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Biosensors & Bioelectronics 16 (2001) 85–95

BIOSENSORS & BIOELECTRONICS

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Microgravimetric DNA sensor based on quartz crystal microbalance: comparison of oligonucleotide immobilization methods and the application in genetic diagnosis

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Received 7 February 2000; received in revised form 29 September 2000; accepted 26 October 2000

Abstract

We report on the study of immobilization DNA probes onto quartz crystal oscillators by self-assembly technique to form variety types of mono- and multi-layered sensing films towards the realization of DNA diagnostic devices. A 18-mer DNA probe complementary to the site of genetic β-thalassaemia mutations was immobilized on the electrodes of QCM by covalent bonding or electrostatic adsorption on polyelectrolyte films to form mono- or multi-layered sensing films by self-assembled process. Hybridization was induced by exposure of the QCMs immobilized with DNA probe to a test solution containing the target nucleic acid sequences. The kinetics of DNA probe immobilization and hybridization with the fabricated DNA sensors were studied via in-situ frequency changes. The characteristics of QCM sensors containing mono- or multi-layered DNA probe constructed by direct chemical bonding, avidin–biotin interaction or electrostatic adsorption on polyelectrolyte films were compared. Results indicated that the DNA sensing films fabricated by immobilization of biotinylated DNA probe to avidin provide fast sensor response and high hybridization efficiencies. The effects of ionic strength of the buffer solution and the concentration of target nucleic acid used in hybridization were also studied. The fabricated DNA biosensor was used to detect a set of real samples. We conclude that the microgravimetric DNA sensor with its direct detection of amplified products provide a rapid, low cost and convenient diagnostic method for genetic disease.

Keywords: Quartz crystal microbalance; DNA hybridization; Biosensor; Monolayer; Multilayer; β-thalassaemia

1. Introduction

Sequence-specific hybridization between nucleic acids in solution and immobilized on fixed supports are widely used, powerful methods for the detection and analysis of genetic material (Ausubel et al., 1993). Applications of this technology include tests for genetic disease; detection and characterizations of viruses, bacteria, and parasites; establishing identities in forensic and environmental cases; and assays in the oncogenic field (Symons, 1989). There is increasing interest in the detection of specific DNA sequences using biosensor methods, which do not require the use of labels such as radioisotopes, enzymes, and fluorophores (Downs, 1991). Biosensor systems based on nucleic acids not only eliminate the need for such labels but also offer the potential advantage of rapid, real-time solution monitoring of DNA hybridization, as well as high sensitivity and specificity. The basis of operation for a DNA biosensor is the complementary coupling between the specific DNA sequences within target analytes and the specific DNA sequences immobilized onto the solid support (i.e. transducer).

Methods used for the direct detection of DNA binding through base pairing (without using specific labels) have been reported based on electrochemical (Palecek, 1988), optical (Jost et al., 1991; Piscevic et al., 1995) and piezoelectric (Yamaguchi et al., 1993; Su and Thompson, 1995; Caruso et al., 1997b; Su et al., 1997; Bardea et al., 1998) techniques. Piezoelectric transducer offers the advantages of a solid-state construction,

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0956-5663/01/$ - see front matter
PII: S0956-5663(00)00136-6

chemical inertness, durability, and ultimately the possibility of low cost mass production. The AT-Cut quartz crystals as piezoelectric materials can function in a 'microbalance' mode, and is known as quartz crystal microbalance (QCM). The QCM is an extremely sensitive mass sensor, capable of measuring sub-nanogram levels of mass changes. The potential for the detection of DNA hybridization based on QCM devices has recently been demonstrated (Yamaguchi et al., 1993; Su and Thompson, 1995; Caruso et al., 1997b; Su et al., 1997; Bardea et al., 1998; Zhou et al., 2000). The QCM sensors were used to kinetically measure DNA hybridization in solution (Su et al., 1997), quantitatively study sequence-specific binding of peptides to a duplex DNA (Okahata et al., 1998), and directly monitor DNA polymerase chain reaction (Niikura et al., 1998). More recently, the QCM sensors have been applied in genetic detection of a Tay-Sachs genetic disorder (Bardea et al., 1998) and in the study of UV-damage (Zhang et al., 1998).

Currently, DNA biosensors are fabricated by immobilization of the nucleic acids on transducer surfaces by covalent binding. This puts limitations on using different types of materials as transducer surfaces because the surfaces should contain chemical groups capable of linking the fragments of nucleic acids. Moreover, the chemical reactions between the nucleic acid and the transducer surfaces may change some physical properties of the transducers and also may damage the biological properties of the nucleic acids. (It is noted that in many cases where biomolecules have been directly bound to solid surface through adsorption, they have been denatured in the process, thereby losing their biospecific activity, Sukhorukov et al., 1996a). The chemical bonding immobilization method is also time-consuming. Thus, there is a need to develop methods of immobilization of nucleic acids while saving the nucleic acid structure. For this purpose it seems promising to use mono- and multilayer nucleic acid-containing ultrathin films prepared by the Langmuir–Blodgett technique (Decher et al., 1992) or by the self-assembly successive deposition of nucleic acids and polyelectrolytes (Lvov et al., 1993; Sukhorukov et al., 1996a). Electrostatic attachment at different points makes the adsorbed nucleic acid layers stable. Although the LB process can provide well-oriented, densely packed mono- and multilayered structures useful for many applications, e.g. non-linear optics, microelectronics, and sensor coatings, these products and their uses have certain disadvantages. The layers in the ultrathin layered product of LB are mechanically unstable, being held together primarily by van der Waals forces. Also, the products that can be produced by the LB method are somewhat limited because the organic materials for building up the layers must be spreadable on the water surface. Some structural details of the DNA-containing multilayer LB and self-assembled films have been studied by X-ray analysis and spectroscopies (Lvov et al., 1993; Sukhorukov et al., 1996b; Montrel et al., 1997). Moreover, it is reported that the formation of multilayered biomolecules films on the surface of sensors can increase the detection limits of the biosensor (Caruso et al., 1997a).

β-Thalassaemia is a heterogeneous inherited disorder characterized by reduced or absent β-globin gene expression. This disease has a high frequency in Mediterranean Basin, Africa, South-East Asia and the Indian subcontinent (Weatherall and Clegg, 1981). There are over 160 known mutations in β-thalassaemia, affecting 150 million people worldwide. This requires life-long blood transfusion and high level iron chelating therapy for survival. The 4-bp deletion in codon 41/42 (-TTCT) in the β-globin gene is a common mutation that causes β-thalassaemia. Prenatal diagnosis of β-thalassaemia has been carried out using globin chain biosynthesis (Alter et al., 1980), allele-specific oligonucleotide (ASO) hybridization after DNA amplification (Tan et al., 1993), and amplification refractory mutation system (ARMS) (Tan et al., 1994; Thong et al., 1996). All of these diagnosis methods are time-consuming and are not easy to handle. There is a need for development of a rapid diagnostic method for rapid detection of β-thalassaemia disease.

In this report, we propose a new diagnostic method based on QCM device for the detection of genetic β-thalassaemia using DNA hybridization technique. The DNA diagnostic QCM sensors were fabricated by immobilization of a specific nucleotide as a probe molecule on the QCM electrode. Methods for immobilization of DNA probe on the QCM electrode by chemical bonding or electrostatic adsorption to form monolayer or multilayer DNA sensing films are explored. The kinetics of DNA immobilization and hybridization of the immobilized DNAs were studied via in-situ frequency changes. Hybridization was conducted by exposure of the DNA probe immobilized QCM to a test solution contains PCR product. The effects of ionic strength of the buffer solution and the concentration of target nucleic acid used in hybridization were also studied. The characteristics of QCM sensors containing mono- and multi-layered DNA probe were compared. The fabricated DNA sensors were applied to test a set of real samples.

2. Experimental section

2.1. Chemicals and materials

Sodium poly(styrenesulfonate) (PSS, MW 70 000, Aldrich Co.) at concentration of 3 mg/ml, poly(allylamine)hydrochloride (PAAH, MW 50 000–65 000,

Aldrich Co.) at a concentration of 3 mg/ml were prepared with pure water. 3′,3′-dithiopropionic acid ethanolamine was obtained from Sigma and was used as obtained. Avidin from hen egg white, *N*-hydroxysuccimide (NHS), and *N*-ethyl-*N*′-(3-(dimethyl)aminopropyl)carbodiimide hydrochloride (EDC) were purchased from Fluka. HEPES buffer (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) and MES buffer (2-(*N*-morpholino)ethanesulfonic acid) were obtained from Aldrich. Solubilizing buffer and hybridization buffer was 0.05 M HEPES, 0.2 M NaCl, and pH 7.5, referred as 'HEPES buffer'. MES buffer of pH 6.0 was prepared by 0.05 M MES, and 0.2 M NaCl. All other chemicals were of at least reagent grade and were used as received. Deionized water from Milli-Q system was used to prepare all solutions.

The 18-mer single-stranded oligonucleotide having the sequence 5′-CAG AGG TTG AGT CCT TTG-3′ that is complementary to the 41/42 mutation in β-thalassaemia sequence were synthesized by GENSET CA, France. 5′-biotinylated oligonucleotides were prepared using biotinylating amidite (Biodite, Pharmacia Biotech AB). The 18-mer oligonucleotide probe having a disulfide group at 5′-phosphate end was synthesized with a DNA synthesizer(Perkin-Elmer; Model 391 PCR-MATE EP) by introducing the (β-hydroxyethyl)dithioethoxy group to the 5′-phosphate end with water-soluble carbodimide. All the resultant DNA probe was purified with a NAP column (Pharmacia), and their concentrations were determined by an optical density measurement taken at 260 nm.

2.2. DNA extraction and purification

DNA from blood and Chorionic-Villi (CV) were extracted in Tris–EDTA (pH 8) using sodium-dodecylsulphate and proteinase *K* at 37°C overnight. DNA was then purified using phenol–chloroform–isoamyl-alcohol digestion and precipitated in sodium acetate and ethanol.

2.3. PCR protocol

The 443 base pair target DNA sequence (41/42β) that has 4bp deletion is the PCR product with codon 41/42 mutation in β-thalassaemia. DNA amplification was carried out using 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% gelatin in a total volume of 25 μl. About 10–20 pmol of PCR primers and 1 μg of DNA was added and the mixture was denatured at 95°C for 5 min. Enzyme Taq polymerase (0.5 U final concentration) was added and the mixture was overlaid with 30 μl of mineral oil. The PCR consisted of 30 cycles at 93°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min, followed by a final extension step at 72°C for 3 min. Amplified DNA (15 μl) was visualized after electrophoresis in 1% agarose and ethidium bromide staining to determine the target of the right size (M.W. $= 1.51 \times 10^4$ d). All primers were annealed at 65°C. DNA sequence without mutation (41/42N) is taken as negative control. The concentration of 41/42 mutation sample (i.e. 41/42β) and 41/42 normal sample (i.e. 41/42N) PCR product is 0.525 and 0.5875 μg/μl, respectively. The codon 41/42 PCR primer has the sequence 5′-ACC TCA AAA TGT GAG GCC AC-3′ (Forward) and 5′-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3′ (Reverse).

2.4. QCM apparatus

The quartz crystals employed in this study were commercially available 10 MHz, AT-cut type (diameter 13.67 mm) with Au electrodes (5.1 mm diameter) on both sides, purchased from International Crystal Manufacturing Co., Oklahoma City, OK USA. The electrodes from the crystal were connected to a TTL oscillating circuit based on IC 74LS04, similar to that described in the literature (Nomura et al., 1985). The output frequency was measured using a universal counter (Thurlby-Thandar Ltd., Cambridgeshire, UK, model TF830) attached to a personal computer. PZ-Tools (Universal Sensors, USA) was used for measurements, data storage and evaluation). The following equation has been established for an AT-cut shear mode QCM (Sauerbrey, 1959):

$$\Delta f = -2f_0^2(\rho_q\mu_q)^{-1/2}\Delta m/A, \quad (1)$$

where Δf is the measured frequency shift due to the added mass in hertz, f_0 the fundamental oscillation frequency of the dry crystal, $\Delta m/A$ the surface mass loading in grams per square centimeter, ρ_q the density of quartz (2.65 g/cm^3), and μ_q the shear modulus (2.95×10^{11} dyne/cm^2). For the 10 MHz quartz crystals used in this work, Eq. (1) predicts that a frequency change of 1 Hz corresponds to a mass increase of 0.902 ± 0.01 ng on the electrode.

For in-situ detection of frequency change, the quartz crystal was fixed vertically between two blocks of Plexiglas of a detection cell in which only one side of the quartz crystal was allowed to contact with the aqueous sample solution. The frequency response was stable within ± 1.0 Hz in air over periods of 3–4 h, when contacted with solution, the response was less stable but typically better than ± 2.0 Hz.

2.5. Sensing film preparation

The DNA probe was immobilized on the QCM electrodes by the following methods to form sensing films that have different structures: (I) immobilization of biotinylated DNA via interaction with avidin which is covalently bonded to the electrode (sensing film 1);

(II) immobilization of biotinylated DNA via interaction with avidin which is electrostatically adsorbed on polyelectrolyte (sensing film 2 and 3); (III) direct immobilization of DNA probe on polyelectrolyte films by electrostatic adsorption (sensing film 4 and 5); and (IV) direct immobilization of DNA probe that has disulfide group on the Au electrodes by chemisorption (sensing film 6). Fig. 1 illustrated the structures of the fabricated DNA sensing films.

2.5.1. Immobilization of biotinylated DNA via interaction with avidin which is covalently bonded to the electrode (sensing film 1)

The gold electrodes of 10 MHz crystal was cleaned with hot Piranha solution (30% H_2O_2:H_2SO_4/1:3, *Caution:* this mixture reacts violently with organic materials, and it should not be stored in closed containers), and then thoroughly rinsed with distilled water and ethanol. The cleaned quartz crystal was soaked in an aqueous solution (2 ml) of 3′,3′-dithiopropionic acid (1 mM) at room temperature for 1 h, followed by water rinsing. Before drying, 5 μl of 100 mg/ml EDC ethanolic solution was then placed on the surface, followed immediately by 5 μl of 100 mg/ml NHS aqueous solution. These solutions were allowed to interact with 3′,3′-dithiopropionic acid monolayer formed on the QCM electrode for 30 min in a 100% humidity environment to prevent solution evaporation. Then the QCM having the activated carboxyl groups was immersed in 1 ml HEPES buffer of 0.1 mg/ml avidin for at least 1 h, and rinsed with water again. The QCM was then immersed in the aqueous solution of ethanolamine (1 mM, pH 8.0, adjusted using HCl) for 30 min to convert the residual carboxyl group to β-hydroxyethylamide. After rinsing the QCM electrodes with water, the avidin-immobilized QCM was immersed in 1 ml of HEPES buffer of biotinylated olidonucleotide (10 μg/ml). The resonance frequency was monitored as a function of time until immobilization was completed, as indicated by a constant and maximum frequency shift. After rinsing with water, drying in air, the frequency change of the QCM was measured.

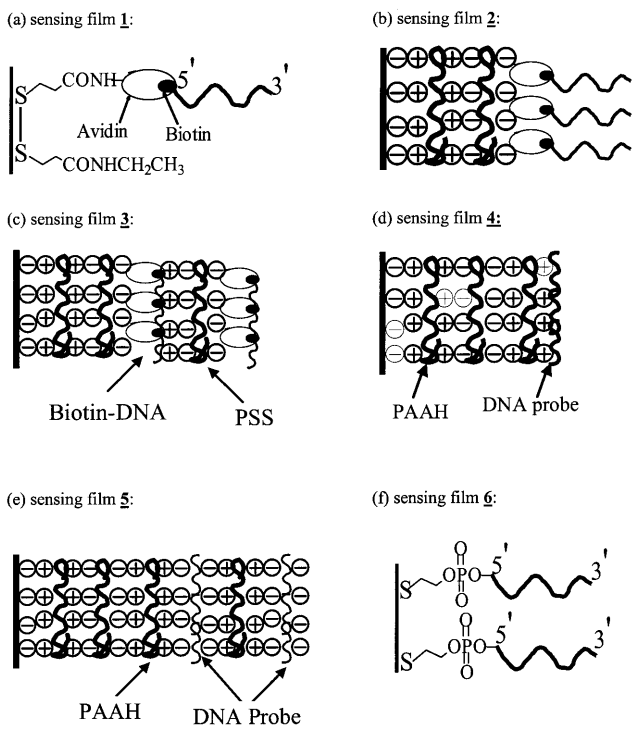

Fig. 1. Structure of sensing film: (a) Biotin-DNA immobilized via interaction with avidin which is covalently bonded on the QCM electrode (sensing film 1); (b) Biotin-DNA immobilized via interaction with avidin which is adsorbed on (PAAH/PSS) precursor film by electrostatic adsorption to form monolayer sensing film (sensing film 2); (c) biotin-DNA immobilized with avidin/PSS multilayer films constructed by alternate deposition of avidin and PSS on (PAAH/PSS) precursor film coated QCM electrodes (sensing film 3); (d) DNA probe immobilized on the outer layer of PAH/PSS/PAH film as monolayer by electrostatic adsorption (sensing film 4); (e) DNA probe immobilized on the outer layer of PAH/PSS/PAH film as multilayer sensing film by alternate deposition of avidin and PSS (sensing film 5); (f) DNA probe immobilized directly on QCM electrode by chemical bonding (sensing film 6).

2.5.2. Immobilization of biotinylated DNA via interaction with avidin that is electrostatically adsorbed on polyelectrolyte films (sensing film 2 and 3)

The cleaned crystal was first modified with 3′ ,3′-dithiopropionic acid/aqueous solution for 1 h to form a self-assembled monolayer film with the carboxyl groups outside, which are then able to interact with the polyelectrolytes under certain conditions. The precursor film of (PAAH/PSS) on modified QCM electrode was prepared by repeating two alternate adsorption cycles of PAAH and PSS. Polyelectrolyte adsorption was performed as follows. The crystal were immersed in 5 ml of 3 mg/ml PAAH solution (pH 8.0, adjusted by adding NaOH) for 5 min, followed by washing with water. The PAAH-coated crystal was then exposed to 5 ml of 3 mg/ml PSS aqueous solution of 1 M $MnCl_2$ and 0.01 M HCl, pH $\sim$2) for 5 min. This surface was then washed with deionized water. Avidin was then adsorbed onto the outer PSS layer from HEPES buffer solution of 0.1 mg/ml avidin for the subsequent immobilization, after which the surface was rinsed with water. The avidin-coated QCM was then exposed to 1 ml of HEPES buffer solution of biotin-DNA (10 μg/ml) to form sensing film 2.

Multilayered biotin-DNA film (sensing film 3) was fabricated by the successive solution deposition of avidin and PSS (up to a total of five layers) on the (PAAH/PSS) precursor film and subsequent exposure of this thin film to a solution of biotinylated DNA. Experiments were carried out as follows. The avidin-coated QCM fabricated as above was exposed to 1 ml of a PSS solution (3 mg/ml of PSS contained 0.01 M

$MnCl_2$, 0.01 M HCl, pH ~2) for 2 min. This surface was rinsed with pure water, and then exposed to HEPES buffer solution of 0.1 mg/ml avidin for the subsequent immobilization. This procedure was repeated until a total of five layers (three avidin and two PSS) were deposited. The multilayer film, which was kept wet to avoid avidin denaturation, was placed into HEPES buffer solution of biotin-DNA (10 μg/ml) for 50 min. The QCM was then removed from the solution, rinsed and was either used immediately for hybridization experiments or stored at 4°C.

2.5.3. Direct immobilization of DNA probe on polyelectrolyte films by electrostatic adsorption (sensing film 4 and 5)

The QCM substrate modified with 3′ ,3′-dithiopropionic acid monolayer was deposited with PAAH/PSS/PAAH precursor layer by alternating adsorption as described above. DNA probe was adsorbed onto the outer PAAH layer from MES buffer solution of 10 μg/ml DNA probe for 30 min to form sensing film 4. The multilayer DNA/PAAH sensing film 5 was further performed by alternative adsorption of DNA/PAAH pair from 10 μg/ml of DNA probe solution and PAAH solution, respectively.

2.5.4. Direct immobilization of DNA probe on QCM electrodes by chemisorption (sensing film 6)

The direct immobilization of DNA probe onto the QCM electrodes by chemisorption was carried out by immersing the cleaned QCM in 1 ml aqueous solution (contained 0.3 M NaCl) of 10 μg/ml DNA probe having disulfide group for 1 h. The immobilization amount was followed by a frequency decrease in solution until equilibrium was reached. The QCM was then removed from solution, rinsed, and used in the hybridization experiments.

2.6. Hybridization experiments

Hybridization was conducted by immersing a QCM immobilized with DNA probe into detection cell containing 1 ml HEPES buffer solution at the room temperature, and the resonance frequency of the QCM was defined as zero after the equilibrium. The frequency change of the QCM responding to the addition of 10–100 μl of aqueous solution of target DNA in the detection solution was recorded. Hybridization was evidenced by two modes: (i) stepwise; and (ii) in-situ frequency change. In stepwise mode, the QCM sensor was removed from the solution after hybridization, rinsed and dried and the in-air frequencies was recorded. For in-situ experiments, one side of the quartz crystal is in contact with solution and the frequency change in solution due to hybridization was monitored as a function of time. Non-complementary (i.e. non-specific) binding was assessed by reacting non-complementary sample with the DNA probe immobilized QCM at the same condition.

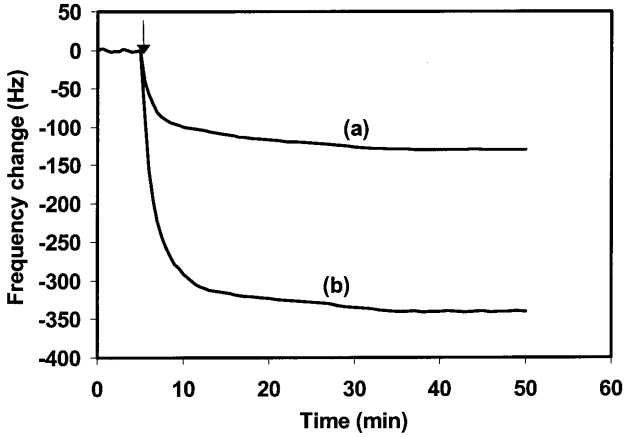

Fig. 2. QCM frequency change as function of time for the immobilization of avidin from 0.1 mg/l aqueous avidin solution: (a) avidin binding to 3′,3′-dithiopropionic acid/EDC monolayer; (b) avidin adsorbing electrostatically on the outer negatively charged PSS layer of the (PAAH/PSS) precursor film. The arrow indicates the time at which avidin was injected into water solution.

3. Results and discussion

3.1. DNA sensing film preparation

The avidin immobilization plays the key role in the immobilization of Biotin-DNA probe via avidin–biotin interaction. Fig. 2, curve (a) shows the frequency change of avidin immobilization onto 3′,3′-dithiopropionic acid/EDC monolayer from an aqueous solution of 0.1 mg/ml avidin. The in-solution frequency change (ΔF_{sol}) is about 130 Hz, and adsorption is completed within 30 min. However, the ΔF_{sol} value cannot be directly transposed to mass change because in liquid phase the QCM does not necessarily behave as predicted by the Sauerbrey equation due to viscoelastic effects arising from the solvent and the adsorbed layers (Kanazawa, 1990; Karpovich and Blanchard, 1994). In addition, water entrapped within the layers also contributed to the observed in-solution frequency changes. In order to study the avidin adsorption, the avidin adsorption after drying is recorded. The in-air frequency change (ΔF_{air}) for the avidin adsorption after drying is 70 ± 7 Hz (average $\pm$ standard deviation for triplicate experiments). The factor of ~2 difference between the (ΔF_{sol}) and (ΔF_{air}) values can be ascribed to water entrapped within the protein layers (Ebara and Okahata, 1993; Caruso et al., 1997a). Converting the ΔF_{air} value to a mass using 0.902 ng/Hz for our QCM system (Zhou et al., 1997a,b; Ng et al., 1998) yields 63 ± 6 ng for avidin adsorbed on the QCM. The exper-

imental mass changes of 63 ± 6 ng is very close to the calculated value of 69 ng for a mono-molecular layer of close packed avidin in a flat orientation (i.e. occupying an area 33 nm^2, avidin dimensions are 6.0 nm × 5.5 nm × 4.0 nm (Caruso et al., 1997b).

In this work, avidin layers were also constructed as monolayer or multilayer on the precursor layer of PAAH/PSS that was self-assembled on the 3′,3′-dithiopropionic acid modified QCM electrodes. In these films, avidin is electrostatically immobilized on the outer, negatively charged polyion surfaces. The isoelectrostatic point of avidin is 10.5, it was positive charged in the pH 7.5 HEPES buffer solution, and thus can be adsorbed on the negative charged polyion surface. Fig. 2, curve (b) shows a typical frequency change for avidin adsorption in solution onto the negatively charged PSS. A frequency change of 340 Hz is obtained at 25 min, by which time adsorption is completed. Drying of this layer resulted in $(\Delta F_{air}) = 210$ Hz. The quantity of avidin (~ 189 ng) on the surface suggests that multilayers/aggregates of avidin are formed and/or that penetration of the precursor film occurs (as mentioned in above, 69 ng is expected for a monomolecular layer of close packed avidin adsorption on QCM electrode).

Multilayer films of avidin were fabricated by the alternate deposition of avidin and PSS onto the (PAAH/PSS) coated QCM electrodes for the purpose of making biotin-DNA multilayer by exposing them to a solution of biotin-DNA. The principle of the multilayer buildup is based on the electrostatic attraction between oppositely charged species. This method has been extensively used to fabricate multilayer films of polyelectrolytes and biomolecules of alternating charges (Lvov et al., 1995; Caruso et al., 1997a). Fig. 3 shows the (ΔF_{air}) for avidin and PSS adsorption as a function of the number of layers and confirms the buildup of an avidin/PSS multilayer. The avidin and PSS were alternatively adsorbed on the precursor (PAAH/PSS) layer, with pure water rinsing, drying and frequency measurement between intermediate steps. From Fig. 3, we can see that the layer buildup is essentially linear after the first avidin/PSS layer deposition cycle. The average frequency change for avidin and PSS pair, after the first avidin/PSS layer deposition, is 580 ± 50 Hz (450 Hz for avidin, 130 Hz for PSS). The large frequency change for avidin deposition most likely represents aggregation and multilayers of avidin on the surface. For the avidin coated QCM used for further biotinylated DNA immobilization and hybridization experiments, drying of avidin layer was avoided because of avidin denaturation.

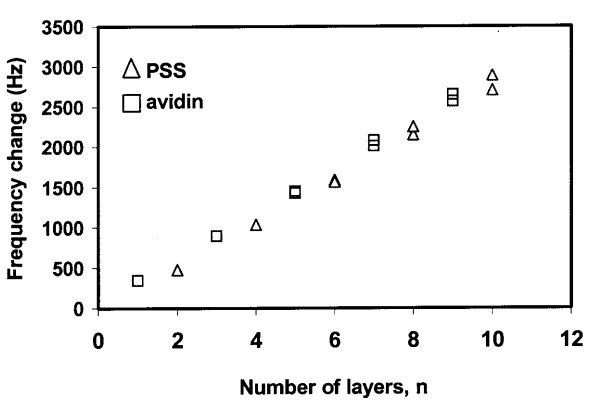

Fig. 3. QCM frequency change for the construction of alternating avidin/PSS multilayer films formed on the (PAAH/PSS) precursor film on 3′,3′-dithiopropionic acid modified electrodes. The odd layer numbers correspond to avidin deposition and the even layer numbers to PSS. Experimental data points for two separate experiments are shown.

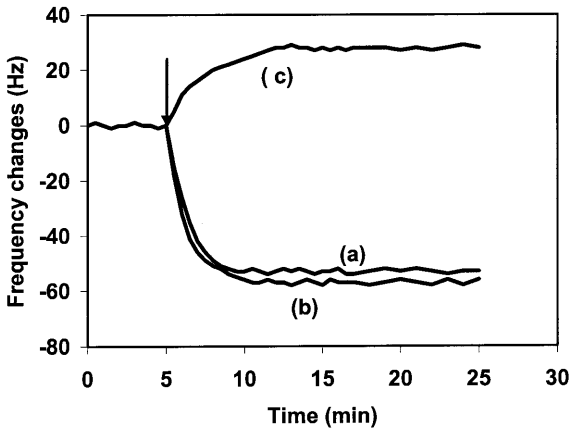

Fig. 4. QCM frequency change versus time for the immobilization of biotin-DNA from HEPES buffer on avidin modified electrodes where: (a) avidin is covalently bonded on electrodes (sensing film 1); (b) avidin is electrostatically adsorbed on (PAAH/PSS) precursor film (sensing film 2); and (c) avidin/PSS multilayer film was constructed by alternate deposition of avidin and PSS on (PAAH/PSS) coated QCM electrodes (sensing film 3). The arrow indicates the time at which biotin-DNA was injected into the HEPES solution.

Fig. 4, curves (a), (b), (c) shows the QCM frequency response with time for the subsequent immobilization of biotin-DNA via interaction with avidin which was immobilized on the QCM by different methods to form sensing film 1, 2 and 3, respectively. Immobilizations of biotin-DNA probes are confirmed by the decrease in frequency, $(\Delta F_{sol}) = 52$ and 56 Hz for sensing film 1 and 2, respectively. The immobilization processes are completed within ~ 5 min as indicated by Fig. 4, curves (a) and (b). The average frequency change (± standard deviation) for biotin-DNA immobilization for triplicate experiments in forming sensing film 1 is 52 ± 2 Hz, indicating that biotin-DNA is reproducibly immobilized. Immersion of the avidin-coated QCM crystal in HEPES buffer, followed by injection of a 5 μg/ml aqueous biotin solution and then biotin-DNA solution, showed no frequency change for the biotin-DNA step, indicating that biotin (from the biotin only solution) blocked the avidin binding sites. This result shows that biotinylated DNA is immobilized onto the avidin-

coated QCM only via interaction with avidin. The immobilization of biotin-DNA from HEPES buffer solution onto avidin layer that was electrostatically adsorbed onto the (PAAH/PSS) precursor film (sensing film 2) yielded $\Delta F_{sol} = -58$ Hz. As shown in Fig. 4, curve (b), the adsorption is completed within ~ 5 min. In this case, the frequency change and the adsorption time are the same, within experimental error, as those obtained for immobilization of biotin-DNA onto avidin in forming sensing 1. This suggests that, despite the large amount of avidin adsorbed on the precursor (PAAH/PSS) layer, the equivalent of only a monolayer of avidin is actually interacting with biotin-DNA for its immobilization.

Multilayer films of biotin-DNA (sensing film 3) were constructed in order to increase the DNA hybridization capacity of the sensing film. These films were fabricated by immersing the QCM coated with avidin/PSS multilayer film into a solution of biotin-DNA. The QCM frequency was found to increase rather than decrease (Fig. 4, curve (c)). The frequency increase which indicated desorption was occurred from the QCM surface may be attributed to avidin removal from the avidin/PSS multilayer film with biotin-DNA immobilization because of the weakly adsorbed/immobilized avidin may be solublized by avidin-DNA.

The DNA probe was also immobilized on the Au electrode directly by immersing the QCM into an aqueous buffer solution of oligonucleotide having S–S groups (sensing film 6). The immobilization amount was followed by a frequency decrease of 38 Hz (Fig. 5, curve (a)). After drying, the frequency change for the immobilization of DNA probe is 30 Hz. The immobilized amount of 27 ng on the Au electrode (0.204 cm^2) is calculated to be 31% coverage of single strand nucleotide (area per molecule ~ 2.2 nm^2) on the electrode. The immobilized DNA probe, however, was hardly

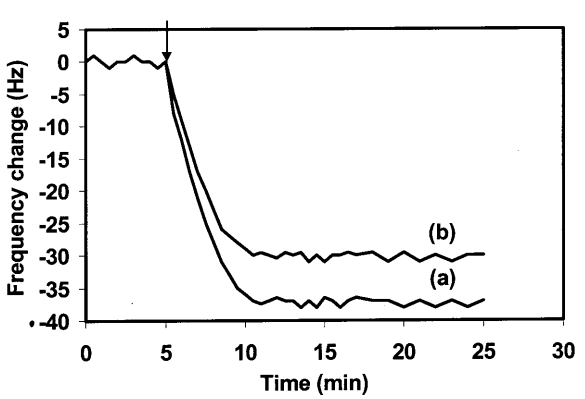

Fig. 5. QCM frequency change versus time for the immobilization of: (a) DNA probe having disulfide group on the QCM electrode by chemisorption from aqueous solution (sensing film 6); (b) DNA probe immobilized on the PAAH/PSS/PAAH precursor film by electrostatic adsorption (sensing film 4).

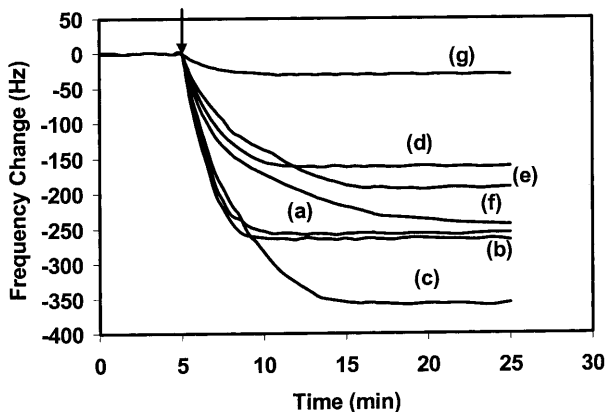

Fig. 6. Frequency change versus time for the hybridization experiments on QCM sensor with different sensing films in HEPES buffer solution of 52.5 μg/ml 41/42β DNA: (a) sensing film 1; (b) sensing film 2; (c) sensing film 3; (d) sensing film 4; (e) sensing film 5; (f) sensing film 6; (g) hybridization on sensing film 1 with 58.75 μg/ml of 41/42N DNA. The arrow indicates the time at which the target PCR product was injected into water solution.

released from the QCM during soaking in buffer for 1 day ($\Delta m \leq 4$ ng). Fig. 5, curve (b) shows the immobilization of DNA probe onto outer film of PAAH/PSS/PAAH polyelectrolyte layer by electrostatic adsorption (sensing film 4). The immobilization was followed by a frequency decrease of 30 Hz in solution and 21 Hz after dry in air (immobilized amount is calculated of 19 ng). The immobilized amount of 19 ng on the Au electrode is calculated to be 22% coverage of single strand nucleotide on the electrode. The buildup of multilayer DNA sensing film 5 via DNA/PAAH pair on PAAH/PSS/PAAH precursor film gives frequency change of 158 ± 15 Hz (52 Hz for DNA and 106 Hz for PAAH). This large amount of DNA adsorbed on the DNA/PAAH pair indicated the polyelectrolyte film has increased the surface area. In these DNA/PAAH complex films, the amino groups of the polyelectrolyte (PAAH) interact electrostatically with phosphate groups and form hydrogen bonds with nucleic bases and oxygen of polynucleotide chains (Sukhorukov et al., 1996b). These interactions provide the stability of the DNA/PAAH complex films.

3.2. Hybridization measurement

Fig. 6, curves (a)–(g) shows the frequency changes of QCM immobilized with DNA probe by different methods when it was exposed to the HEPES buffer solution, followed by injection of a solution of complementary 41/42β DNA (final concentration, 52.5 μg/ml). The interaction between 41/42β DNA and immobilized biotin-DNA/avidin which is either chemically bonded (sensing film 1) or electrostatic adsorbed (sensing film 2) as mono-sensing layer is rapid, being complete within ~ 5 min, and gives the similar frequency decrease of

~250 Hz (Fig. 6, curve (a)–(b)). A duplicate experiment of QCM with sensing film 1 yield $\Delta F_{sol} = 252$ Hz, showing that the values are reproducible. Exposure of a similarly prepared QCM with sensing film 1 to a solution of 41/42N non-complementary PCR product (final concentration, 58.75 μg/ml) for hybridization produced a frequency change of only 10 Hz (Fig. 6, curve (g)). This confirms that the 41/42β is hybridized by biotin-DNA that was immobilized on QCM via biotin-avidin interaction.

Fig. 6, curve (c) shows the QCM frequency response of biotin-DNA immobilized on QCM as a multilayered sensing film 3 exposure to HEPES buffer, and subsequent injection of 41/42β target DNA (final concentration, 52.5 μg/ml). A frequency change of 356 Hz is obtained, suggesting binding the target DNA through hybridization. A repeat experiment yielded a frequency change of 354 Hz. To check that the target DNA sequence was not non-specifically bound to avidin or PSS, the sensor was reacted with the PCR product 41/42N at the same condition, a frequency change of 30 Hz was observed. The experiments confirmed the presence of the immobilized biotin-DNA in the multilayer film although frequency increase was observed during the immobilization of biotin-DNA onto the avidin/PSS multilayer (see Fig. 4, curve (c)). The kinetics of hybridization for duplicate experiments were identical, with hybridization being complete within ~15 min, which was much slower than that for the corresponding monolayer sensing film (see earlier sensing film 1, and 2). This result indicated that the target DNA might penetrate into the multilayered sensing film. In addition, the frequency change is ~1.5 times that observed for the monolayer sensing film. This shows that the sensitivity can be enhanced for a given concentration of target DNA in solution by constructing DNA-containing multilayers.

The methods of immobilization of DNA probe directly on the QCM, either by covalent bonding (sensing film 6), or electrostatic adsorption on polyelectrolytes layer to form monolayer sensing film (sensing film 4) or multilayer sensing film (sensing film 5), were simpler than that to immobilize DNA probes by avidin–biotin interaction. Fig. 6, curve (d)–(f) shows the frequency changes due to hybridization on the QCM with sensing films 4, 5, 6, respectively. Hybridization with DNA probes adsorbed directly on the polyelectrolyte film (sensing film 4) produced ~140 Hz frequency change (Fig. 6 curve (d)), which was much lower than that for sensing film 2, with biotin-DNA immobilized via biotin–avidin interaction. Hybridization on multilayered DNA probe (sensing film 5) also gave much lower frequency change (Fig. 6, curve (e)) than that for multilayered biotin-DNA/avidin film (sensing film 3). This indicates that DNA probe adsorbed directly on the polyelectrolyte film has some mobility, and some adsorbed DNA probe do not undergo hybridization. The binding of non-complementary PCR product 41/42N onto the QCM with sensing film 4 and sensing film 5 produced ~40 Hz frequency change, which was higher than that on the sensing film 1, 2 and 3. This results indicate some non-specific binding may be included in the sensing films 4 and 5.

The spacer length of the chemical group for immobilization of DNA probe on QCM electrode also affects the hybridization. For avidin–biotin method (sensing film 1), the DNA probe was immobilized with a long spacer group (~5 nm) of a large avidin on the Au electrode. For the direct immobilization method (sensing film 6), a DNA probe was bound directly at the *S–S* group linked to the 5′-end phosphate group of the probe (spacer length ~0.6 nm). Fig. 6, curve (a) and (f) show typical time dependencies of hybridization processes on sensing film 1 and 6, respectively. Although the frequency changes in response to hybridization for both sensing films are almost identical, there is obvious difference of time scale for the hybridization equilibrium. In the direct immobilization method (sensing film 6), hybridization was much slower, taking ~30 min to reach the binding equilibrium compared with ~5 min when the probe was immobilized with a long spacer of avidin–biotin linkage.

The above results indicate that the sensing film 1 and 2 fabricated by immobilization of biotin-DNA to avidin which is covalently bonded or electrostatically adsorbed on polyelectrolytes layer can provide fast sensor response and high hybridization efficiencies. The sensing film 1 and 2 are the optimal choices for the fabrication of QCM sensor for the diagnosis of β-thalassaemia. The multilayered DNA sensing films can increase the sensor response, although, they also increase the sensor response time.

3.3. Effect of ionic strength on hybridization

Hybridization between the 41/42β target DNA and the QCM sensor with sensing film 1 and 2 were studied at different NaCl concentrations. For the purpose to compare the frequency changes of hybridization under different ion strength, the background frequency (F_b) of the fabricated QCM sensor was measured in pure water. After hybridization with the target samples, the QCM sensor was rinsed with water, and immersed into the pure water. The frequency (F_t) of the QCM sensor in pure water was measured again. The frequency change of ΔF was calculated by ($F_b - F_t$). Table 1 shows the frequency changes after hybridization was completed. Hybridization could hardly be observed without NaCl in the hybridization buffer. The apparent binding amount increased gradually with increasing ionic strength. The hybridization percentage increased gradually with increasing NaCl concentrations, because

Table 1
Effect of ionic strength on hybridization behavior between QCM sensor with 41/42β and 41/42N DNA sample at 25°C[a]

[NaCl] (M)	Sensing film 1			Sensing film 2		
	ΔF[b] (Hz)	Hybridization[b] (%)	ΔF[c] (Hz)	ΔF[b] (Hz)	Hybridization[b] (%)	ΔF[c] (Hz)
0.05	33 ± 3	12	30	38 ± 3	13	14
0.1	148 ± 10	57	40	168 ± 16	62	26
0.3	224 ± 20	86	37	240 ± 24	88	30
0.5	254 ± 26	100	47	266 ± 26	100	41
0.7	258 ± 26	100	34	264 ± 26	100	35

[a] *Condition:* 0.05 M HEPES solution, pH 7.5. [Target nucleotide] = 52.5 μg/ml. Procedures for frequency measurements are described in text.
[b] 41/42β sample, the maximum frequency change in the experiment is taken as 100% hybridization.
[c] 41/42N sample.

of the decrease in electric repulsion between nucleotides, and 100% hybridization was observed at 0.5 M NaCl. With the high ionic strength condition, the hybridization ability increased due to the fast binding and the slow dissociation process. Negligible frequency changes were observed on the hybridization of 41/41 N sample under different ionic strength with these two sensing films (data not shown).

3.4. Effect of the concentration of target DNA

Hybridization experiments were conducted with DNA probe immobilized QCM exposure to the target DNA sequence at different concentrations. Results are shown in Fig. 7. It indicated that the binding of target DNA sequence was somewhat inhibited when an excess amount of target DNA sequence complementary to the DNA probe was present in the buffer solution. This is due to the formation of double-strand in the solution by the two single strands of the target DNA at high concentration. The optimum concentration range of the target DNA sequence is 50–70 μg/ml for hybridization with sensing films 1 and 2.

3.5. Detection of β-thalasaemia samples

The fabricated DNA sensor with sensing film 1 was applied to detect a set of real patent sample. Initially, three samples that have 41/42β mutations and five samples that do not have 41/42β mutations were used as positive and negative control test to identify the performance of the fabricated QCM sensor. The fabricated sensor provided frequency background (F_b), after hybridization with the test samples the sensor provided frequency of (F_t). The frequency change of ΔF for each sample was calculated as ($F_b - F_t$). As can been seen in Fig. 8, the control samples that have 41/42β mutation showed large frequency changes compared to the normal samples. The average frequency change and the standard deviation (SD) were calculated for those negative samples. The cut-off threshold value was set up as the average frequency change of the samples added three SD. It was found that the average value of the negative reference samples plus 3 times of the their SD, 75 Hz, was sufficient in value to include all negative samples and the frequency changes of all known positive samples obtained were large than that value. Another six unknown samples were employed to justify the diagnostic sensitivity and specificity of the QCM sensor. Two samples were justified positively (with 41/42 mutation) and the other four samples were negative. These results of the tested samples were confirmed by amplification refractory mutation system (ARMS). The results indicated that the fabricated QCM sensor could be used as diagnostic test for the β-thalasaemia genetic mutation.

4. Conclusion

This study has demonstrated the possibility of using QCM technique as DNA genetic sensor for the diagnosis of β-thalassaemia. The results showed that the QCM sensor fabricated by immobilization of biotin-DNA

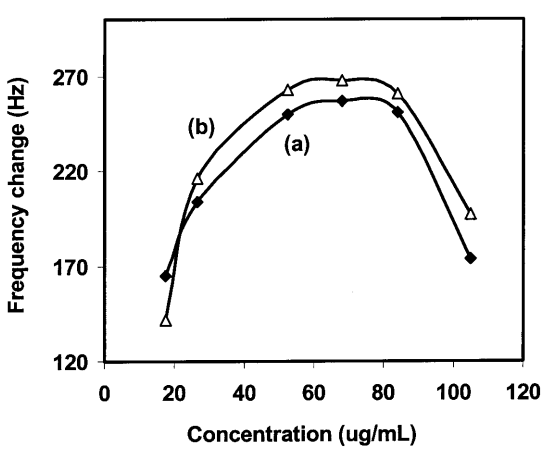

Fig. 7. Effects of target DNA concentration on sensor response. Hybridization was conducted on 0.05 M HEPES buffer solution with 0.5 M NaCl.

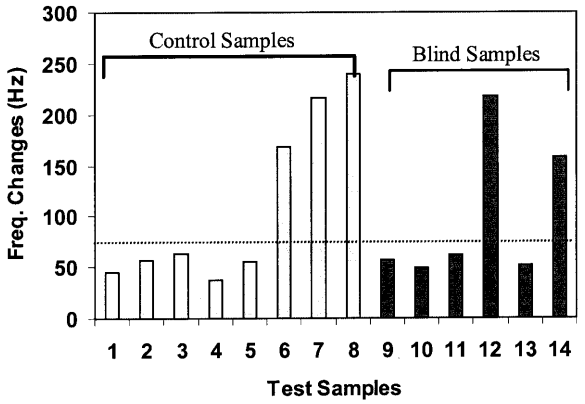

Fig. 8. Classification of positive and negative samples by QCM sensor coated with sensing film 1.

probe to avidin that is covalently bonded or electrostatically adsorbed on polyelectrolyte layers can provide fast sensor response and high hybridization efficiency to the PCR product. The multilayered DNA sensing films can increase the sensor sensitivity, and also increase the sensor response time. Results indicated that the optimum concentration range of the target DNA sequence is 50–70 μg/ml for hybridization, and 100% hybridization could be obtained at 0.5 M NaCl in the target DNA sample. The QCM genetic diagnostic sensor can provide many advantages compared with conventional fluorescence-labeling or radiolabeling methods: (i) pre- and post-treatments are not required to modify DNA probes; (ii) in-situ monitoring of hybridization is possible; (iii) speedy measurements and inexpensive instrument are used; (iv) small instrumental set-up and multiple sensor systems can be prepared on which different DNA probes are immobilized on each QCM substrate. The present study has presented practical approaches to develop oligonucleotide (DNA) sensor for diagnosis of genetic disease.

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