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Quartz crystal microbalance-based biosensor for the detection of α-thalassemia 1 (SEA deletion)

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Abstract

Background: DNA piezoelectric biosensors have become a promising tool in molecular medicine since they do not require any label or staining. Here, a DNA piezoelectric biosensor based on a quartz crystal microbalance (QCM) was created to identify abnormal genes causing α-thalassemia 1 (SEA deletion).

Methods: The functionalized gold electrode of the quartz crystal was coated with avidin and the biotinylated DNA probe was attached. The target gene causing α-thalassemia 1 was amplified and hybridized with the immobilized probe. DNA hybridization was indicated by changes in the quartz resonance frequencies. Diagnostic ability of the new α-thalassemia 1 biosensor was validated using both known and unknown blood samples. Specificity was tested using samples of β-thalassemia and α-thalassemia 2. Stability of the sensor was also evaluated.

Results: The new biosensor could clearly identify α-thalassemia 1 (SEA deletion), both carrier and disease states, from the normal genotype. Identification accuracy was compatible to the standard gel electrophoresis. It was specific only to α-thalassemia 1 since no cross reaction was found with β-thalassemia and α-thalassemia 2. The sensor could be kept at room temperature up to 6 months with consistent identification accuracy.

Conclusions: The label free QCM based biosensor was successfully developed to diagnose an abnormal human globin gene causing α-thalassemia 1 (SEA deletion). Its accuracy, specificity and sensitivity were comparable to the standard method. Its stable diagnostic potency up to 6 months implied its field application in thalassemic control program.
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Introduction

DNA biosensor technology using a quartz crystal microbalance (QCM) has become more and more attractive in clinical diagnosis. Its principle is a linear relationship between deposited mass and frequency changes (1). Therefore, it is a label-free detection system (2, 3). However, the DNA probe adsorbed on the functionalized quartz surfaces must retain its native conformation and hybridization specificity (4, 5). Hybridization between the DNA probe and the amplified target gene on the quartz gold electrode results in changes in the deposited mass. Detection can be accomplished by following frequency changes of the quartz crystal (1, 6, 7).

Thalassemias are inherited hematologic disorders due to defects in the synthesis of one or more of the globin chains of hemoglobin (8, 9). The most severe form is homozygous α-thalassemia 1 or Hb Bart's hydrops fetalis. Infants born with this syndrome either die in utero or soon after birth (9, 10). Accurate identification of carriers of α-thalassemia 1 is the most important step in the prevention and control of this deadly genetic disease (10, 11). Many molecular methods based on polymerase chain reaction (PCR) with different post PCR steps (12–14) have been developed to detect α-thalassemia 1. Here, we used the QCM biosensor to detect the PCR products of an abnormal gene causing α-thalassemia 1 (Southeast Asian deletion). The sensor was constructed using a specific avidin-biotin interaction to immobilize the biotinylated oligonucleotide probe on the functionalized gold electrode of the quartz surface. Complementary hybridization between an immobilized DNA probe and the target gene on quartz surface could be simply verified by an in-house oscillation frequency counter.

Materials and methods

Reagents and apparatus

N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Fluka Chemical Corp, NY, USA. Hydrogen peroxide, disodium ethylenediaminetetra-acetic acid (disodium EDTA), ethanol and all the reagents for buffers were purchased from Merck KGaA, Darmstadt, Germany. Three different buffers were used (HEPES buffer: 0.05 M HEPES and 0.2 M NaCl, pH 7.5; probe immobilization buffer: 300 mM NaCl, 20 mM Na_2HPO_4 and 0.1 mM EDTA, pH 7.4 and hybridization buffer: 150 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM

EDTA, pH 7.4). All other reagents were purchased from Sigma-Aldrich Corp, St. Louis, MO, USA.

Oligonucleotide probe and primers were synthesized by 1st BASE Pte. Ltd., Gemini Singapore Science Park II, Singapore. The target DNA was amplified by PCR using three primers as described previously (15): A7 (5′-CTCTGTGTTCTCAGTATTGGAG-3′), A1B (5′-GGTTCCCTGAGCCCCGACACG-3′) and A9 (5′-ATATATGGGTCTGGAAGTGTATC-3′). The DNA probe used to identify the abnormal gene causing α-thalassemia 1 (SEA deletion) was designed using Primer 3 software (16) and tagged with biotin at the 5′ end (5′-biotin-TTTTTTGAAGGAGGGGAGAAGCTGAG-3′).

The 12 MHz AT-cut quartz crystals with 4 mm gold electrode (0.1257 cm^2 area) fabricated on both sides were purchased from Kyocera-Kinseki Corp, Kyoto, Japan. For each measurement, only one side of the crystal was in contact with the reaction solution. The resonance frequency (Hz) was recorded using an in-house oscillation counting device (17). Major components of this device are PIC-microcontroller, oscillator circuit and the read out display.

Preparation of target DNA from blood samples

The study was approved by the Mahidol Ethical Committee on Institutional Review Broad (MU-IRB 2009/045.0904). Genomic DNA was extracted from EDTA blood using a PUREGENE® Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The concentration of extracted DNA was determined by measuring the absorbance at 260 nm (18).

The PCR was performed using PCR Master Mix Solution (*i*-Taq) (iNtRON Biotechnology, Gyeonggi-do, Korea) as described previously (15). All PCR products were verified by electrophoresis on 1.5% agarose gel.

Preparation of α-thalassemia 1 biosensor

The gold electrode surface of quartz crystal was specially cleaned with a hot piranha solution consisting of H_2O_2 (30%) and H_2SO_4 in a 1:3 ratio for 20 s. It was then washed thoroughly with distilled water and dried overnight in a desiccator. The baseline resonance frequency of the cleaned quartz crystal (f_0) was recorded.

The cleaned gold electrode surface was soaked in 10 mM of mercaptopropionic acid (MPA) at room temperature for 1 h, followed by water rinsing and air-drying. The MPA coated surface was further treated with 5 μL of 20 mM EDC and 50 mM NHS, respectively. These solutions were allowed to interact with the MPA coated surface at room temperature for 30 min in a humidified chamber to prevent the evaporation of solution. The gold surface covered with activated carboxyl groups was washed with distilled water and 10 μL of 0.1 mg/mL avidin and was immobilized for 1 h before rinsing with distilled water.

In order to calculate the number of avidin molecules immobilized on the quartz surface, the surface was air-dried overnight in a desiccator. The resonance frequency shift (f_1) was measured as an indicator of the number of deposited avidin molecules.

In the next step, the wet quartz surface coated with avidin was exposed to 10 μL of 1 mM ethanolamide hydrochloride for 30 min to convert the residual carboxyl group to β-hydroxyethanolamide. After rinsing with distilled water, the avidin-coated quartz surface was immersed in 10 μL of 1 μM biotinylated oligonucleotide probe for 30 min. This was washed with distilled water, dried in a desiccator and the resonance frequency shift (f_2) was measured. The number of deposited probes was calculated from the resonance frequency shift. This immobilized probe on the quartz surface was maintained in a sealed cap at room temperature.

Evaluation of diagnostic ability of the α-thalassemia 1 biosensor

To be applied for clinical diagnosis, the hybridization buffer was added on the biotinylated oligonucleotide probe of the sensor. Next, the PCR-amplified target DNA was denatured at 95°C for 10 min and hybridized with the probe at room temperature in a humidified chamber for 30 min. After hybridization, the sensor was rinsed with distilled water and dried overnight in a desiccator. The resonance frequency shift (f_3) was measured.

Blood samples were collected from normal individuals (n = 3), α-thalassemia 1 carriers (SEA deletion) (n = 3), Hb Bart's hydrops fetalis patients (n = 3), α-thalassemia 2 carriers (n = 3) and β-thalassemia carriers (n = 3). The target DNA was amplified using three primers as described. The amplified products were hybridized with the biotinylated probe of the sensor and compared to the control containing no DNA.

To test the sensitivity of the α-thalassemia 1 biosensor, target DNA amplified from normal, α-thalassemia 1 carrier (SEA type), and Hb Bart's hydrops fetalis were diluted in hybridization buffer to various concentrations from 0, 25, 50, 100, 200, to 400 μg/mL. Next, the diluted target DNA was detected by either the α-thalassemia 1 biosensor, as described above, or by 1.5% agarose gel electrophoresis.

To test the specificity of the α-thalassemia 1 biosensor, similar procedures were performed to detect the amplified DNA from carriers of either α-thalassemia 2 (n = 3) or β-thalassemia (n = 3).

To test the stability of the α-thalassemia 1 biosensor, the sensor was stored in a sealed cap at room temperature for 0, 15, 30, 45, 60, 75, 90, 120, 150, and 180 days. The changes in resonance frequency of the sensor were measured after storage. The diagnostic ability of the stored α-thalassemia 1 biosensor was also evaluated by hybridization with the known target DNA amplified from normal, α-thalassemia 1 carriers (SEA deletion), and Hb Bart's hydrops fetalis patients.

Clinical application of the newly developed α-thalassemia 1 biosensor

The clinical diagnostic ability of the α-thalassemia 1 biosensor was tested using 44 unknown blood samples. All samples had low mean corpuscular volume (MCV < 80 fL). The DNA was extracted and the target amplified as described above. Hybridization reaction on the α-thalassemia 1 biosensor was performed and frequency shifts were measured. Results were compared with those of 1.5% agarose gel electrophoresis.

Statistic analysis

All data shown in this study were means of triplicate experiments. All comparison data were analyzed using a two-tailed Student's t-test. p-Values less than 0.05 were deemed to be statistically significant.

Results

Primers and probe

In this study, the DNA fragment of the α-globin gene was purified and then amplified using three primers as shown in Figure 1. The forward (A7) and normal reverse primers (A1B) were used to amplify a fragment of the normal

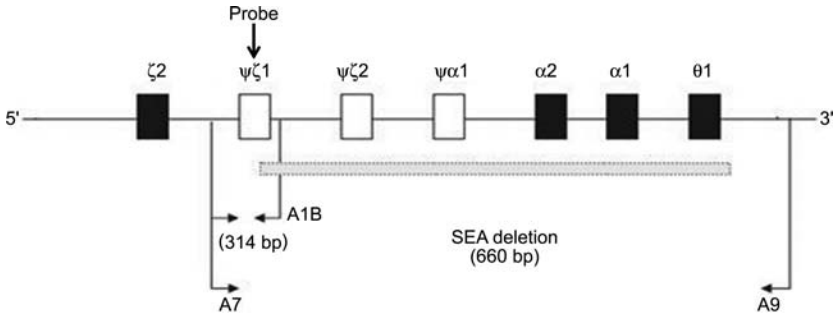

Figure 1 Position and orientation of primers and probe on the α-globin genes used in the present study. Arrows indicate the orientation of primer A7, A1B, and A9.

α-globin gene. A specific gene deletion (20.5-kb) causing α-thalassemia 1 (SEA deletion) was amplified using forward (A7) and thalassemic reverse primers (A9). The complementary DNA probe was designed using Primer 3 software and synthesized by tagging biotin at the 5′ end (Figure 1). These primers and probe were used in all experiments.

Preparation of target DNA from clinical blood samples

The target DNA fragments of the α-globin gene from blood of normal individuals, α-thalassemia 1 carriers (SEA deletion) and patients with Hb Bart's hydrops fetalis were amplified using three primers and analyzed by gel electrophoresis (Figure 2). A single DNA fragment of 314 bp was expected from the normal α-globin gene. A specific gene deletion (20.5-kb) causing α-thalassemia 1 (SEA deletion) was expected to get a PCR product at 660 bp. Blood from

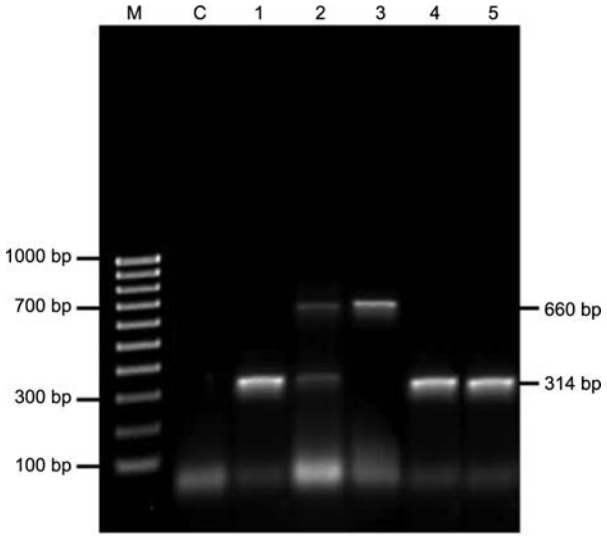

Figure 2 Agarose gel electrophoresis of the amplified α-globin gene.
Lane 1, 2, 3, 4 and 5 were the amplified products from normal genotype, α-thalassemia 1 carrier (SEA deletion), Hb Bart's hydrops fetalis, α-thalassemia 2 carrier, and β-thalassemia carrier, respectively. C was the control without genomic DNA and M was molecular weight markers (100 bp DNA ladder; BioRad).

α-thalassemia 1 carriers was expected to produce two products at both positions.

Preparation of the α-thalassemia 1 biosensor

The biotinylated probe was immobilized on the gold electrode surface of a quartz crystal based on the specific interaction between avidin and biotin. The gold surface was sequentially treated with MPA, EDC and NHS to add the ester group on the quartz surface in order to bind with the amine group of avidin.

After the attachment of avidin at optimal concentration (0.1 mg/mL) the resonance frequency changes were measured (139.8 ± 4.60 Hz, $n = 3$). These frequency changes could be used to calculate the deposited mass on quartz surface using the Sauerbrey equation (1), and the mass changes could be further used to calculate the number of attached avidin molecules. There were $4.94 \pm 0.16 \times 10^{11}$ molecules of avidin attached on the gold electrode quartz surface.

The avidin coated surface was treated with ethanolamide hydrochloride to block the non-specific binding sites. The biotinylated probe at the optimal concentration of biotinylated probe (1 μM) was bound to the freshly treated quartz surface and the frequency change was re-measured (79.0 ± 12.12 Hz, $n = 3$). By similar calculations based on the Sauerbrey equation (1), there were $1.97 \pm 0.30 \times 10^{12}$ molecules of biotinylated probe attached to the gold surface of the quartz crystal.

Evaluation of the diagnostic ability of α-thalassemia 1 biosensor

The α-thalassemia 1 biosensor was verified for its diagnostic ability using a known specimen prepared from blood from patients with normal genotype, α-thalassemia 1 carriers (SEA deletion), and patients with Hb Bart's hydrops fetalis. The amplified target DNA in hybridization buffer was diluted to various concentrations ranging from 0, 25, 50, 200 to 400 μg/mL. Next, 10 μL of each concentration was hybridized with the biotinylated probe and changes in resonance frequencies were measured. As shown in Figure 3, the α-thalassemia 1 biosensor could identify Hb Bart's hydrops fetalis from normal genotypes or α-thalassemia 1 carriers at 25 μg/mL of DNA. But a significant differential diagnosis

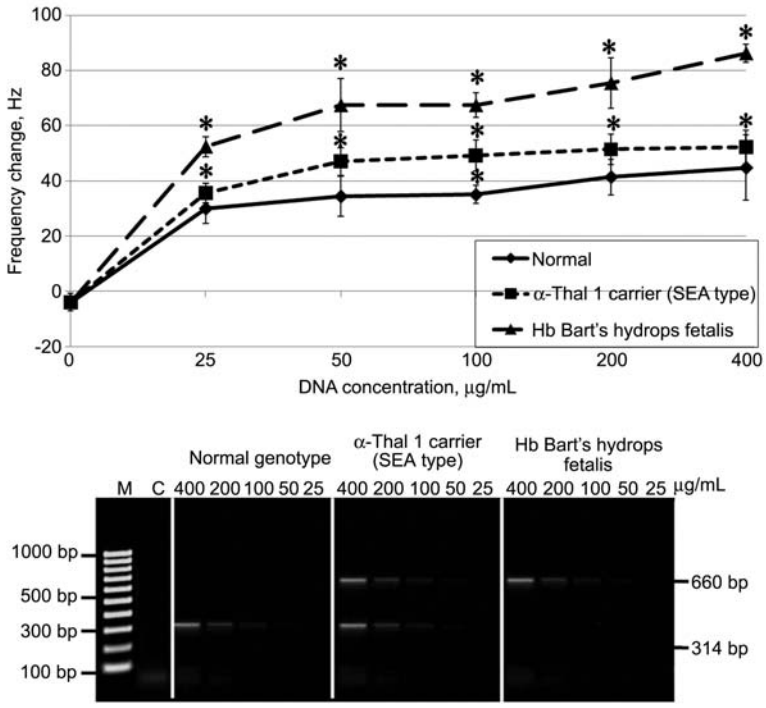

Figure 3 Sensitivity of the α-thalassemia 1 biosensor (above) compared to that of gel electrophoresis (below). Various concentrations of the amplified target DNA were tested by both methods. (*indicate the significant difference compared to normal genotype.)

($p < 0.05$) between the normal genotype and α-thalassemia 1 carriers required 100 μg/mL of DNA which obtained the resonance frequency changes 34.7 ± 4.04 Hz and 50.3 ± 2.52 Hz, respectively.

Figure 3 shows the sensitivity of the α-thalassemia 1 biosensor. The lowest DNA concentration that could diagnose Hb Bart's hydrops fetalis was 25 μg/mL. However, analysis by agarose gel electrophoresis required at least 100 μg/mL of DNA to be clearly seen after staining with ethidium bromide. Therefore, the α-thalassemia 1 biosensor was four times more sensitive than conventional gel electrophoresis.

In addition, to sensitivity, the α-thalassemia 1 biosensor was also tested for specificity. The same experiments were performed using blood samples from carriers of either α-thalassemia 2 or β-thalassemia. Figure 4 shows that the changes in resonance frequency of both samples were similar to that of the normal genotype. These results indicated that the α-thalassemia 1 biosensor was highly specific for diagnosing only globin gene defects causing α-thalassemia 1. There was no misdiagnosis with α-thalassemia 2 and β-thalassemia.

The α-thalassemia 1 biosensor was tested for storage stability at room temperature. Every 15 days after storage, the sensors were used for the differential diagnosis of the normal genotype from α-thalassemia 1 carriers and patients with Hb Bart's hydrop fetalis and compared with the freshly prepared sensor. As shown in Figure 5, the diagnostic ability of the α-thalassemia 1 biosensor was stable up to 180 days ($p < 0.05$).

Clinical application of the developed α-thalassemia 1 biosensor

Since thalassemia carriers always have microcytic red blood cells, blood samples with low mean corpuscular volume (MCV < 80 fL) were selected for evaluation of the diagnostic potency of the new biosensor. DNA from 44 blind blood samples were purified and amplified by PCR, and then diagnosed either with the α-thalassemia 1 biosensor or standard gel electrophoresis. As shown in Figure 6, the diagnostic ability of the α-thalassemia 1 biosensor was 100% consistent with the gel electrophoresis in the identification of 12 of 44 samples as being α-thalassemia 1 carriers (SEA type).

All results from both known and clinical samples indicated the diagnostic potential of the newly developed α-thalassemia 1 biosensor as compared to standard agarose gel electrophoresis.

Discussion

Complementary base pairing forms the principle of DNA biosensor technology, which is often based on the hybridization of target DNA with a specific probe on a bio-sensing surface (2–5). DNA complementary binding will change the transducer signal (5). Different transducers have been reported including optical procedures based on surface plasmon resonance (19, 20), electrochemical (21, 22) and piezoelectric devices (2–4, 7). The functionalized quartz crystal gold

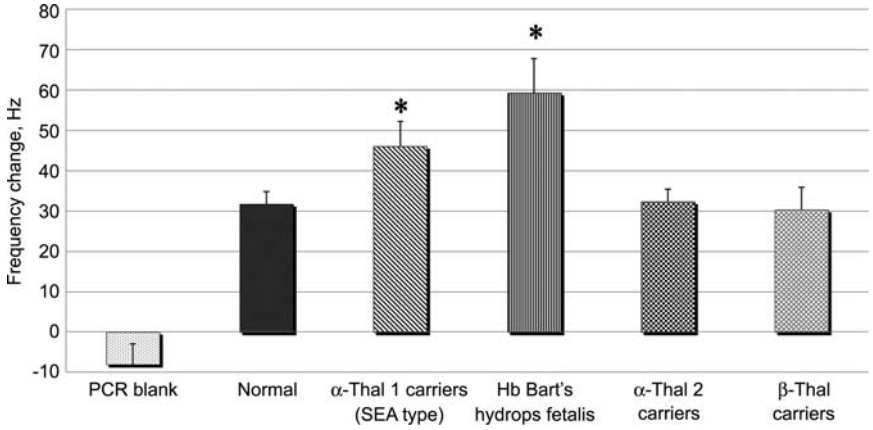

Figure 4 Specificity of the α-thalassemia 1 biosensor.
Target DNA from carriers of either α-thalassemia 2 or β-thalassemia were hybridized to the biotinylated probe of the biosensor and changes in resonance frequency were measured. Results were compared to those with normal genotypes, α-thalassemia 1 carriers (SEA type) and patients with Hb Bart's hydrops fetalis. (*indicate the significant difference compared to normal genotype.)

surface is one of the most common piezoelectric transducers used as a QCM (4–7, 23, 24). Measurement of the oscillation of the quartz crystal allows quantitative determination of the hybridized DNA fragment. However, the label-free QCM has a major limitation of non-specific quartz surface adsorption, which might be avoided by appropriate immobilization chemistry (25–27).

Homozygous α-thalassemia 1 or Hb Bart's hydrops fetalis is the fatal form of thalassemia (9). Couples who are carriers of α-thalassemia 1 have a 25% chance of producing babies born with this deadly disease. An important prevention program is the accurate diagnosis of carriers and family planning. Many methods have been used to screen carriers, but the definitive diagnosis requires identification of the abnormal gene(s) (11–13). PCR has been widely used to amplify a fragment of abnormal DNA. In this paper, we reported the coupling of PCR with a QCM and an optimized procedure to detect an abnormal α-globin gene causing α-thalassemia 1 (SEA deletion).

QCM has been applied in the diagnosis of α-thalassemia 1 (SEA deletion) (28). However, this previous work used a 5-MHz AT-cut quartz crystal (2.54 cm in diameter) with a gold electrode (1.33 cm^2 area). In the present study, the sensitivity was improved using 12-MHz AT-cut quartz crystal (26, 29, 30). In terms of cost effectiveness, the small quartz crystal (4 mm in diameter and a gold area of 0.1257 cm^2) was utilized since the big crystal costs approximately $100 while the small one costs only $2–$3. In addition, the primers used to amplify the target genes were also improved to obtain products that were not bigger than 500 bp, since long DNA fragment could result in steric hindrance during hybridization (30–33). In this study, the PCR product amplified from nor-

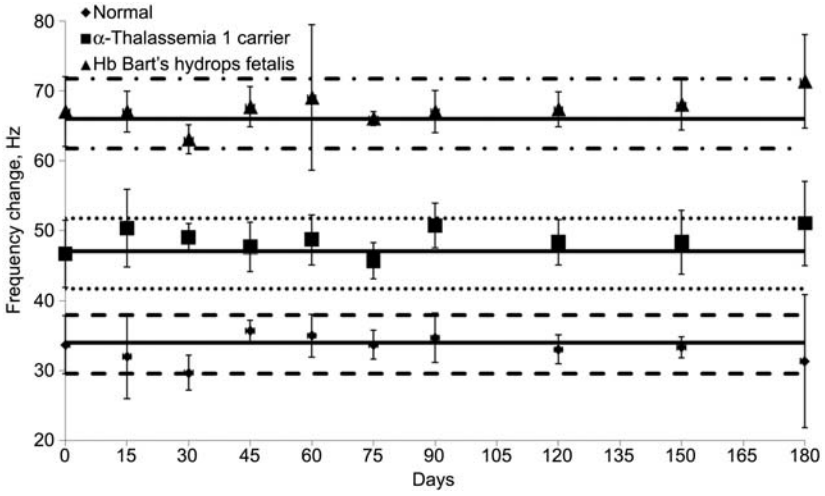

Figure 5 Storage stability of the α-thalassemia 1 biosensor.
The biosensor was stored at room temperature. At each time interval, it was used to test samples from normal genotypes, α-thalassemia 1 carriers (SEA type) and patients with Hb Bart's hydrops fetalis and compared to the freshly prepared sensor (day 0).

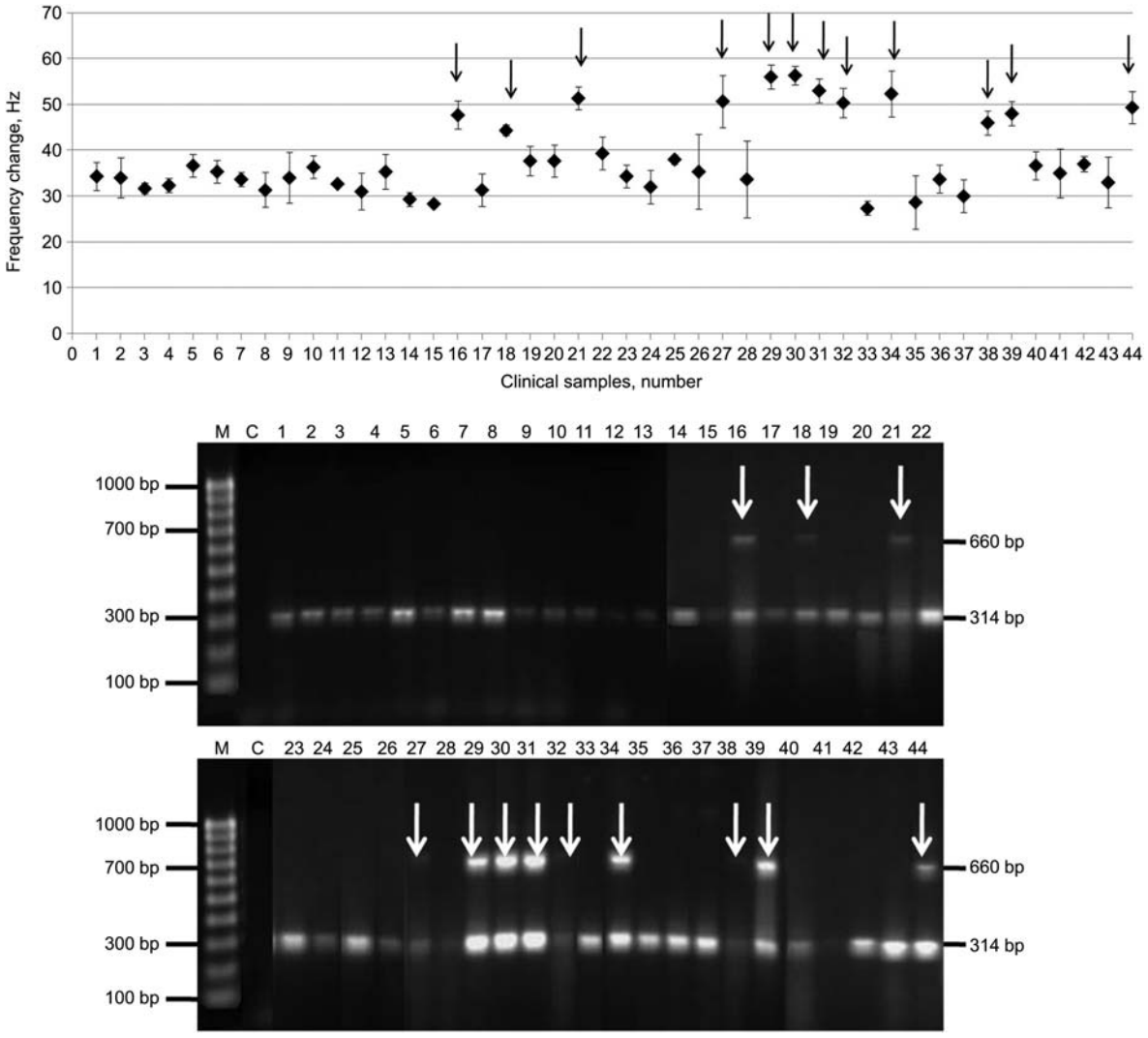

Figure 6 Clinical application of the α-thalassemia 1 biosensor compared to that of agarose gel electrophoresis.
Arrows indicate 12 positive samples carrying abnormal globin gene of α-thalassemia 1 trait (SEA deletion). C is the PCR control and M is the molecular weight markers (100 bp DNA ladder; BioRad).

mal gene was 314 bp, and that of Hb Bart's hydrops fetalis was 660 bp. The carrier was heterozygous and showed two bands at both positions. The steric hindrance effect was also reduced by adding 6-dT as a spacer to the 5′-end of the biotinylated probe (30, 32).

Immobilization of probe to the quartz gold surface was based on avidin-biotin interaction (25) since its affinity constant is very high (10^{15} M^{-1}). The strong binding could ensure stability during biosensor development which required multiple washing, drying and binding steps (26, 27).

The quartz gold surface was sequentially treated with immobilization reagents MPA, EDC and NHS in order to functionalize the surface for attachment of avidin (25). However, the immobilization of MPA, EDC and NHS could not be directly verified because the resonance frequency changes of each reaction were too low to be detected (30). The success of these steps could be indirectly validated at the step of avidin attachment by the sum of resonance frequency changes (139.8 ± 4.60 Hz). The frequency change has a linear relationship to the deposited mass according to the Sauerbrey equation (1). Therefore, the number of attached avidin molecules could be estimated ($4.94 \pm 0.16 \times 10^{11}$ molecules). However, the theoretical number of immobilized avidin molecules bound as a monolayer should not exceed 5.18×10^{11} molecules, calculated on the basis of the molecular size of avidin ($6.0 \times 5.5 \times 4.0$ nm) (34) and area of gold surface (0.1257 cm^2). Therefore, $4.94 \pm 0.16 \times 10^{11}$ molecules of avidin could be immobilized on the quartz gold surface as a monolayer.

The biotinylated probe, $1.97 \pm 0.30 \times 10^{12}$ molecules, was attached to the avidin coated quartz gold surface. The binding ratio of avidin to biotin was 3.99. In this case, the avidin was immobilized. Thus, the ratio might be too high and indicate some non-specific adsorption which is a limitation of QCM (25–27). However, the immobilized probe could still be used as a biosensor to diagnose α-thalassemia 1.

In terms of detection limit, the biosensor was more sensitive than gel electrophoresis for identifying Hb Bart's hydrops fetalis (25 μg/mL vs. 100 μg/mL), but the differential diagnosis between the normal genotype and α-thalassemia 1 carriers using the biosensor needed required higher concentrations (100 μg/mL) (Figure 3). This might be due to competition between DNA fragments of 314 bp and 660 bp of α-thalassemia 1 carriers for hybridization with the immobilized probe. The shorter fragments might hybridize better than longer ones. Therefore, the low deposited mass (25 μg/mL) could not discriminate α-thalassemia 1 carriers from non-carriers. However, the detection limit at 100 μg/mL is close to that previously reported in DNA piezoelectric biosensor for β-thalassemia (35). The sensitivity could be increased if a labeled DNA probe was used. Recently, Lien et al. developed an automated platform based on a microfluidic system and used a fluorescent-labeled DNA probe to detect α-thalassemia 1. The detection limit of this system was 12 ng/mL (36).

Figure 4 shows the high specificity of the α-thalassemia 1 biosensor for identifying only the abnormal α-globin gene causing α-thalassemia 1. There was no cross reactivity with diagnosis of either α-thalassemia 2 or β-thalassemia. The specificity was comparable to standard gel electrophoresis.

In addition, to sensitivity and specificity, the α-thalassemia 1 biosensor was tested for its diagnostic potential after storage (Figure 5). The biosensor was stable up to 180 days at room temperature. The results indicated its capability for transportation for use in remote areas. The storage time might be longer if kept at 4°C, as described previously (37).

Since most thalassemias are associated with microcytic red blood cells, blood samples with MCV values <80 fL were selected to validate the new biosensor compared to standard gel electrophoresis. As shown in Figure 6, the diagnostic ability of the α-thalassemia 1 biosensor was consistent with that of gel electrophoresis for identifying 12 of 44 samples as α-thalassemia 1 carriers (SEA deletion). The 12 samples (27.3%) are in the range of the incidence of α-thalassemia carriers in Thailand, as reported previously (38).

Conclusions

It is essential to differentiate α-thalassemia 1 carrier from the normal genotype since two carriers carry a high risk of producing a baby with homozygous α-thalassemia 1 or Hb Bart's hydrops fetalis. In this study, the α-thalassemia 1 biosensor was developed based on the DNA hybridization on a piezoelectric surface of quartz crystal without a labeling system. This new biosensor showed better analytical sensitivity than standard gel electrophoresis. Its diagnostic potential was compatible to other DNA detection methods. It was stable at room temperature with storage stability of up to 6 months. This stability allows the biosensor to be transported and used in remote area of thalassemic control sites in order to prevent the birth of new cases of Hb Bart's hydrops fetalis.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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