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Differential Pulse Voltammetric Determination of DNA Hybridization using Methylene Blue on Screen Printed Carbon Electrode for the Detection of *Mycobacterium tuberculosis*

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Abstract: The aim of the present study was to apply a small dimension, low cost and simple electrochemical biosensor for the detection of nucleic acid related to tuberculosis (TB) using Methylene Blue (MB) as the electroactive intercalator. The MYC Probe was immobilized on the Screen-Printed Carbon Electrode (SPCE) surface via non covalent attachment. The electrochemical biosensor of the DNA hybridization was evaluated using Differential Pulse Voltammetry (DPV) for measuring the interaction of MB with DNA oligonucleotides on SPCE using a PalmSens Electrochemical Portable Apparatus. Parameters affecting the response of the voltammetric signals towards detecting nucleic acid were optimized including concentration of MYC Probe, immobilization time of MYC Probe, hybridization time of immobilized MYC Probe, concentration of MB and time of MB accumulation. Various synthetic oligonucleotides of *Mycobacterium tuberculosis* (*M. tuberculosis*) were used such as MYC Probe, MYC Target, MYC Non-complementary and MYC Mutation for the DNA hybridization detection on SPCE. Differences in the voltammetric signals of MB were observed between MYC Probe and Polymerase Chain Reaction (PCR) amplified products for the detection of *M. tuberculosis* DNA. It is concluded that MB could be used as an effective electroactive hybridization indicator in the development of DNA biosensors for detection of *M. tuberculosis* DNA.

Key words: DNA biosensor, immobilization, hybridization, indicator, tuberculosis

INTRODUCTION

Tuberculosis (TB) is known as a 'killer' disease and one of the major causes of death worldwide. Determination and identification of TB becomes more important nowadays. Researchers are developing new, simple, direct, sensitive and specific diagnostic techniques for detecting tuberculosis (Rock *et al*., 2008).

DNA biosensor based on nucleic acid recognition process has an important role and can be applied in detection from environmental, pharmaceutical and clinical samples. The protocols related to the electrochemistry have an advantage in research and development in providing a rapid, simple and inexpensive testing of infectious disease (Wang *et al*., 1997; Erdem *et al*., 2002). Hybridization of Polymerase Chain Reaction (PCR) amplicons has also been employed in DNA biosensor (Yean *et al*., 2008). PCR has been used as a diagnostic tool for detection of tuberculosis (Haron *et al*., 2008). Application of PCR and electrochemistry technique would further enhance the detection of infectious diseases.

There are electrochemical studies reporting the interaction of redox indicators such as echinomycin and cobalt phenanthroline for the determination of DNA oligonucleotides sequences (Karadeniz *et al*., 2006). Methylene Blue (MB) is well-known as an effective electroactive hybridization indicator. It is an aromatic heterocyclic organic dye belonging to the phenothiazine family. The cationic charge of MB will intercalate directly to the guanine bases and binding affinity of the DNA is due to the electrostatic interaction with negative charge of phosphate backbone (Kara *et al*., 2002). Ozkan *et al*. (2002a, b) were using the redox marker of MB for detection of nucleic acid sequences.

In this study, the electrochemical method using DPV was tested to study the interaction of MB with DNA oligonucleotides on SPCE surface via non covalent attachment. The results obtained from this experiment were applied to detect the amplified PCR products of *M. tuberculosis*.

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MATERIALS AND METHODS

This study was conducted in January till December 2009. Differential pulse voltammetry measurements were carried out using a PalmSens Electrochemical Portable Apparatus controlled by a PalmsensPC. Screen Printed Carbon Electrodes (SPCE) were used, consisted of working electrode, reference electrode and an auxiliary electrode (Fig. 1). The convective transport was provided by a magnetic stirrer.

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

- MYC Probe: 5'-CTC gTC CAg CgC CgC TTC gg-3'
- MYC Target: 5' -CCg AAg Cgg CgC Tgg ACg Ag -3'
- MYC Non-complementary: 5' -TTT GGT ATT ATT GTT CAT GT -3'
- MYC Mutation: 5'-CTC gTC CAg CgC CIC TTC gg-3'

The 20-base sequence of MYC Target is complementary to 20-base sequence of MYC Probe. These DNA oligonucleotides solution were prepared in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0 (TE solution) and kept frozen at -20°C until use. In the hybridization protocol, diluted solutions of DNA oligonucleotide probe were prepared with either 0.50 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). The PCR samples of *M. tuberculosis* were prepared as described by Haron *et al*. (2008). Deionized water was used in all solutions. Experiments were performed at room temperature (27.0±0.5°C).

SPCE pretreatment: The surface of the working electrode was activated by applying +1.4 V for 1 min in 0.5 M acetate buffer solution (ABS) containing 20 mM NaCl (pH 4.8) without stirring (modified from Kerman *et al*. (2004). The electrode was washed with deionized water.

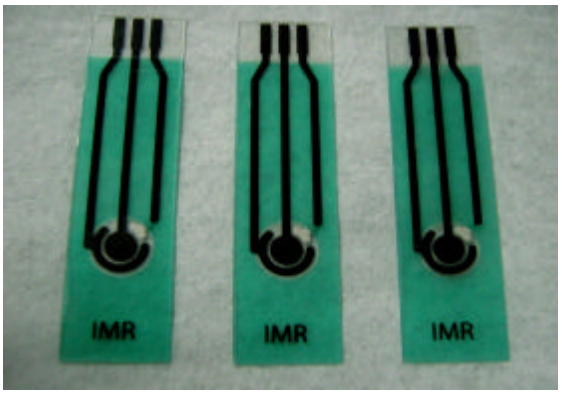

Fig. 1: Screen printed carbon electrode (SPCE)

DNA oligonucleotide immobilization: A solution of 20 μL of MYC Probe (10 μg mL^{-1} in 0.5 M ABS) was immobilized on a pretreated SPCE for 25 min without applying any potential (modified from Kerman *et al*., 2004). After immobilization, the immobilized MYC Probe on SPCE was washed with 0.5 M ABS containing 20 mM NaCl (pH 4.8) for 5 sec. The method was repeated for immobilization of MYC Target on SPCE.

Hybridization with MYC Target and MYC Non-complementary: A solution of 20 μL of MYC Target (15 μg mL^{-1} in 20 mM Tris-HCl containing 20 mM NaCl, pH 7.0) was dropped onto the MYC Probe immobilized on SPCE surface for 6 min (modified from Kerman *et al*., 2004). Then, the surface was washed by immersing the hybridized MYC Target-MYC Probe immobilized on SPCE into 0.02 M Tris-HCl containing 20 mM NaCl (pH 7.0). The method was repeated for hybridization of MYC Non-complementary with MYC Probe immobilized on SPCE.

Hybridization with TB PCR samples: The TB PCR blank [1:40 diluted in 0.05 M phosphate buffer solution (PBS), pH 7.4] was denatured by heating at 95°C for 5 min and immediately freezed in ice bath to prevent reannealing (modified from Kerman *et al*., 2004). A solution of 20 μL of denatured TB PCR sample was dropped onto the MYC Probe immobilized on SPCE for 6 min. The electrode was washed with 2 X SSC for 5 sec. The method was repeated using other TB PCR samples (negative control, negative sample, positive control and positive sample) as described above.

Accumulation of MB: The MB was accumulated to the hybridized MYC Target-MYC Probe immobilized on SPCE surface by immersing the electrode into solution of 20 μM MB (pH 7.0) in 20 mM Tris-HCl containing 20 mM NaCl (modified from Kerman *et al*., 2004). The condition was continuosly stirred, at the potential of +0.50 V for 5 min. The electrode was washed with solution of 20 mM Tris-HCl (pH 7.0) for 5 sec. The assay was repeated using hybridized MYC Mutation -MYC Target immobilized on SPCE.

Differential Pulse Voltammetry (DPV) measurement: The DPV of MB reduction signals was measured in solution of 20 mM Tris-HCl (pH 7.0) without applying any potential, an amplitude of 10 mV and scan rate at 20 mV sec^{-1} (modified from Kerman *et al*., 2004). Repetitive measurements (n = 3) were carried out for the assay format.

RESULTS AND DISCUSSION

The detection of immobilization and hybridization for non covalent attachment on SPCE were accomplished using DPV measurements of MB. Figure 1 shows the SPCE used in this study. Interaction of MB with MYC Probe was investigated at SPCE surface under several optimizations conditions to study the effects of the parameters (Fig. 2a-f). Figure 3 shows the histograms for the MB reduction signals after hybridization. Figure 4 shows the decrease in DPV measurements during the binding of guanine bases with MB. Figure 5 shows DPV measurements of 20 μM MB as hybridization indicator of DNA oligonucleotides on SPCE surface. Figure 6 shows the decrease in DPV measurements after hybridizations with PCR amplified products.

Kara *et al.* (2002) reported that, the critical ionic strength at which the electrostatic interaction equals the intercalative interaction was at 10 mM NaCl showing a constant potential signal and after this electrostatic interaction range, the potential signal values would increased rapidly. The constant potential value indicated that the ionic shielding of the negative charges on the DNA was achieved and thus MB could no longer interact

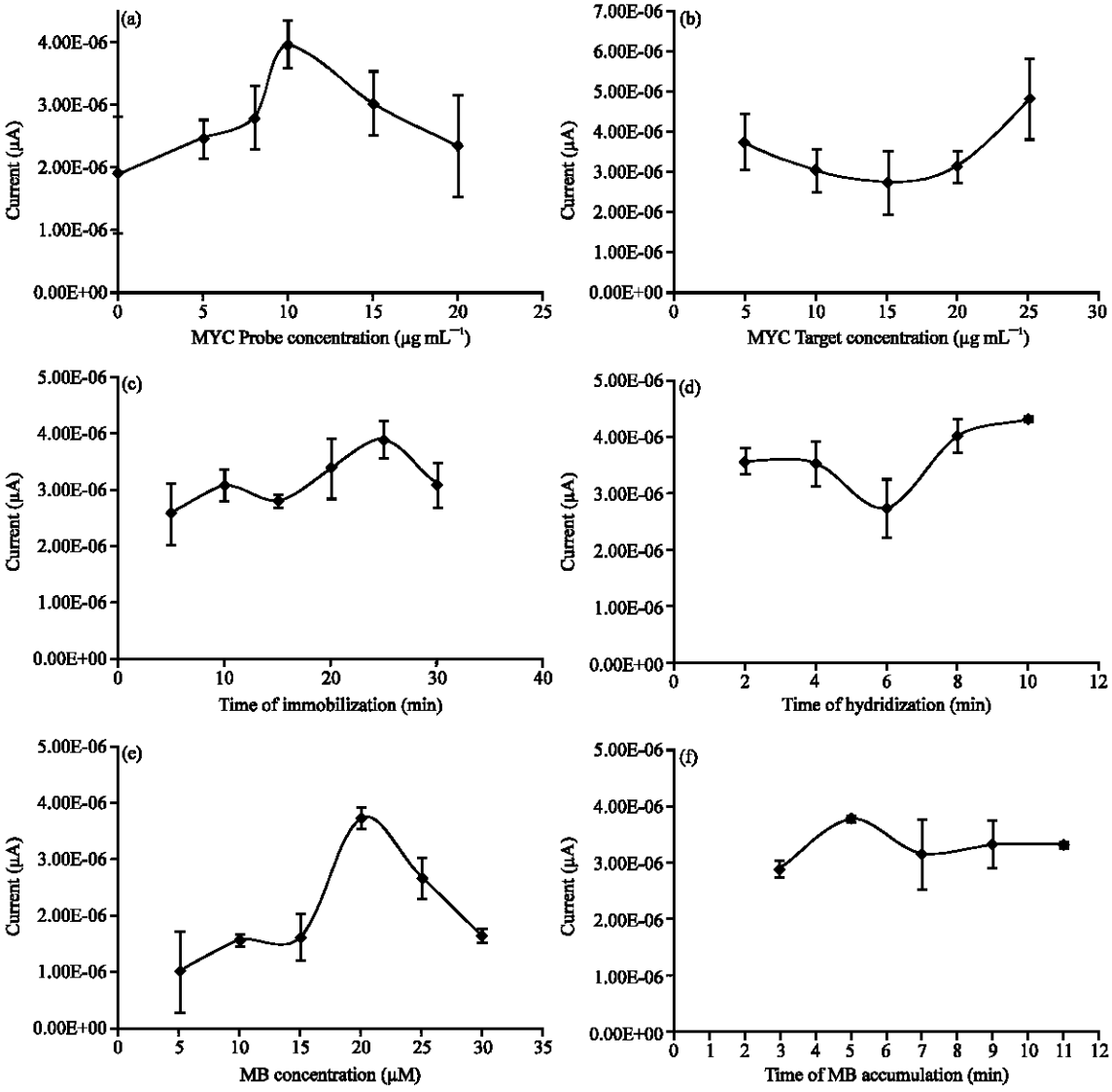

Fig. 2: Differential pulse voltammograms effects (n = 3) of (a) MYC Probe concentration, (b) MYC Target concentration, (c) immobilization time of MYC Probe, (d) hybridization time of MYC Target, (e) MB concentration and (f) MB accumulation time

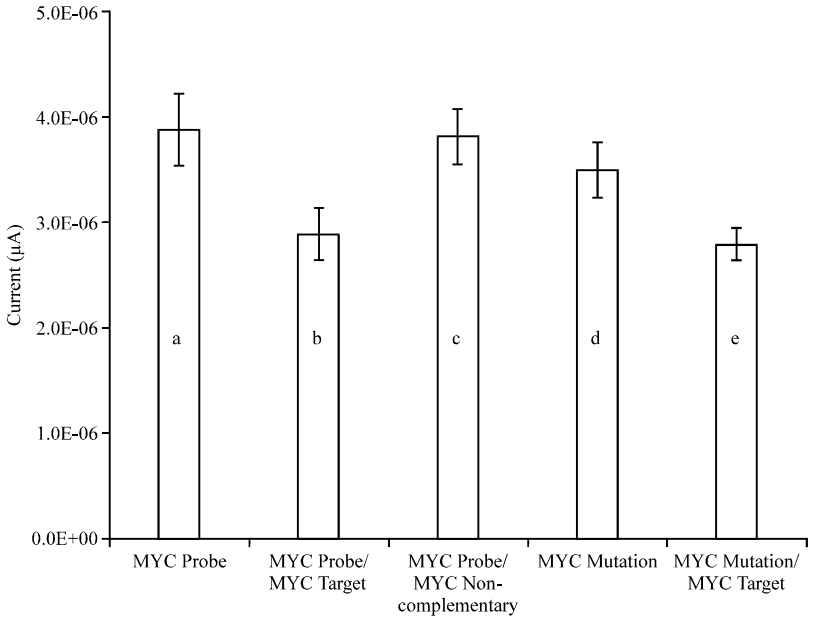

Fig. 3: Histograms for the DPV measurements of MB reduction signals (n = 3): (a) Immobilized MYC Probe, (b) Hybridization of MYC Probe with MYC Target, (c) Hybridization of MYC Probe with MYC Non-complementary, (d) Immobilized MYC Mutation and (e) Hybridization of MYC Mutation with MYC Target

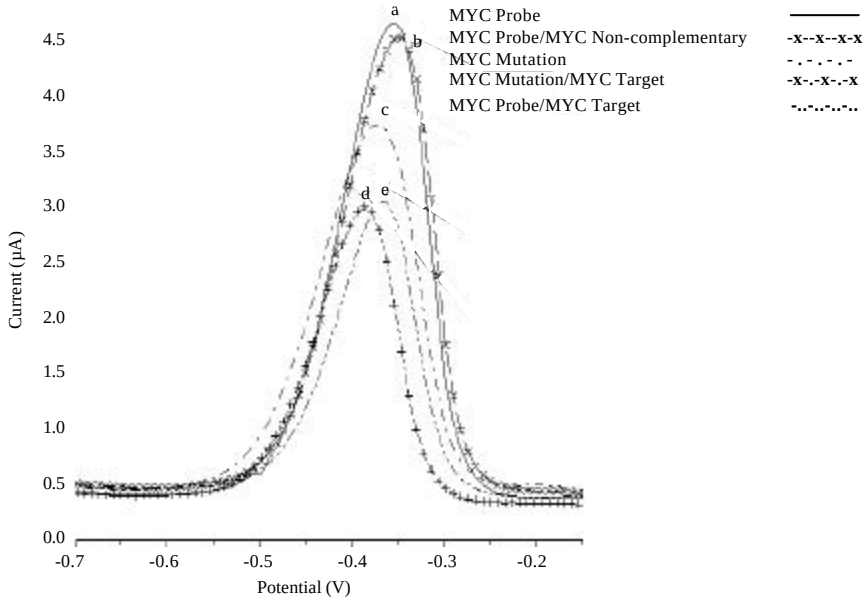

Fig. 4: Differential pulse voltammograms (n = 3) of 20 µM MB as hybridization indicator at: (a) 10 ppm of MYC Probe, (b) 10 ppm of MYC Probe after hybridization with 15 ppm of MYC Non-complementary, (c) 10 ppm of MYC Mutation, (d) 10 ppm of MYC Probe after hybridization with 15 ppm of MYC Target, (e) 10 ppm of MYC Mutation after hybridization with 15 ppm of MYC Target. SPCE pretreatment was done for 1 min at + 1.40 V in 0.5 M of ABS; DNA immobilization was done for 25 min by dropping 20 µL of 10 ppm of MYC Probe in 0.5 M ABS; DNA hybridization was done for 6 min by dropping 20 µL of 15 ppm MYC Target or MYC Non-complementary in 20 mM Tris-HCl containing 20 mM NaCl, pH 7.0; MB accumulation was done for 5 min at + 0.5 V potential containing 20 µM MB (pH 7.0) in 20 mM Tris-HCl and 20 mM NaCl

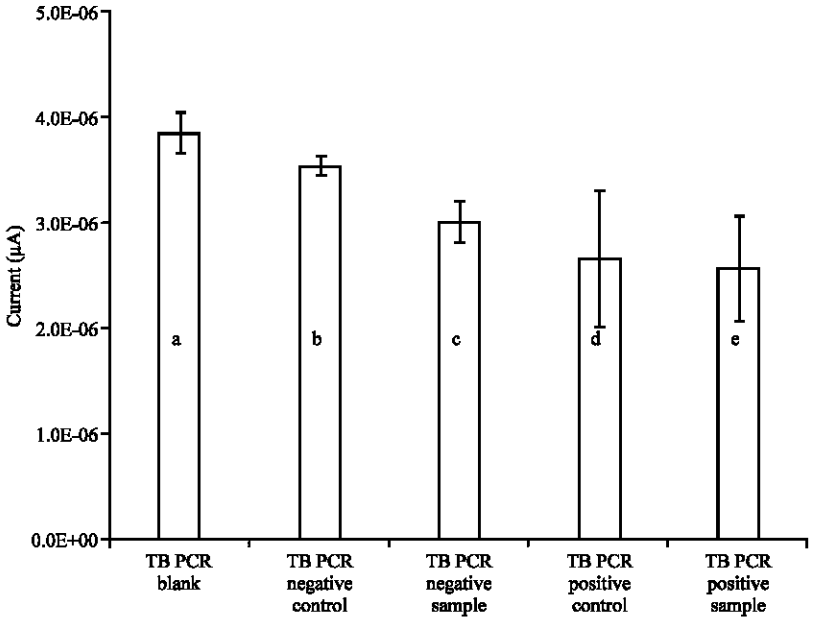

Fig. 5: Histograms of DPV measurements for the MB reduction signals (n = 3) after hybridization for MYC Probe with: (a) TB PCR blank, (b) TB PCR negative control, (c) TB PCR negative sample, (d) TB PCR positive control and (e) TB PCR positive sample

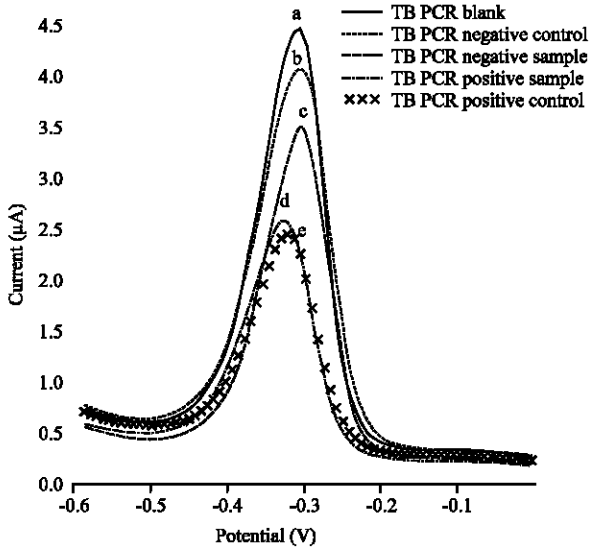

Fig. 6: Differential pulse voltammograms of 20 µM MB as electroactive indicator for MYC Probe on SPCE after hybridization with: (a) TB PCR blank, (b) TB PCR negative control, (c) TB PCR negative sample, (d) TB PCR positive sample and (e) TB PCR positive control. Hybridization was set for 6 min by adding 20 µL of PCR amplified products (concentration: 1/40) directly onto the MYC Immobilized on SPCE surface

with DNA electrostatically. At 10 mM NaCl, the dsDNA had attracted the oxidized and reduced forms of MB with equal strength. However in this experiment, the 20 mM NaCl was chosen as the optimum ionic strength for both dsDNA and ssDNA electrodes. At 20 mM NaCl conditions, the electrochemical signal was derived from the intercalated MB into dsDNA on the SPCE. While, on the ssDNA on the SPCE at 20 mM NaCl, the negative charge of ssDNA was shielded and the electrochemical signal was derived from the interacted MB with guanine bases.

Figure 2a and b showed the DPV effect on different concentration of MYC Probe and MYC Target immobilized on the SPCE surface. The optimum concentration was 10 ppm of MYC Probe (Fig. 2a) and concentration of probe corresponded with the concentration used in the study of multiple point mutation detection of *M. tuberculosis* (Kara *et al*., 2009). MB has two free amino groups at both sides of the aromatic planar ring, which might have caused the binding of MB to the guanine bases (Kerman *et al*., 2004). The accessible guanine bases were 200 times less reactive in the dsDNA compared to the ssDNA. Hence, the duplex formation had protected the guanine moiety from the attack of the incoming positively charged of MB (Erdem *et al*., 2001). It can be observed that there was a decrease in the DPV signal after immobilization with 10 ppm MYC Probe. This indicated that the surface of the SPCE had been immobilized with MYC Probe. Meanwhile, it was observed that the optimum concentration was 15 ppm of MYC Target (Fig. 2b). However, the DPV did show a response at concentration of 5 ppm MYC Target. The lowest DPV of MB signals was observed at concentration of 15 ppm MYC Target. The decreased in the DPV of MB signal indicated that the double helix DNA had formed on the SPCE surface. The surface of the SPCE was immobilized with the MYC Target. These results showed considerable agreement with the recent report for the oligonucleotide concentrations in the range of 1-20 ppm (Siddiquee *et al*., 2009). An increased DPV of MB signal was observed at 25 ppm concentration of MYC Target. This indicated that fouling effect of the surface had occurred between the single-stranded MYC Target and the SPCE surface since the guanine signal was detected at 1.0 V but not at -0.3 V (Erdem *et al*., 2001). The MB acted as an indicator for the determination of the DPV of the immobilized DNA oligonucleotides on the SPCE surface.

The effects of time on the immobilization were investigated; it was observed that the DPV of MB signal increased with immobilization time of 15 to 25 min (Fig. 2c). Thus, 25 min was chosen as the optimum time for immobilization of MYC Probe to the SPCE surface. The effects of hybridization time were also investigated (Fig. 2d). It was observed that the DPV of MB signal increased with hybridization time of 6 min. Hence, 6 min was the optimum hybridization time for MYC Target to bind with MYC Probe on the SPCE surface through the formation of hydrogen bond. Meanwhile, the concentrations of the MB as indicator binding reaction were observed. For the effects of MB concentration (Fig. 2e), it showed that the optimum concentration for accumulation of MB was 20 μM. According to Fig. 2f, 5 min is the optimum time for positively charged of MB to intercalate with the negatively charged of guanine bases on the electrode surface.

The DPV of MB signal can be observed at the 20-mer MYC Probe immobilized on SPCE surface (Fig. 3a) and this is the highest DPV of the MB reduction signal observed. The DPV of MB signal was lower after hybridization of 20-mer MYC Target with MYC Probe on the SPCE (Fig. 3b). A voltammetric reduction signal is as expected since the MYC Probe -MYC Target has hybridized to form a duplex and MB acted as an intercalator. The expression of the MB signal after hybridization indicates that the voltammetric signal due to intercalation was small as compared to the signal from direct interaction with the guanine bases. The MB had accumulated at the hybridized MYC Target-MYC Probe on the SPCE surface. The response of the hybridized MYC Non-complementary-MYC Probe on the SPCE can be observed in Fig. 3c. A slight decrease in the DPV was due to the available binding of the complementary bases between MYC Non-complementary and MYC Probe oligonucleotides. This experiment demonstrated that the hybridization had occurred between MYC Non-complementary and MYC Probe but lower DPV measurement was observed as compared with hybridized MYC Target-MYC Probe. Hybridization of MYC Mutation on the SPCE surface was performed as a control experiment for accessing the hybridization response (Fig. 3d). It was observed that, the DPV of MB signal of hybridized MYC Mutation was lower than compared to the hybridized MYC Probe. This may be due to the oligonucleotide sequence of MYC Mutation was similar to the MYC Probe and having single mismatch of an inosine replacement for guanine. Hence, intercalation had occurred between MB and the free guanine bases. Meanwhile, the DPV measurement for the hybridization of MYC Target/MYC Probe was similar to the MYC Target/MYC Mutation (Fig. 3e). A series of three repetitive DPV measurements of the reduction MB gave reproducible results with a relative standard deviations (RSD) of 8.74 % for MYC Probe (Fig. 3a), 8.61% for MYC Probe/MYC Target (Fig. 3b), 6.86% for MYC Probe/MYC Non-complementary (Fig. 3c), 7.66% for MYC Mutation (Fig. 3d) and 5.37 % for MYC Mutation/MYC Target (Fig. 3e). The reduction signal of MB towards DNA oligonucleotides using DPV measurements can be observed in Fig. 4.

The Fig. 5a-e displays the Differential Pulse Voltammetry (DPV) of Methylene Blue (MB) obtained from the MYC Probe on the SPCE after hybridization with TB PCR products (1:40 diluted in PBS) for the detection of *M. tuberculosis* DNA. The MB signal obtained for

hybridization with TB PCR positive control (Fig. 5d) and TB PCR positive sample (Fig. 5e) was lower than the hybridization with TB PCR blank, TB PCR negative control and TB PCR negative sample as shown in Fig. 5a-c. The TB PCR blank consisted of master mix and distilled water, without template DNA, hence no binding had occurred and a higher DPV measurement was observed. Meanwhile, lower DPV measurements were observed for the MB signal of TB PCR negative control and TB PCR negative sample. These were attributed to the present of template DNA other than *M. tuberculosis* DNA in the amplified samples. The *M. tuberculosis* DNA was not present in the TB PCR negative control and TB PCR negative sample. The DNAs from other organisms were present in the both TB PCR negative control and TB PCR negative sample (Haron *et al*., 2008) which had formed as non complementary strand and hybridizing to the MYC Probe. During the hybridization process, MB will directly intercalate towards free guanine bases and produced voltammetric reduction signal of which can be the same as the signal of MYC Probe. The slight decrease in the voltammetric response of the TB PCR negative sample is attributed to the possible binding of some of the complementary bases during the hybridization reaction between MYC Probe and DNA present in the TB PCR negative sample. Meanwhile, DPV measurements for both of TB PCR positive control and TB PCR positive sample were lower. This indicated that hybridization had occurred between MYC Probe with the *M. tuberculosis* DNA in the TB PCR positive control and TB PCR positive sample (Fig. 5d and e). The Relative Standard Deviations (RSD) of DPV measurements are 5.23% for TB PCR Blank (Fig. 5a), 2.51% for TB PCR negative control (Fig. 5b), 6.50% for TB PCR negative sample (Fig. 5c), 24.24% for TB PCR positive control (Fig. 5d) and 19.79% for TB PCR positive sample (Fig. 5e). The DPV measurements of MB were observed at MYC Probe immobilized on SPCE after hybridization with TB PCR blank, TB PCR negative control, TB PCR negative sample, TB PCR positive sample and TB PCR positive control. The reduction signal of MB relied on the hybridization between probe and the PCR amplified products, similar to described by Siddiquee *et al.* (2009). The reduction signal of MB towards TB PCR samples using DPV measurements can be observed in Fig. 6.

CONCLUSION

The DNA oligonucleotide immobilization and hybridization on SPCE were investigated using electrochemical method and MB as an effective electroactive indicator. Differences in the DPV of MB signal were observed on the MYC Probe on SPCE for detection of *M. tuberculosis* DNA from PCR amplified products. Hence, MB could be used as an effective electroactive indicator in the development of DNA biosensors for detection of tuberculosis. This study described a simple, sensitive and economical electrochemical biosensor for detection of *M. tuberculosis* DNA. Our ongoing research will focused on using *N*-hydroxysulfosuccinimide (NHS) and *N*-(3-dimethyl) aminopropyl-*N'* -ethylcarbodiimide hydrochloride (EDC) as coupling agents for covalent attachment as compared to non covalent attachment on SPCE.

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REFERENCES

Erdem, A., K. Kerman, B. Meric and M. Ozsoz, 2001. Methylene blue as a novel electrochemical hybridization indicator. Electroanalysis, 13: 219-223.

Erdem, A., K. Kerman, B. Meric, D. Ozkan, P. Kara and M. Ozsoz, 2002. DNA Biosensor for *Microcystis* spp. sequence detection using methylene blue and ruthenium complex as electrochemical hybridization labels. Turk. J. Chem., 26: 851-862.

Haron, S., R. Issa, N.M. Sidik and N.M. Zin, 2008. The usefulness of PCR amplification for direct detection of *Mycobacterium tuberculosis* DNA from clinical samples. Biotechnology, 7: 100-105.

Kara, P., Cavusoglu, C., S. Cavdar and M. Ozsoz, 2009. Direct electrochemical genosensing for multiple point mutation detection of *Mycobacterium tuberculosis* during the development of rifampin resistance. Biosensors Bioelectronics, 24: 1796-1800.

Kara, P., K. Kerman, D. Ozkan, B. Meric, A. Erdem and M. Ozsoz, 2002. Electrochemical genosensor for the detection of interaction between methylene blue and DNA. Electrochem. Commun., 4: 705-709.

Karadeniz, H., B. Gulmez, A. Erdem, F. Jelen, M. Ozsoz and E. Palecek, 2006. Echinomycin and cobalt-phenanthroline as redox indicators of DNA hybridization at gold electrodes. Bioscience, 11: 1870-1877.

Kerman, K., D. Ozkan, P. Kara, H. Karadeniz and Z. Ozkan *et al*., 2004. Electrochemical detection of specific DNA sequences from PCR amplification on carbon and mercury electrodes using meldola's blue as intercalator. Turk. J. Chem., 28: 523-533.

Ozkan, D., A. Erdem, P. Kara, K. Kerman, J.J. Gooding, P.E. Nielsen and M. Ozsoz, 2002a. Electrochemical detection of hybridization using peptide nucleic acids and methylene blue on self-assembled alkanethiol monolayer modified gold electrode. Electrochem. Commun., 4: 796-802.

Ozkan, D., P. Kara, K. Kerman, B. Meric and A. Erdem *et al*., 2002b. DNA and PNA sensing on mercury and carbon electrodes by using methylene blue as an electrochemical label. Bioelectrochemistry, 58: 119-126.

Rock, R.B., M. Olin, C.A. Baker, T.W. Molitor and P.K. Peterson, 2008. Central nervous system tuberculosis: Pathogenesis and clinical aspects. Clin. Microbiol. Rev., 21: 243-261.

Siddiquee, S., N.Y. Azah, A.S. Bakar, A.B. Fatimah and Y.L. Heng, 2009. Electrochemical DNA biosensor for the detection of specific gene related to *Trichoderma harzianum* species. Bioelectrochemistry, 10.1016/j.bioelechem.2009.10.004

Wang, J., G. Rivas, X. Cai, N. Dontha, H. Shiraishi, D. Luo and F.S. Valera, 1997. Sequence-specific electrochemical biosensing of *Mycobacterium tuberculosis* DNA. Analytica Chimica Acta, 33: 41-48.

Yean, C.Y., K. Balqis, D.A. Ozkan, L.S. Yin and P. Lalitha *et al*., 2008. Enzyme-linked amperometric electrochemical genosensor assay for the detection of PCR amplicons on a streptavidin-treated screen-printed carbon electrode. Anal. Chem., 80: 2774-2779.