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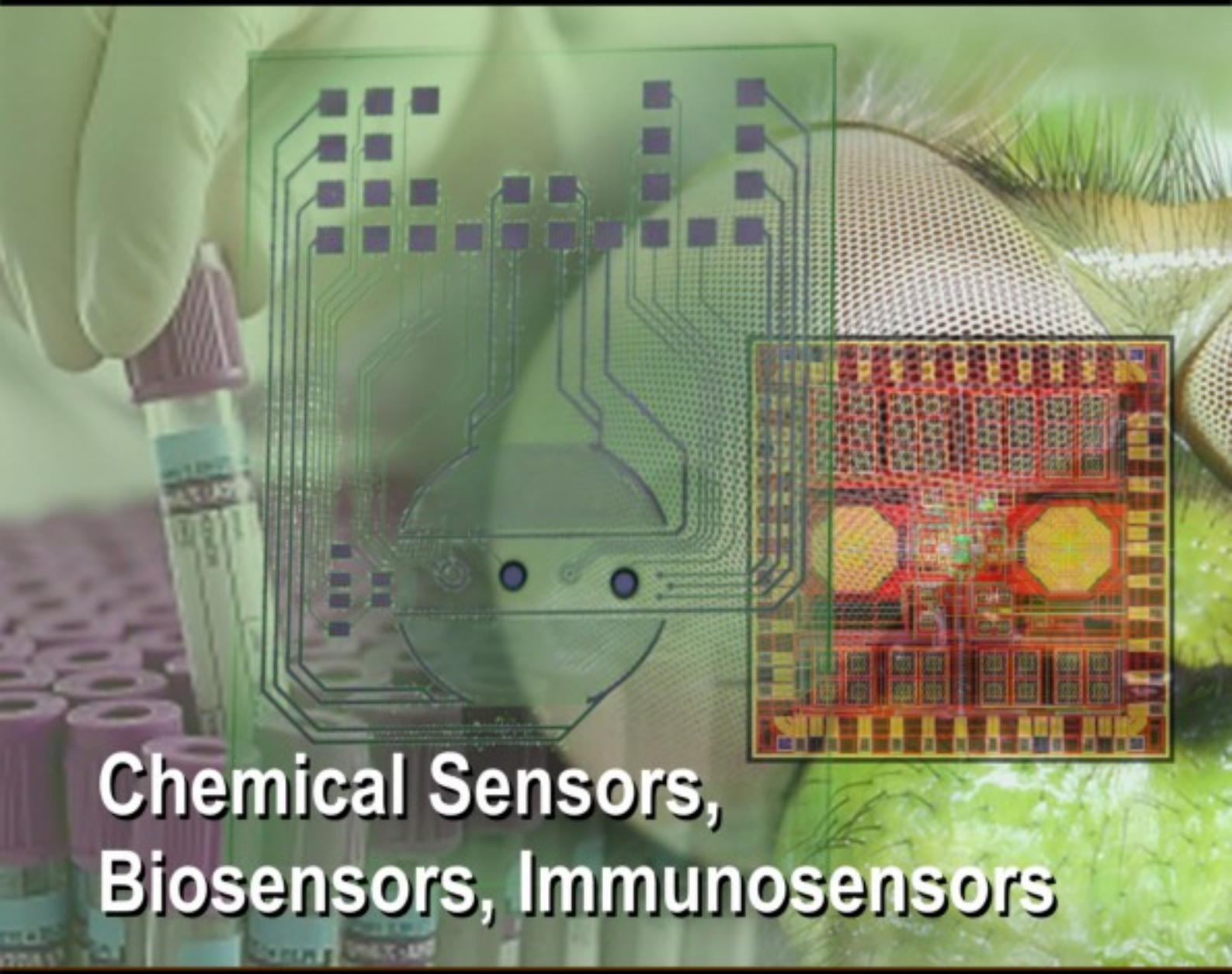

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Development of Piezoelectric DNA-Based Biosensor for Direct Detection of *Mycobacterium Tuberculosis* in Clinical Specimens

[1]Thongchai KAEWPHINIT, [2]Somchai SANTIWATANAKUL, [3]Chamras PROMPTMAS and [1]Kosum CHANSIRI

[1]Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand
Tel.: +66 2260-2122 ext 4605, fax: +66 2260-0125.
E-mails: tkaewphinit@yahoo.com, kosum@swu.ac.th
[2]Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand
E-mail: titi41@yahoo.com
[3]Department of Clinical Chemistry, Faculty of Medical Technology, Mahidol University, Prannok, Bangkok, 10700, Thailand
E-mail: mtcpm@mahidol.ac.th

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Abstract: This study was focused on establishment of piezoelectric biosensor for direct detection of *Mycobacterium tuberculosis* (MTB) in clinical specimens. The quartz crystal immobilized via 3-mercaptopropionic acid (MPA)/avidin/DNA biotinylated probe on gold surface and hybridization of the DNA target to DNA biotinylated probe. The optimal concentration of MPA, avidin and 5'-biotinylated DNA probe for immobilization of specific DNA probe on gold surface were 15 mM, 0.1 mg/ml and 1.5 μM, respectively. The detection of genomic DNA digestion in the range from 0.5 to 30 μg/ml. The fabricated biosensor was evaluated through an examination of 200 samples. No cross hybridization were observed against *M. avium* complex (MAC) and other microorganism. This target DNA preparation without amplification will reduce time consuming, costs, and the tedious step of amplification. This study can be extended to develop the new method which is high sensitivity, specificity, cheap, easy to use, and rapid for detection of MTB in many fields. *Copyright © 2010 IFSA.*

Keywords: *Mycobacterium tuberculosis*, Piezoelectric biosensor, Hybridization, Digestion genomic DNA

1. Introduction

Tuberculosis (TB) is a disease caused by bacteria called *Mycobacterium tuberculosis* (MTB). It is among the top ten causes of global mortality and morbidity which makes it becomes the important public health problem among developing countries. It is a slow-growing bacterium that needs 1-2 months for growing in the culture [1, 2]. The standard method of laboratory diagnosis is based on cultivation which takes about 2-4 months to get the result. The acid fast stain for direct specimen examination is also conventional diagnostic tools but lack of sensitivity [3]. Polymerase chain reaction (PCR) [4] is sensitive for detection of TB by using specific primers but involves in the use of ethidium bromide staining which is carcinogenic agent in gel electrophoresis. The analysis of restriction fragment length polymorphism (RFLP) of PCR product is an alternative DNA detection system and it has been successfully applied to species differentiation [5].

There has been increasing interest in biosensor technology for rapid and sensitive detection among them, especially piezoelectric biosensor. This biosensor has its own advantages that the detection method is label-free from radioactive or fluorescent tags [6, 7]. In addition, this technique could tenuously exhibit the qualitative and quantitative analysis. It shows high sensitivity to mass on the surface of the quartz crystal with the high specificity of a bioreaction [6, 8, 9]. The thin oscillating gold quartz crystal surface generates the intrinsic resonance frequency by mass attached, adhered or deposited onto the piezoelectric active surface [10]. At present, biosensors for detection of MTB were examined by DNA /DNA and antigen/ antibody hybridization. In 1995, Joseph W and coworkers reported the use of electrochemical biosensor for the determination of short sequences from MTB DNA [11]. In 2002, He and Zang reported the use of immuno-piezoelectric biosensor for diagnosis of MTB which showed sensitivity at 0.5 mg/ml [12]. However, the specific binding between MTB and anti-TB in this biosensor was larger than that without precoated protein A. In 2004, Mac Sweeney and coworkers has developed the optical biosensor for detection of multidrug resistant tuberculosis. To complete the biosensor system, the individual elements of the biosensor was optimized to its maximum sensitivity for the electrochemiluminescent optical signal, which was produced during the DNA hybridization event [13]. In 2005, Diaz-Gonzalez and coworkers demonstrated that the immunoelectrochemical biosensor could detect MTB, with a detection limit of 1.0 ng/ml [14].

This study was focused on development of the piezoelectric DNA-based biosensor for direct detection of MTB. The method involved in immobilization of specific synthetic biotinylated probe that was designed from IS*6110* gene-specific for MTB [15, 16] to the surface of quartz crystal. The high sensitivity of hybridization was utilized gold nanoparticle as mass enhancement but we were presented capture protein (avidin) to primer blocking for direct detection this study. This biosensor was used for detecting the target DNA by measuring the frequency change. The oscillation counting device was used for measuring the resonant frequency of the quartz crystal in all of experiments in this study.

The advantage of this study is using a non-amplified genomic bacterial DNA target. This target DNA preparation without amplification will reduce time consuming, costs, and the tedious step of amplification.

2. Materials and Methods

2.1. Reagents and Oligonucleotides

*Bst*DSI (*Btg*I) restriction enzyme and all oligonucleotides used in this study were synthesized by Pacific Science Company (Bangkok, Thailand). All oligonucleotides used in this study were designed based on the nucleotide sequence of IS*6110* gene retrieving from NCBI. The DNA biotinylated probe

for MTB was designed from the nucleotide sequence of IS*6110* gene (Genbank accession number AJ242908.1). The DNA probe was 5'-biotin-TTTTTTGTGGCCATCGTGGAAGCGA-3' and the synthetic complementary DNA target was 5'-TCGCTTCCACGATGGCCAC-3'. The blockings (Block 1: 5'-ATCGTGGTCCTGCGGGCTTTTTTTTT-biotin-3', Block 2: 5'-CCTGCGAGCGTAGGCGTCGG-3') were used for annealing with the ssDNA of bacterial target sequence after denatuaration method which biotin at 3' end of Block 1 was captured with avidin as simple amplification signal.

4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 30 % hydrogen peroxide, 98 % sulfuric acid, sodium chloride, hydrogen chloride, sodium hydroxide and 6-mercaptohexanol were purchased from Sigma Aldrich (USA). DNAzol® was purchased from Invitrogen™. Other chemicals used were at analytical reagent grade, and distilled water (18.2 MΩ) was used throughout.

2.2. Samples

Standard strain from cultivation including MTB (H37RVKK11-20) was provided from Department of Communicable Disease, Ministry of Public Health Thailand. *M. avium* complex (MAC), *P. aeruginosa* (PA), *E. coli* (EC), *S. aureus* (SA) and *E. faecalis* (EF) were collected from Department of Pathology, Faculty of Medicine, Srinakharinwirot University. The clinical sputum 200 samples; 150 samples as positive infection of MTB and 50 samples as negative of non- MTB and other bacteria were obtained from Department of Pathology, Faculty of Medicine, Srinakharinwirot University and Bureau of Tuberculosis, Ministry of Public Health Thailand.

2.3. Apparatus

The 12 MHz AT-cut quartz crystal wafer with gold electrode was used for preparing piezoelectric DNA-based biosensor. The gold electrode that fabricated on the quartz crystal wafer has diameter of 4 mm (area of gold electrode as 0.125 cm^2) and thickness of 1000 Angstroms (Kyocera-Kinseki Company, Thailand). The in-house resonance frequency counting was used for all measurement in this study (Fig. 1). It was used for measuring the frequency change of the quartz crystal after the addition of immobilization material. The major component of this device composes of AVR-microcontroller, oscillation circuit and read out recorded by a computer with the aid of LabVIEW interface software. The frequency shifts were reported as the difference between two stable frequency values (±1 Hz) based on Sauerbrey equation [17].

Fig. 1. Piezoelectric DNA-based biosensor system.

2.4. Extraction and Fragmentation of Genomic DNA

The samples were extracted in 1 mL DNAzol® reagent by inverting the tube several times prior to centrifugation at 4,000xg for 10 minutes. The DNA in supernatant was precipitated by adding 0.5 mL of cold absolute ethanol. The supernatant was discarded and the DNA pellet was washed twice with 1.0 mL of 70 % ethanol by inverting the tubes 3 times. The mixture was then centrifuged at 13,000 xg for 5 minutes to allow DNA to settle and ethanol was removed by decanting. The genomic DNA was air-dried, distilled water was added and kept at 4°C, and immediately used.

The quantity and purity of the DNA were investigated by measurement of the absorbance at 260 nm and 280 nm.

Genomic DNA of partial IS*6110* gene of purified MTB DNA was performed by using *Bst*DSI restriction enzyme. All reactions were manipulated in 50 μL containing genomic DNA in 5 μL of 10X buffer, and 10 units of *Bst*DSI restriction enzyme. Sterile distilled water was added to adjust volume to 50 μL. The *Bst*DSI digestion was allowed to proceed at 37 °C for 14 hours. The reaction was inactivated by heating at 65 °C for 20 minutes immediately used.

2.5. Preparation of Piezoelectric DNA-based Biosensor

The method used to prepare DNA sensor in this study was modified by Zhou and colleague [7]. Initially, the gold electrode surface was cleaned with hot Piranha solution consisting of H_2O_2 (30 %) and H_2SO_4 in a 1:3 ratio for 30 seconds. The crystals were thoroughly washed with distilled water, and air-dried. The initial resonance frequency (f_0) was recorded as the baseline. The cleaned quartz crystal was soaked in the optimal concentration of 3-mercaptopropionic acid or MPA (Sigma, USA) ethanolic solution for 1 hour, rinsed with absolute ethanol, washed with distilled water, and air-dried. To activate the monolayer, 10 μL of 200 mM 1-ethyl-3(3-dimethylaminopropil) carbodiimide or EDC (Fluka, Switzerland) aqueous solution was placed on the surface. Then, 10 μL of 50 mM *N*-hydroxysuccinimide or NHS (Fluka, Switzerland) aqueous solution was immediately added and left to react on the surface of gold electrode to form MPA monolayer for 30 minutes followed by water rinsing and air-dried. An aliquot of 10 μL containing the optimal concentration of avidin (Sigma, USA) in HEPES buffer (0.05 M HEPES, 0.2 M NaCl, pH 7.5) was placed on the electrode surface for at least 1 hour before washing, air-drying, and measuring for the resonance frequency (f_1). The residual carboxyl groups on gold surface were blocked by 1 mM ethanolamine for 30 minutes.

After rinsing the quartz crystal with distilled water, 5'- biotinylated probe was immobilized by interact with avidin for 20 minutes. The quartz crystal was rinsed with immobilization buffer and distilled water respectively, the resonant frequency (f_2) was measured as the amount of added DNA probe. Then, the quartz crystal was exposed to a 1 mM ethanolamine HCl (Fluka, Switzerland) for 30 min, rinsed with distilled water and immobilization buffer (300 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM EDTA, pH 7.4).

2.6. DNA Hybridization

The DNA hybridization was performed by adding 10 μL of the synthetic complementary DNA target on to the surface of Au/MPA/EDC/NHS/avidin/DNA biotinylated probe quartz crystal. The hybridization reaction was left for 20 minutes at room temperature. Each quartz crystal was washed well with hybridization buffer (150 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM EDTA, pH 7.4) to

remove the unbound oligonucleotides followed by washing with distilled water, and air-dried. The new frequency (f_3) was recorded. The frequency shift ($\Delta f = f_2 - f_3$) was related to the amount of target DNA hybridized to the DNA biotinylated probes immobilized on the quartz crystal surface.

2.7. Optimization of Quartz Crystal for Detections

Self assembly monolayer of MPA on gold surface of quartz crystal was performed at room temperature for 1 hour by placing various concentration of MPA solution. The concentration of MPA at 0, 1, 5, 10, 15, and 25 mM were applied on the gold surface. Then, 10 μL each of EDC and NHS were consecutively placed over the gold surface to activate the monolayer. Ten microlitters of 0.2 mg/mL avidin was applied over this activated gold surface at room temperature for 1 hour. The frequency change was measured after the surface was completely dry.

Each avidin solution at the concentration of 0, 0.025, 0.05, 0.1, 0.15, and 0.2 mg/mL was placed on the optimal MPA modified quartz crystal at room temperature for 1 hour. Then, the different of resonant frequency was monitored for effectiveness of avidin immobilization. The lowest concentration of avidin giving the highest change of resonant frequency was optimized.

The concentration of DNA biotinylated probe at 0, 0.5, 0.75, 1, 1.5, and 2 μM were selected to study the optimal concentration for probe immobilization. Each concentration was applied on the quartz crystal at room temperature for 20 minutes with avidin binding to MPA/EDC/NHS monolayer. Then, the resonant frequency of crystal was read to evaluate the amount of immobilized probe.

2.8. Study of the Responses of the Synthetic Complementary DNA Target

The optimum concentrations of MPA, avidin, and DNA biotinylated probe were applied for preparation of quartz crystal sensor. The different concentrations of the synthetic complementary oligonucleotide to the probe immobilized on the quartz crystal were performed for hybridization assay at room temperature for 20 minutes. This experiment used oligonucleotide target at the concentration of 0, 0.25, 0.5, 0.75, 1 and 2 μM, respectively. The experiment was tested for optimization of piezoelectric biosensor.

2.9. Development of Hybridization Assay

Generally, simple thermal treatment of bacterial target DNA is sufficient to give a significant analytical signal when amplified bacterial DNA fragment is used in DNA biosensor technique. But this treatment was not enough for non-amplified genomic DNA because of reannealing of bacterial target DNA. Minnuni and colleague [7, 18] used the blocking oligonucleotides for blocked the bacterial target DNA after simple thermal treatment. This method increases the efficiency of hybridization between DNA probe and non-amplified genomic DNA. The DNA target enhanced signal by using 0.1 mg/mL avidin capture biotin at 3'-end of block 1 primer (1 μM).

The optimal concentration of the DNA targets loaded into the piezoelectric biosensor was determined. The amount of 0.5, 1, 5, 10, 20, and 30 μg/ml of digestion DNA target were diluted in hybridization buffer to a total volume of 10 μL before being added into the biosensor system.

2.10. Detection of Genomic DNA Target

The piezoelectric DNA-based biosensor in this study for identification of MTB, 150 AFB positive and 50 AFB negative from sputum samples were detected individually using the piezoelectric biosensor assay and PCR technique by using primer from PCR targeting 123 bp of IS*6110* gene which reported the highest sensitivity and specificity in diagnosis of tuberculosis in clinical samples [16].

2.11. Statistical Analysis

Each experiment was performed triplicate with different piezoelectric devices. All data were presented as the mean ± standard deviation (S.D.).

3. Results

3.1. Optimization of Quartz Crystal for Detections

The optimum amount of MPA on the surface of quartz crystal was shown in Fig. 2A. It was found that the highest frequency change was obtained at 15 mM of MPA.

The optimum amount of avidin on the quartz crystal was shown in Fig. 2B. The data revealed that the range of optimum concentration of avidin could be 0.1 – 0.2 mg/mL. However, the higher amount of avidin (0.2 mg/mL) showed no longer increase in immobilized mass.

The optimization of DNA biotinylated probe concentration was shown in Fig. 2C. The highest frequency change was obtained from 1.5 μM of probe.

3.2. Study of the Responses of the Synthetic Complementary DNA Target

Fifteen mM of MPA, 0.1 mg/mL of avidin, and 1.5 μM of DNA biotinylated probe were applied in process of preparation of piezoelectric DNA based biosensor. The result revealed that the optimal frequency change was obtained when the amount of synthetic complementary oligonucleotide was 1 μM (Fig. 2D).

3.3. Development of Hybridization Assay

MTB (H37RVKK11-20) was chosen for studying the denaturation method plus primer blocking compared primer blocking capture with avidin (0.2 mg/mL). All samples were hybridized with 1.5 μM of 5'-biotinylated probe for 20 minutes at room temperature. After air dried, the frequency shift of each denaturation method (n=3) was presented as mean ± S.D. as seen in Fig. 3. The frequency shift of thermal treatment plus primer blocking capture with avidin (0.2 mg/mL) was higher than the frequency shift of thermal treatment plus primer blocking only.

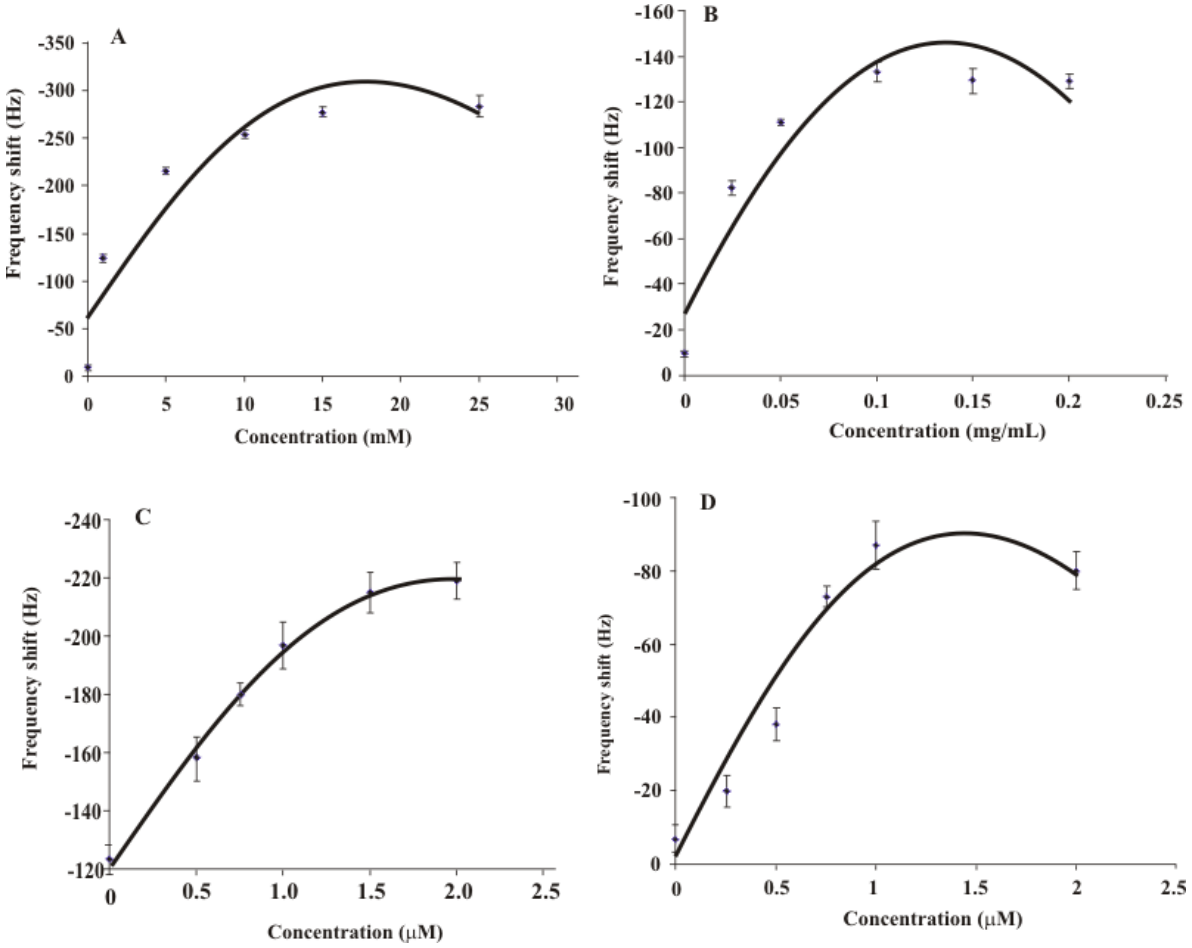

Fig. 2. Optimization of piezoelectric biosensor. (A), The frequency shift were avidin immobilization after MPA monolayer forming. (B), the frequency changes were monitored after various concentrations of avidin were covalently bound on the surface. (C), the frequency changes were monitored after various concentrations of probe immobilized on the surface and (D), after the synthetic complementary target DNA hybridized with the probe. The experiments were independently performed for 3 times.

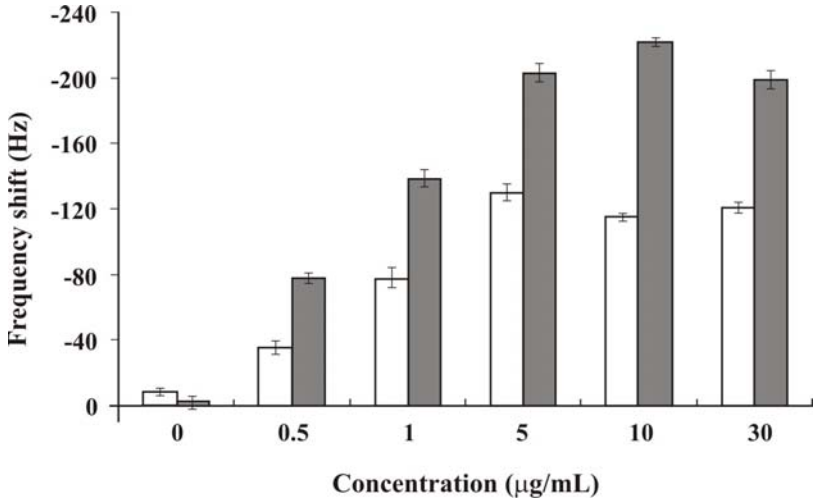

Fig. 3. The frequency shifts were monitored after target of *Bst*DSI-digestion MTB genomic DNA by using blocking oligonuclotide (■), and blocking oligonuclotide capture with avidn (□) at indicated concentrations were used. Means and SD ranges were presented as bars for the frequency shift from each concentration.

3.4. Evaluation of Piezoelectric DNA-based Biosensor System Specificity

The specificity was detected by using MTB, MAC, PA, EC, SA, and EF. The hybridization buffer was the negative control (BK). All samples were denatured genomic DNA digestion by thermal denaturation plus primer blocking capture with avidin (0.2 mg/mL) and hybridized with 1.5 μM of biotinylated probe for 20 minutes at room temperature. After air-dried, the frequency change of each sample (n=3) of denaturation methods was presented as mean ± S.D. as seen in Fig. 4. The NC gave the lowest frequency change. MTB gave frequency changes with higher frequency change than of MAC, PA, EC, SA, and EF of denaturation plus primer blocking capture with avidin methods. Therefore, the thermal denaturation plus blocking capture avidin for preparation of target DNA can differentiate MTB from MAC and other microorganism.

This result, the frequency change of MTB is lower than the frequency shift of MAC and other microorganism. This result was the similar to reported previous study [5, 6, 17] which used IS*6110* gene target for detection of MAB by PCR technique. The specific IS*6110* gene target was used to differentiate MTB from non-MTB. The result showed that MAC had no cross hybridization to MTB as shown with non-significant change in frequency signal in the Fig. 4.

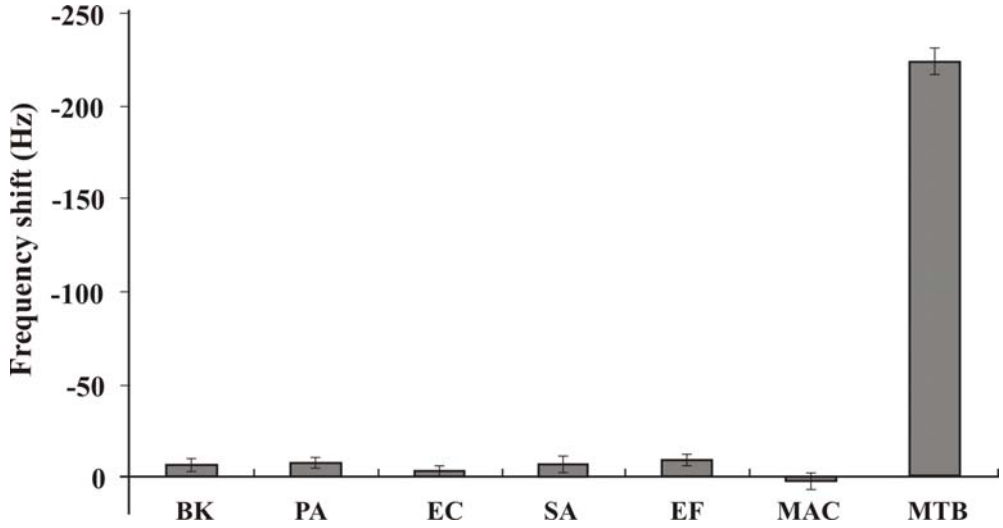

Fig. 4. Specificity test of DNA-piezoelectric biosensor on *Bst*DSI-digested DNA of MTB (positive control), MAC and other microorganisms (negative control) against hybridization buffer (BK).

3.5. Direct Detection of DNA Target from Clinical Specimens

*Bst*DSI-digested genomic DNA from 150 specimens of MTB and 50 specimens of MAC and other microorganism were tested by using piezoelectric biosensor in comparison to the result of PCR technique (Table 1). The PCR targeting 123 bp of IS*6110* was analyzed by using gel electrophoresis to confirm the successful amplification of PCR products (not data shown). The data revealed that the results obtained from piezoelectric biosensor were corresponded to those of PCR techniques. However, the detection of MTB IS*6110* gene by using this specific piezoelectric DNA based biosensor provides many advantages including the label free DNA hybridization reaction (no toxic compounds are required, i.e. ethidium bromide).

Table 1. Samples identified with piezoelectric DNA-based biosensor, tested with specific sensors carrying the IS*6110* gene probes compared with the PCR method.

Methods	Number of clinical specimens		
	Positive	Negative	Total
PCR technique	150	50	200
Piezoelectric biosensor technique	150	50	200

4. Discussions

The piezoelectric biosensor method is a well established technique for the measurement of the mass changes that are based on the relationship between changes in mass of materials attached to the crystal and oscillation frequency of the crystal. The DNA-piezoelectric biosensor was selected on the basis of the successful achievement of DNA hybridization for the rapid direct detection of genomic DNA of IS*6110* gene MTB.

There are two major issues that must be dealt with in order to produce a sensitive and specific DNA-piezoelectric biosensor for detection of the target of interest: (i) the immobilization of DNA probe on the quartz crystal and (ii) the optimization of experimental conditions to minimize nonspecific hybridization. In this study, the DNA biotinylated probe immobilization was attached on avidin via MPA formed self assembly monolayer to the gold surface. Immobilization not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilizing it for the reuse [20]. This immobilization technique is widely reported to be one of the most efficient and simple immobilization methods available [7, 21].

The efficiency of piezoelectric DNA based biosensor was checked by using Sauerbrey equation [17]. The advantage of the DNA piezoelectric biosensor is that it could simultaneously measure the frequency shift and the mass change. At the step of washing, quartz crystals must not be over rinsed since this could deteriorate the surface of gold electrode. The concentrations of MPA, avidin, and DNA biotinylated probe immobilized onto the quartz crystal had been determined for the optimal conditions (Fig.2A, 2B, and 2C). The excess of MPA led to saturation response of the avidin immobilization or the steric hindrance of MPA to avidin immobilization. Whereas, the excess of avidin immobilization can produce the saturation response of the avidin immobilization or interfere by the steric hindrance for the hybridization between DNA probe and synthetic complementary DNA target in Fig. 2D.

The detection of genomic DNA target by using a quartz crystal piezoelectric normally has less probability to hybridize with DNA probe due to the steric hindrance effect of DNA secondary structure and lower amount of specific DNA sequence than the detection limit of general analytical technique. The alternative solution to solve this problem is restricted enzymatic digestion of the DNA samples without any previous amplification step to separate small DNA fragments [19, 22].

The frequency shift was reported as the difference between two stable frequency values based on Sauerbrey equation [17]. The calculations suggested that a frequency change of 1 Hz corresponds to a mass increase of 0.38 ng/cm^2 for 12 MHz AT-cut. The limit detection of this DNA based sensor was 0.5 μg/mL genomic DNA digestions, the observed frequency shift was 78±3.4 Hz.

However, non-amplified genomic DNA, the blocking oligonucleotides were capped with avidin enhancement in this denaturation step in Fig. 3. This blocking can prevent the reannealing of single-stranded DNA to increase the efficiency of DNA target hybridization. Therefore, the blocking oligonucleotides were used for development of DNA target hybridization. The thermal plus blocking oligonucleotides capture with avidin (0.2 mg/mL) gave the frequency shift (in decreasing the resonant frequency) higher than thermal denaturation plus blocking primer only. This result shows the similarity with previous studies [7, 18] which the thermal plus blocking oligonucleotides gave the frequency shift higher than the frequency shift of thermal denaturation only.

The amount of non-amplification (DNA target) could effect the determination of specific IS*6110* gene sensor. The dilutions of digested genomic DNA could be performed to reach the appropriate concentration within the limit of this sensor. The binding of target DNA sequences was inhibited when an excess amount of target DNA sequence complementary to the DNA probe. This is due to the formation of double-stranded in the solution by the two single strands of the DNA target at high concentration. Hence, blocking oligonucleotides were used for protection of the DNA fragments form self-assembly. The specificity of specific IS*6110* gene sensor is also an important key for this determination.

This result, the frequency shift of MTB is lower than the frequency change of MAC and other microorganism in Fig. 4. This result was the similar to reported previous study [16] which used IS*6110* gene target for detection of MTB by PCR technique. The specific IS*6110* gene target was used to differentiate MTB from non-MTB.

The direct detection of 150 AFB positive was decreased in frequency value (Hz) after the hybridization reaction with DNA probe. The interaction with 50 of AFB negative samples did not result in a significant measurable frequency and also did not observe the band of PCR amplicon. The data revealed that the results obtained from piezoelectric biosensor were corresponded to those of PCR techniques in Table 1. It can be concluded that specific IS*6110* gene sensor with DNA probe had high specificity for detection. For the clinical samples detection, the sensitivity resulted in 100 % and specificity resulted in 100 % compared with the PCR assay.

Upon sensitivity examination to improve the sensitivity of the tool, the modification of quartz crystal combine with gold nanoparticle for amplification signal will be considered. In addition, the determination on specific IS*6110* gene piezoelectric biosensor found that non-MTB samples provided response frequency shift less than 1 Hz. This result is comparable to other systems, when the negative samples provided response less than 5 Hz [11, 21, 23].

Finally, this study can be extended to develop the new method which is sensitivity, specificity, cheap, easy to use, and rapid for detection of MTB in many fields of work such as clinical diagnosis, epidemiology study, and bioterrorist weapon survey.

5. Conclusions

The piezoelectric DNA-based biosensor appears to be a suitable and convenient tool for monitoring hybridization of complementary stands of oligonucleotides compared to other biosensor methods. Label free is the principle feature of the piezoelectric DNA-based biosensor which could directly detect the target DNA specimens without PCR amplification. The method demonstrated the sensitivity and specificity of the detection. The addition of blocking oligonucleotides in denaturation step can improve the hybridization efficiency of direct detection of non-amplified genomic DNA at concentration as low as 0.5 μg/mL. This sensor can be tested with IS*6110* gene also for differentiation

of MTB from non MTB. This study will help the selection of gene which is more suitable for detection of MTB.

Moreover, piezoelectric biosensor in liquid-flow system may be developed for improvement the time and tedious step of washing and drying of this sensor preparation.

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