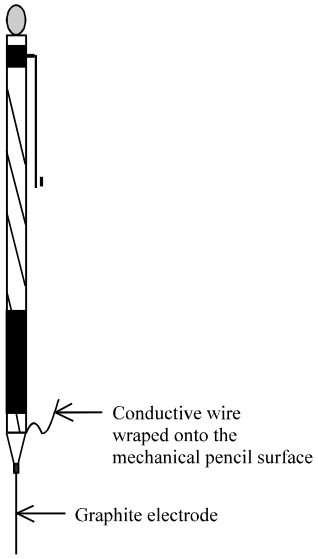

Fig. 1: The structure of Pencil Graphite Electrode (PGE)

- M. Non-complementary: 5'-TTT ggT ATT ATT gTT CAT gT-3'
- M. Mutation: 5'-CTC gTC CAg CgC CIC TTC gg-3'

DNA oligonucleotides stock solution were prepared in 10 mM Tris-HCl containing 1 mM EDTA (TE buffer, pH 8.0) and kept frozen at -20°C until used. Diluted solution of DNA oligonucleotides were prepared with either 0.5 M acetate buffer solution containing 20 mM NaCl (pH 4.8) or 20 mM Tris-HCl buffer solution containing 20 mM NaCl (pH 7.0). Meanwhile, the PCR samples of *M. tuberculosis* were prepared as described by Haron *et al.* (2008). All stock solutions were prepared using ultrapure and autoclaved water and experiments were conducted at room temperature (27.0±0.5°C).

Procedures: The procedures as described below were referred to Kara *et al.* (2007) with a slight modification.

Preparation of Pencil Graphite Electrode (PGE): PGE was prepared by cutting 6 cm lead into 3 cm long sticks. A marker was used to separate the section of PGE. The PGE with diameter 0.5 mm, was held vertically with 1.5 cm of the PGE was immersed in the solution.

Activation of PGE surface: The PGE surface was activated by immersing 1.5 cm lead into 0.5 M acetate buffer solution containing 20 mM NaCl (pH 4.8) and applying +1.4 V for 60 sec. The procedure was repeated until no interferences were observed.

Immobilization of M. Probe onto the pretreated PGE surface: About 10 μg mL^{-1} of M. Probe was prepared in 0.5 M acetate buffer containing 20 mM NaCl (pH 4.8) was immobilized on a pretreated 15 mm PGE surface for 25 min without applying any potential. Then, M. Probe immobilized PGE was washed with the same buffer solution for 5 sec. This method was repeated for M. Mutation immobilized PGE.

Hybridization of M. Probe immobilized PGE with M. Target and M. Non-complementary: About 15 μg mL^{-1} of M. Target which was prepared in 20 mM Tris-HCl containing of 20 mM NaCl (pH 7.0) was hybridized onto the M. Probe immobilized PGE surface for 9 min. After hybridization process, the surface was washed by immersing the hybridized M. Target-M. Probe-PGE into 0.02 M Tris-HCl containing 20 mM NaCl (pH 7.0) for 5 sec. The method was repeated for hybridization with M. Non-complementary.

Hybridization of M. Probe immobilized PGE with TB PCR products: The diluted TB PCR blank [1:40 in 0.05 M phosphate buffer solution (PBS, pH 7.4)] was denatured to form single stranded DNA by heating in the termomixer at 95°C for 5 min. Then, the denatured TB PCR blank was immediately freezed in ice bath to prevent reannealing. About 15 mm of immobilized M. Probe-PGE was immersed into the denatured sample solution for 9 min. The electrode was then washed with 2 X SSC (pH 7.0) for 5 sec. The same procedure was repeated using other TB PCR products which were negative control, negative sample, positive control and positive sample as described above.

Accumulation of Methylene Blue (MB): MB was accumulated onto the hybridized M. Target-M. Probe immobilized on the pretreated PGE surface by immersing the electrode into 20 mM Tris-HCl containing 20 mM NaCl and 20 μM MB (pH 7.0). This procedure was conducted by applying a potential of +0.50 V for 5 min in stirred condition. Then, the PGE was washed with 20 mM Tris-HCl (pH 7.0) solution for 5 sec. The above procedure was repeated using hybridized M. Mutation-M. Target immobilized PGE and hybridized TB PCR samples-M. Probe immobilized PGE.

Differential Pulse Voltammetry (DPV) measurement: The reduction signals of MB was measured in 20 mM Tris-HCl (pH 7.0) solution without applying any potential, with an amplitude of 10 mV and scan rate at 20 mV sec^{-1}. Repetitive measurements (n = 3) were carried out for the above assay format.

RESULTS

Optimization effects: The values are presented as Relative Standard Deviation (RSD). The standard deviation obtained is divided with average (both standard deviation and average are obtained after 3 repetitive measurements) and multiply with 100% to get the value of RSD.

Investigations on several parameters effect were successfully done using Differential Pulse Voltammetry (DPV). According to the Fig. 2a, the optimum concentration of M. Probe chosen for this study was 10 μg mL^{-1} with the Relative Standard Deviation (RSD) of 16.5%. Figure 2b, c and d showed the effect of M. Target concentration, immobilization time of M. Probe and hybridization time of M. Target, respectively. The optimum target concentration selected was 15 μg mL^{-1} with the RSD of 10.1% while 30 min and 9 min time were selected for immobilization and hybridization time, respectively with the RSD of 2.1 and 30.7%. The effect of indicator binding reaction was displayed in Fig. 2e and f include of concentration and accumulation time of MB towards DNA. The results showed that 20 μM was the optimum concentration (10.2%) and 3 min time was chosen as the optimum effect during accumulation of MB with the RSD of 13.8%.

Oligonucleotides analysis of *Mycobacterium tuberculosis*: The results obtained from the optimization effects were applied in the oligonucleotides analysis. As displayed in Fig. 3a, the voltammetric signals for M. Probe before hybridization (with the RSD of 31.5%) was higher compared to after hybridization with M. Target (Fig. 3b, e). The RSD for both M. Probe and M. Mutation hybridized with M. Target (Fig. 3b, e) were 37.9 and 8.7%, respectively. The voltammetric signal for M. Probe hybridized with M. Non-complementary was almost the same as the signal for M. Probe with the RSD of 27.1%. Meanwhile, the voltammetric signal for immobilized M. Mutation onto PGE surface was slightly decrease compared to the voltammetric signal of M. Probe with the RSD of 16.3% (Fig. 3d).

Analysis of TB PCR products: Analysis of TB PCR products was performed using immobilized M. Probe onto the PGE surface, to assess whether the method could respond selectively to the M. Target as the complementary DNA to the M. Probe. TB PCR products contain of TB PCR Positive sample, TB PCR Positive control, TB PCR Negative sample, TB PCR Negative control and TB PCR Blank. The voltammetric signal obtained from M. Probe hybridized with TB PCR Positive sample and TB PCR Positive control as shown in Fig. 5a and b were lower compare to other TB PCR

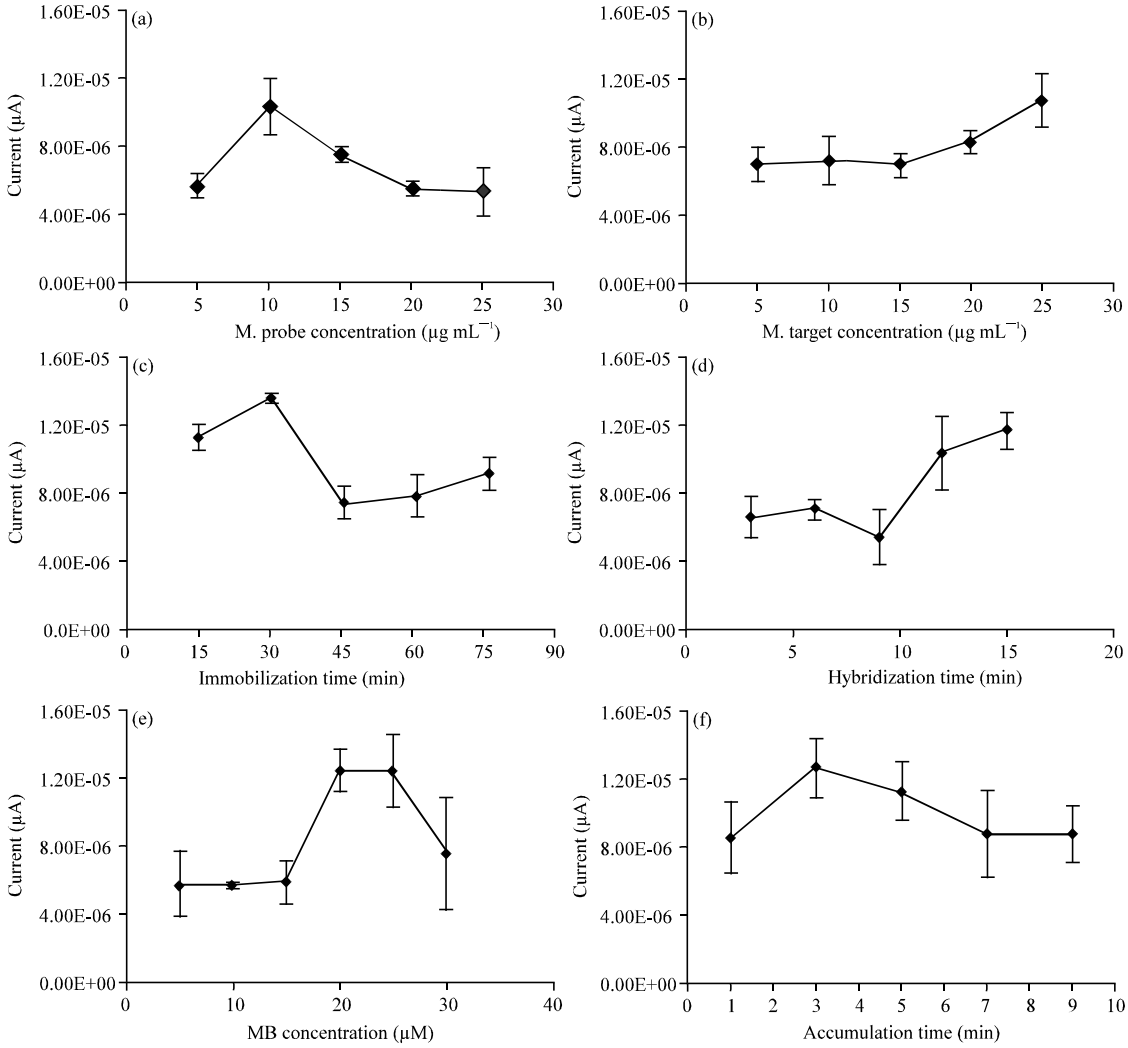

Fig. 2 (a-f): DPV effects (n = 3) of (a) M. Probe concentration, (b) M. Target concentration, (c) immobilization time of M. Probe, (d) Hybridization time of M. Target, (e) MB concentration and (f) accumulation time of MB

products with RSD of 11.5 and 2.9%, respectively. Higher voltammetric signals were observed for M. Probe hybridized with TB PCR Negative sample, TB PCR Negative control and TB PCR Blank (Fig. 5c, d and e), respectively with the RSD of 22.8, 13.8 and 20.3%.

DISCUSSION

Optimization effects: This electrochemical study was performed by using Differential Pulse Voltammetry (DPV) and economical transducer of Pencil Graphite Electrodes (PGEs). Label-based genosensor of Methylene Blue (MB) was used as an effective hybridization indicator. Early study of interaction between MB and DNA was investigated by Erdem *et al.* (2001) using Cyclic Voltammetry (CV). As reported by Kara *et al.* (2002), 20 mM was chosen as the optimum ionic strength. The salt concentration of 10 mM NaCl was found to be the critical ionic strength with

equal interaction between the intercalative interaction and the electrostatic interaction. The potential value remained constant after 10 mM NaCl, indicating that the voltammetric signal was derived from MB and the ionic shielding of the negatively charges of phosphate backbones on the DNA was achieved. MB will no longer interact with DNA electrostatically.

Several optimization effects were explored including the immobilization process of M. Probe, hybridization process with M. Target and indicator binding reaction between MB and DNA. As shown in Fig. 2a, the voltammetric signals of MB increased from 5 to 10 μg mL^{-1} and decreased until 20 μg mL^{-1} before remained constant till 25 μg mL^{-1}. Hence, the probe concentration was chosen as 10 μg mL^{-1}, as it was the optimum concentration for M. Probe to completely accumulate onto the PGE surface. Figure 2b showed the effect of target concentration onto the M. Probe-modified PGE. The optimum M. Target was chosen as 15 μg mL^{-1}. This is due to the lowest voltammetric signal observed when 10 μg mL^{-1} of M. Probe was exposed to 15 μg mL^{-1} of M. Target. The results indicated that the formation of double-stranded DNA was successfully occurred. The voltammetric signals of MB were increased from 15 μg mL^{-1} till 25 μg mL^{-1} as the concentration of M. Target increased. This is due to the excessive M. Target flanking at the PGE surface after hybridization process (Ozkan *et al*., 2002). Furthermore, the result was supported by Erdem *et al*. (2001) which reported that guanine bases were 200 times less reactive in dsDNA in comparison to the ssDNA and hence produced lower voltammetric signal of MB after hybridization with complementary sequence.

Both Fig. 2c and d displayed the effects of immobilization and hybridization time. The optimum time for M. Probe to successfully immobilize onto the PGE surface was selected as 30 min as it was the optimum analytical performance. Meanwhile, 9 min was chosen as the optimum time for hybridization process of M. Target onto the PGE-modified M. Probe through the formation of hydrogen bond. The results were supported according to the previous study by Issa *et al*. (2010). The parameters of indicator binding reactions were also investigated. According to the Fig. 2e, the optimum concentration of MB selected was 20 μM. Meanwhile, 3 min had been selected as it was the optimum accumulation time of MB (Fig. 2f). The estimated RSD for both MB concentration and accumulation time of MB were 10.2 and 13.8%, respectively. MB has two amino groups at both sides of the aromatic ring which might contributed to the binding effect with the negatively charges of phosphate backbones of guanine bases (Kerman *et al*., 2004a).

Oligonucleotides analysis of *Mycobacterium tuberculosis*: Figure 3 displayed the histograms for oligonucleotides analysis of *Mycobacterium tuberculosis*. Higher voltammetric signals were observed for M. Probe and M. Probe-M. Non-complementary (a and c) since there was no binding reaction occurred (Erdem *et al*., 2001; Issa *et al*., 2010). Thus, the formation of double helix DNA was not established. Meanwhile, the voltammetric signals of MB after hybridization with M. Target were lower compared to before hybridization according to the Fig. 3b. This is due to the formation of double helix DNA through hydrogen bond after hybridization process. As reported by Kara *et al*. (2002), MB has higher affinity towards guanine bases and therefore higher voltammetric signal of MB was observed before hybridization with complementary target. Guanine bases in double stranded DNA were known to be 200 times less effective than the guanine bases in the single stranded DNA as reported by Erdem *et al*. (2001). In the other words, the formation of duplex DNA protected the negative charges of guanine bases from attack by the incoming positive charges of MB. M. Mutation as well as M. Non-complementary acted as control experiment to investigate the response in the hybridization process. The MB signals of M. Mutation

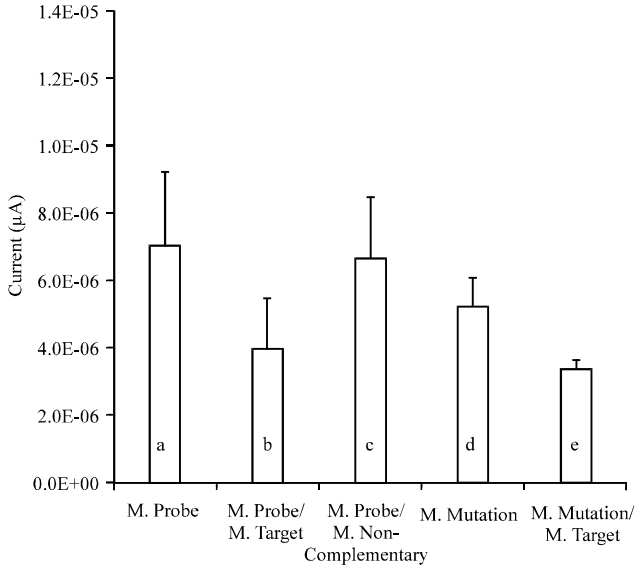

Fig. 3: Histograms for the mean and standard deviation of the MB reduction signals (n = 3): (a) hybridized M. Probe (b) after the hybridization of M. Probe with M. Target (c) after the hybridization of M. Probe with M. Non-complementary (d) hybridized M. Mutation and (e) after hybridization of M. Mutation with M. Target

was lower (d) when compared to immobilized M. Probe. This might be due to the oligonucleotides sequence of M. Mutation which having one single mismatch of inosine base. Thus, fewer amounts of positively charged of MB intercalated towards the guanine bases (Kara *et al.*, 2002). The lower signals of MB was observed after hybridization process between M. Mutation and M. Target, since inosine base can also bind to cytosine by forming two hydrogen bonds. Inosine is also known as an electro-inactive analogue of guanine (Kerman *et al.*, 2004b). Three repetitive measurements (n = 3) were carried out for all DPV analysis including the parameter effects. The results obtained from oligonucleotides analysis were supported by Sabzi *et al.* (2008) which investigated the detection of Human Papilloma Virus (HPP) target DNA using methylene blue and pencil graphite electrode. The differential pulse voltammograms signals of 20 μM MB for DNA oligonucleotides analysis were displayed in Fig. 4.

Analysis of TB PCR products: Analysis of TB PCR products was also performed using DPV measurement as displayed in Fig. 5. The hybridized TB PCR products onto the M. Probe was conducted by diluting the TB PCR products into 1:40 in 0.5 M phosphate buffer solution (pH 7.4). Hybridization with both TB PCR Positive sample and TB PCR Positive control (Fig. 5a,b) gave lower voltammetric signals indicating that the hybridization process was occurred and forming the duplex DNA through formation of either two or three hydrogen bonds. TB PCR Positive sample and TB PCR Positive control contained DNA which was taken from sample of TB patient and cultured TB, respectively (Issa *et al.*, 2010). The decrease in the voltammetric signal indicated that MB could not successfully interact with the bound guanine bases and showed that the hybridization process was occurred. It was attributed to the steric inhibition of the reducable groups of the intercalated

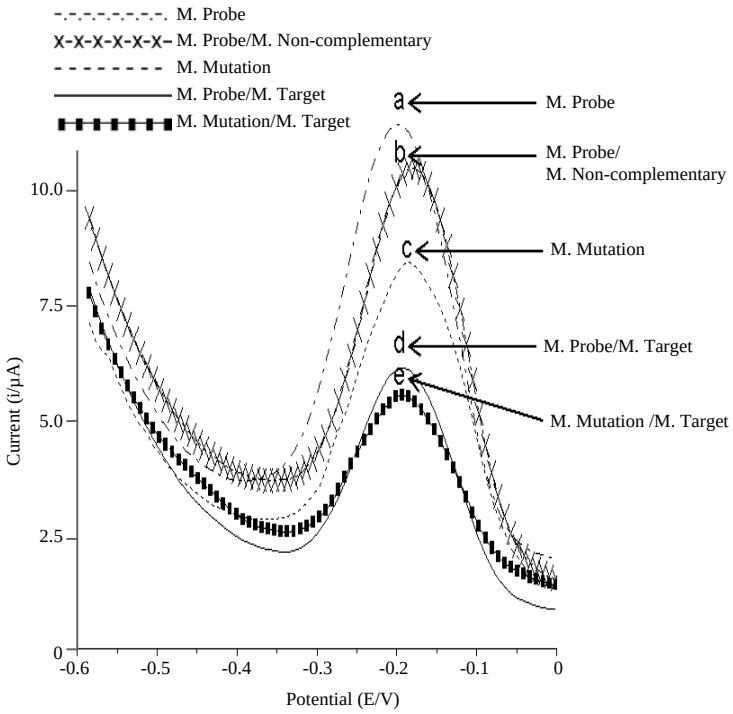

Fig. 4: Differential pulse voltammograms for three repetitive measurement (n = 3) using 20 μM MB as an effective indicator at: (a) 10 μg mL^{-1} of M. Probe (b) 10 μg mL^{-1} of M. Probe after hybridization with 15 μg mL^{-1} of M. Non-complementary (c) 10 μg mL^{-1} of M. Mutation (d) 10 μg mL^{-1} of M. Probe after hybridization with 15 μg mL^{-1} of M. Target and (e) 10 μg mL^{-1} of M. Mutation after hybridization with 15 μg mL^{-1} of M. Target. Activation of PGE surface was done by pretreatment process for 1 min at +1.40 V in ABS; DNA immobilization was done for 25 min by immersing the PGE into 0.5 M ABS containing 10 μg mL^{-1} of M. Probe; Hybridization was done for 6 min by immersing the PGE into 0.02 M Tris-HCl (pH 7.0) containing 15 μg mL^{-1} M. Target or M. Non-complementary; Accumulation of MB was done for 5 min at +0.5 V potential in 0.02 M Tris-HCl (pH 7.0) containing 20 μM MB

MB because of the formation of dsDNA at the electrode surface (Meric *et al.*, 2002b). Meanwhile, higher voltammetric signal was observed for hybridized TB PCR Negative control (Fig. 5c) as it contained other cultured bacteria (Issa *et al.*, 2010). Hence, no binding reaction occured and the formation of duplex was not established. The similar voltammetric signals was also obtained after hybridized with TB PCR Negative sample and TB PCR Blank (Fig. 5d,e). The *M. tuberculosis* DNA was not present in TB PCR Negative sample as it was contain of DNA taken from human free from TB. TB PCR Blank contained the mixture of master mix and distilled water (Issa *et al.*, 2010). Thus, no binding reaction occured and produced higher voltammetric signals after measurement. Figure 6 showed the differential pulse voltammograms of 20 μM MB towards the TB PCR products. The analysis of TB PCR products were also supported as described by Meric *et al.* (2002b) in the detection of TT Virus (TTV) and Hepatitis B Virus (HBV) from Polymerase Chain Reaction (PCR) amplified real samples using electrochemical biosensor and Methylene blue as hybridization indicator.

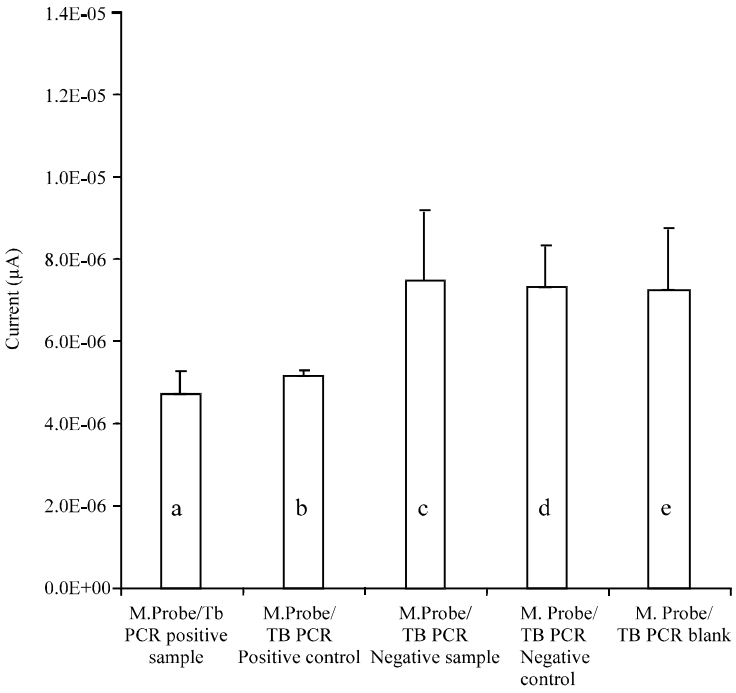

Fig. 5: Histograms for the MB reduction signals (n = 3) after hybridization of M. Probe with: (a) TB PCR posative sample (b) TB PCR positive control (c) TB PCR negative sample (d) TB PCR negative control and (e) TB PCR blank

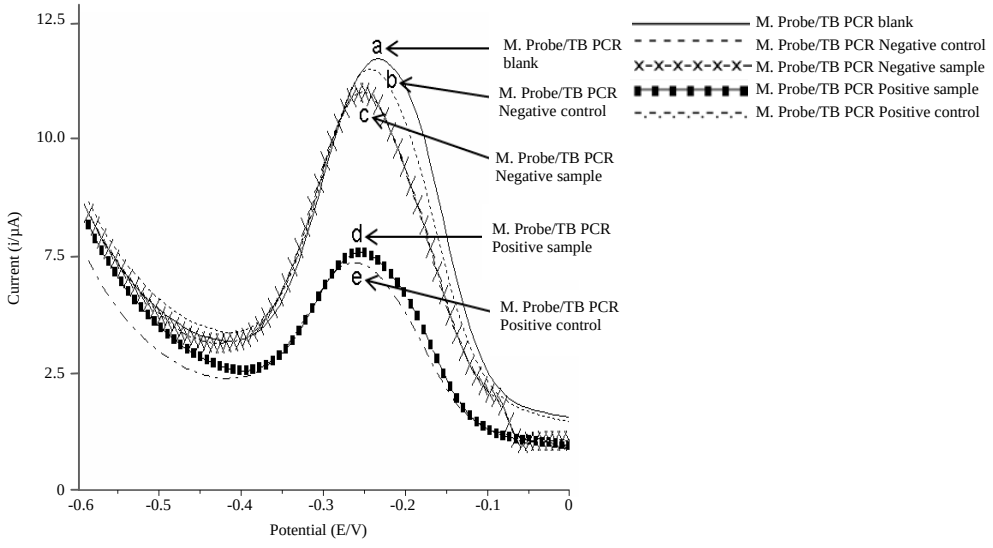

Fig. 6: Differential pulse voltammograms for three repetitive measurement (n = 3) using 20 μM MB as hybridization indicator for 10 μg mL^{-1} Probe modified PGE after hybridization with: (a) TB PCR blank (b) TB PCR negative control (c) TB PCR negative sample (d) TB PCR positive sample and (e) TB PCR positive control. Hybridization process was done for 6 min by immersing the probe modified PGE into PCR amplified products (concentration: 1/40) diluted with 0.05 M PBS (pH 7.4)

CONCLUSION

This research study described a simple, fast and sensitive procedure using electrochemical genosensor for the detection of *M. tuberculosis* from the PCR amplified products. The immobilization and hybridization protocol of DNA oligonucleotides on a PGE surface via non-covalent attachment were successfully optimized and obtained. The reduction signals of MB were observed differently before and after hybridization process. The results indicated that MB has lower affinity towards guanine bases of double stranded DNA compared to single stranded DNA due to the shielding effects. Hence, we can conclude that MB could be used as an effective indicator in the development of electrochemical genosensor procedure for the detection of various types of disease.

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