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Real-time detection of *Escherichia coli* O157:H7 sequences using a circulating-flow system of quartz crystal microbalance

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Abstract

A DNA piezoelectric biosensing method for real-time detection of *Escherichia coli* O157:H7 in a circulating-flow system was developed in this study. Specific probes [a 30-mer oligonucleotide with or without additional 12 deoxythymidine 5′-monophosphate (12-dT)] for the detection of *E. coli* O157:H7 gene *eaeA*, synthetic oligonucleotide targets (30 and 104 mer) and PCR-amplified DNA fragments from the *E. coli* O157:H7 *eaeA* gene (104 bp), were used to evaluate the efficiency of the probe immobilization and hybridization with target DNA in the circulating-flow quartz crystal microbalance (QCM) device. It was found that thiol modification on the 5′-end of the probes was essential for probe immobilization on the gold surface of the QCM device. The addition of 12-dT to the probes as a spacer, significantly enhanced ($P < 0.05$) the hybridization efficiency (*H*%). The results indicate that the spacer enhanced the *H*% by 1.4- and 2-fold when the probes were hybridized with 30- and 104-mer targets, respectively. The spacer reduced steric interference of the support on the hybridization behavior of immobilized oligonucleotides, especially when the probes hybridized with relatively long oligonucleotide targets. The QCM system was also applied in the detection of PCR-amplified DNA from real samples of *E. coli* O157:H7. The resultant *H*% of the PCR-amplified double-strand DNA was comparable to that of the synthetic target T-104AS, a single-strand DNA. The piezoelectric biosensing system has potential for further applications. This approach lays the groundwork for incorporating the method into an integrated system for rapid PCR-based DNA analysis.

Keywords: *E. coli* O157:H7; Quartz crystal microbalance; DNA biosensor; Foodborne pathogens

1. Introduction

Escherichia coli O157:H7 has been an important foodborne pathogen in a variety of foods worldwide in the last 20 years. It is classified as an enterohemorrhagic *E. coli* with the ability to cause hemorrhagic colitis, which includes symptoms, such as bloody diarrhea, hemolytic uremic syndrome and thrombotic thrombocytopenic purpurea (Doyle et al., 1997). Centers for Disease Control and Prevention (CDC, 2005) reported that an estimated 73,000 cases of *E. coli* O157:H7 infection and 61 resultant deaths occur in the United States each year. Consumption of as few as 10 of *E. coli* O157:H7 cells can result in growth of the pathogen in the intestine and production of shiga toxins in susceptible patients. These toxins can kill the cells of the intestinal lining, destroy the kidneys and cause blood clots in the brain, as well as seizures, paralysis and respiratory failure (FDA, 2001; CDC, 2005). Therefore, it is desirable to develop efficient analytical methods with a high degree of sensitivity and specificity to detect this organism in food.

Detecting *E. coli* O157:H7 with conventional procedures can take several days (Meng et al., 2001). A variety of rapid methods have been proposed to detect and screen many microorganisms, including *E. coli* O157:H7. These methods include antibody-based methods (immunofluorescence, immunoimmobilization, enzyme-linked immunosorbent assay, immunomagnetic separation, etc.), nucleic acid-based methods (hybridization and polymerase chain reaction [PCR]), biochemical and enzymatic methods (miniaturized microbiological methods and commercial miniaturized diagnostic kits) and membrane filtration methods (hydrophobic grid membrane filter) (Wu et al., 2004). In recent years, modern bio-techniques, such as real-time PCR (Yoshitomi et al., 2003; Fu et al., 2005), nanoparticles (Zhao et al., 2004; Mao et al., 2006) and biosensing systems (biosensors) (Campbell and Mutharasan, 2005; Simpson and Lim, 2005; Mao

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et al., 2006) have been developed for detection of pathogenic microorganisms. Biosensors are devices that detect biological or chemical complexes in the form of antigen–antibody, nucleic acids, enzyme-substrate or receptor-ligand compounds. Interest in using biosensors to detect foodborne pathogens is on the rise (Hall, 2002; Patel, 2006; Rasooly and Herold, 2006).

DNA probes (i.e. an oligonucleotide sequence immobilized on a fixed support able to hybridize the complementary strand present in solution) are powerful molecular tools for monitoring and detecting specific microorganisms in the environment or in food (Marx, 2003; Mann and Krull, 2004). The standard method of detecting nucleic acid hybrids is by labeling the probe with radioactive nucleotides, chemiluminescent dye or various hapten molecules, such as biotin. Piezoelectric mass-sensing devices based on quartz crystal microbalance (QCM) enable the label free detection of molecules, such as ligands, peptides and nucleic acids (Marx, 2003).

The core of the QCM is a specifically manufactured quartz plate with a fundamental resonance frequency in the range of 5–30 MHz. The crystal is excited to resonance and the effect of molecular absorption monitored (Sauerbrey, 1959). The QCM is comprised of thin film electrodes, usually gold (Au), deposited on each face of a crystal. Voltage is applied across these electrodes to deform the crystal plate, producing relative motion between the two parallel crystal surfaces. The crystal is induced to oscillate at a specific resonant frequency. Changes in the mass of the material on the surface will alter the resonance frequency of the crystal (Marx, 2003). A linear relationship exists between deposited mass and frequency response for quartz crystals. The resonance frequency decreases linearly with the increase of mass on the QCM electrode at the nanogram level or less. This characteristic of QCM can be exploited to develop bioanalytical tools on a 10^{-10} to 10^{-12} g scale (Zhou et al., 2000). QCM-based immunoassay has been designed and applied in several different areas (Su et al., 2000; Kurosawa et al., 2003; Su and Li, 2004). Using QCM sensing, with an appropriate DNA probe immobilized on the sensor surface, it is possible to detect a specific target sequence without additional labeling procedures. The DNA-based sensors can be coupled with the PCR to increase the sensitivity of the systems and offer a viable alternative to gel electrophoresis and other traditional DNA sequences detection methods that require labeled probes. Currently, some QCM devices are capable of detecting label-free oligonucleotides and/or PCR-amplified DNA fragments (Deisingh and Thompson, 2001; Mo et al., 2002; Mannelli et al., 2003; Mao et al., 2006). However, a DNA biosensing system for real-time detection of pathogens, such as *E. coli* O157:H7 in food has yet to be fully developed.

The objective of the study was to develop a DNA piezoelectric biosensor, QCM, in a circulating-flow system for real-time detection and identification of *E. coli* O157:H7 sequences. We evaluated the enhancement of hybridization efficacy by adding an oligonucleotide spacer to the 5′-end of a 30 mer probe. The specificity of the QCM system for synthesized targets of varying lengths (30 and 104 mer) and PCR-amplified DNA fragments (104 bp) from real samples of *E. coli* O157:H7 was explored.

2. Materials and methods

2.1. Reagents and oligonucleotide primers, probes and targets

All oligonucleotides were designed using Primer Express software (Applied Biosystems, Foster, CA) and synthesized by Applied Biosystems (Applied Biosystems). They are specified in Table 1, which includes single-strand DNA (ssDNA) of probes, targets and PCR primers. In the sequence detection for the *E. coli* O157:H7 gene *eaeA* (Genbank U32312) (Call et al., 2001), the specific probe (P) sequences {probe *eaeA*; thiol-linkered tag modification [HS-$(CH_2)_6$, (–SH)] at 5 terminus with or without additional 12 deoxythymidine 5′-monophosphate [12-dT (12T)]: P-30-SH (30 mer) and P-30/12T-SH (42 mer); non-thiolated and with or without additional 12-dT: P-30 (30 mer) and P-30/12T (42 mer)} used in the experiments were designated. The target (T) sequences included complementary target oligonucleotides, T-30AS (30 mer; AS, the anti-sense strand to the probe sequences) and T-104AS (104 mer), and

Table 1
Sequences of the oligonucleotide probes, targets, and primers used in this study

Probe sequences for *E. coli* O157:H7 *eaeA*	
P-30-SH	HS-$(CH_2)_6$-5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG-3′ (30 mer)
P-30/12T-SH	HS-$(CH_2)_6$-5′-TTT TTT TTT TTT AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG -3′ (42 mer)
P-30	5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG-3′ (30 mer)
P-30/12T	5′-TTT TTT TTT TTT AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG -3′ (42 mer)
Short target sequences (30 mer) for *E. coli* O157:H7 *eaeA*	
T-30S	5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG -3′
T-30AS	5′-CAT TGC TGC AGG ATG GGC AAC TCT TGA GCT-3′
Long target sequences (104 mer) for *E. coli* O157:H7 *eaeA*	
T-104S	5′-AAA GTT CAG ATC TTG ATG ACA TTG TAT TTT CTC TTA ATT AAA TTT ATA TTT ACA GA***A GCT CAA GAG TTG CCC ATC CTG CAG CAA TG***T TAT TCC CTG AAA AAT TG -3′
T-104AS	5′-CAA TTT TTC AGG GAA TAA ***CAT TGC TGC AGG ATG GGC AAC TCT TGA GCT*** TCT GTA AAT ATA AAT TTA ATT AAG AGA AAA TAC AAT GTC ATC AAG ATC TGA ACT TT-3′
PCR primers	
E_{157}eae/F	5′-CAA TTT TTC AGG GAA TAA CAT TGC-3′
E_{157}eae/R	5′-AAA GTT CAG ATC TTG ATG ACA TTG-3′

Probe (P) sequences were designed according to the *E. coli* O157:H7 *eaeA* gene and used to detect the sequence of *E. coli* O157:H7. –SH, thiol-linkered tag [HS-$(CH_2)_6$] modification at 5 terminus of probe;/12T, additional 12 mer of dT oligonucleotides to the probes; P-30 (30 mer) and P-30/12T (42 mer), non-thiolated probes with or without additional 12-dT; P-30-SH (30 mer) and P-30/12T-SH (42 mer), thiolated probes with or without additional 12-dT. Target (T) sequences include complementary target oligonucleotides, T-30AS (30 mer; AS, anti-sense strand to the probe sequence) and T-104AS (104 mer), and non-complementary target oligonucleotides, T-30S (30 mer; S, sense strand to the probe sequence) and T-104S (104 mer). The primer pair was used to amplify the 104 bp DNA fragment of *eaeA* gene from *E. coli* O157:H7 genomic DNA.

non-complementary target oligonucleotides, T-30S (30 mer; S, the sense strand to the probe sequences) and T-104S (104 mer). For PCR-amplified DNA from real samples, the primer pair (E_{157}eae/F and E_{157}eae/R) specific for the *E. coli* O157:H7 *eaeA* gene was used for the amplification of target DNA fragments (104 bp).

The buffer and reagents used in the experiments were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO). These include 30% hydrogen peroxide (H_2O_2, Merck), 98% sulfuric acid (H_2SO_4, Sigma–Aldrich), sodium chloride (NaCl, Sigma–Aldrich), sodium phosphate (Na_2HPO_4, Merck), hydrogen chloride (HCl, Merck) and sodium hydroxide (NaOH, Sigma).

2.2. Culture preparation

The target bacteria, *E. coli* O157:H7 (ATCC 43894), were obtained from American Type Culture Collection (Rockville, MD). The culture was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37 °C for 24 h before use and the bacterial counts were determined by conventional spread-plating method using tryptic soy agar (TSA, Difco).

2.3. DNA extraction

Total genomic DNA was extracted from *E. coli* O157:H7 using the Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI). For each DNA preparation, a pellet containing approximately 1×10^6 CFU/ml cells was resuspended in 600 μl of Nuclei Lysis Solution and incubated at 80 °C for 5 min to lyse the cells. Three microliters of RNase Solution was added to the cell lysate and treated for 30 min. Two hundred microliters of Protein Precipitation Solution was added to the RNase-treated cell lysate and the DNA extraction was carried out as according to the manufacturer's protocol for bacteria (Promega). The purified DNA was examined by gel electrophoresis (0.8% agarose) and quantified by determining A260 (OD_{260}) with a SpectraMax 190 spectrophotometer (Molecular Devices Corp., CA).

2.4. PCR conditions

For detection of real samples of *E. coli* O157:H7, a 104-bp DNA fragment within the *E. coli* O157:H7 *eaeA* gene was amplified by the synthetic primers (E_{157}eae/F and E_{157}eae/R) indicated in Table 1. A PTC-100™ thermal cycler (MJ Research Inc., NV) was used with 10× reaction buffer, dNTP (deoxynucleoside triphosphate) concentrated set solution and Super Taq DNA polymerase (all obtained from HT Biotechnology Ltd., Cambridge, England).

PCR reactions contained 3 μl of genomic DNA (10 ng), 1 μl of each primer (10 μM), 5 μl of 10× PCR buffer [100 mM Tris–HCl (pH 9.0), 15 mM $MgCl_2$, 500 mM KCl, Triton X-100 (1%, v/v), gelatin (0.1%, w/v)], 2 μl of 10 mM dNTP, 1 μl of 0.5 U Super Taq DNA polymerase and 38 μl of distilled water resulting in a total volume of 50 μl. Thermal cycler (MiniCycler; MJ Research, Waltham, MA) conditions were as follows: initial denaturation at 94 °C for 5 min; then 32 cycles of 30 s denaturation at 94 °C, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final extension at 72 °C for 3 min. The PCR products were purified using WizardTM PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol and then visualized on 2.5% agarose gels stained with ethidium bromide under UV light. The concentration of DNA in the samples was measured by a UV-absorption spectrophotometer at a wavelength of 260 nm.

The PCR-amplified DNA was sequenced using E_{157}eae/F and E_{157}eae/R as sequencing primers. Both strands of the DNA fragment were sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA Sequencer (Applied Biosystems).

2.5. The circulating-flow QCM system

The piezoelectric quartz crystals, which consist of a 9 MH_Z AT cut quartz crystal slab with a layer of a gold electrode on each side were (0.091 cm^2 in area on each side; the detection limit of the QCM instrument in liquid = 1 Hz) obtained from ANT Technology Co., Ltd. (Taipei, Taiwan). The flow injection and continuous frequency variation recording were operated using Affinity Detection System (ADS; ANT Technology Co., Ltd.). The system has five main components including electronic oscillation circuit, frequency counter, piezoelectric quartz of fixed biosensor molecule (p-chip), circulating-flow system and a computer to demonstrate the curve of frequency change (ΔF) in real time (Fig. 1A). The experimental data were analyzed by P-Sensor software in real time. The sensor unit was composed of resolution: 0.1 Hz, sampling period: 1 s, frequency range: 2–16 MHz, temperature range: 4–60 °C and voltage: 110 V, 50–60 Hz. The reaction cell was one sensor signal channel with 30 μl of reaction cell volume. The circulating-flow system consisted of a temperature controller, sample tubes, pipelines and one tubing pump with a flow rate: 10–200 μl/min and a sample loop volume of 150 μl.

2.6. Gold-QCM device preparations

The gold electrode surface of the QCM device was cleaned with distilled water for 2–3 min. The water droplets on the surface of the electrode were then blown dry using an air gun. The gold electrode surface was subsequently cleaned with a piranha solution consisting of H_2O_2 (30%) and H_2SO_4 in a 1:3 ratio. It was then thoroughly washed with distilled water, dried with the air gun and used immediately afterwards. The cleaning procedures with piranha solution and distilled water removed organic compounds adhering to the gold surface and enhanced the efficiency of immobilization when the thiolated DNA probes covalently attached to the gold surface (Cho et al., 2004; Su and Li, 2004).

2.7. Immobilization of the oligonucleotide probes and hybridization with oligonucleotide targets

The gold-QCM device was inserted into the flow injection ADS. The importing and exporting pipeline ends were placed

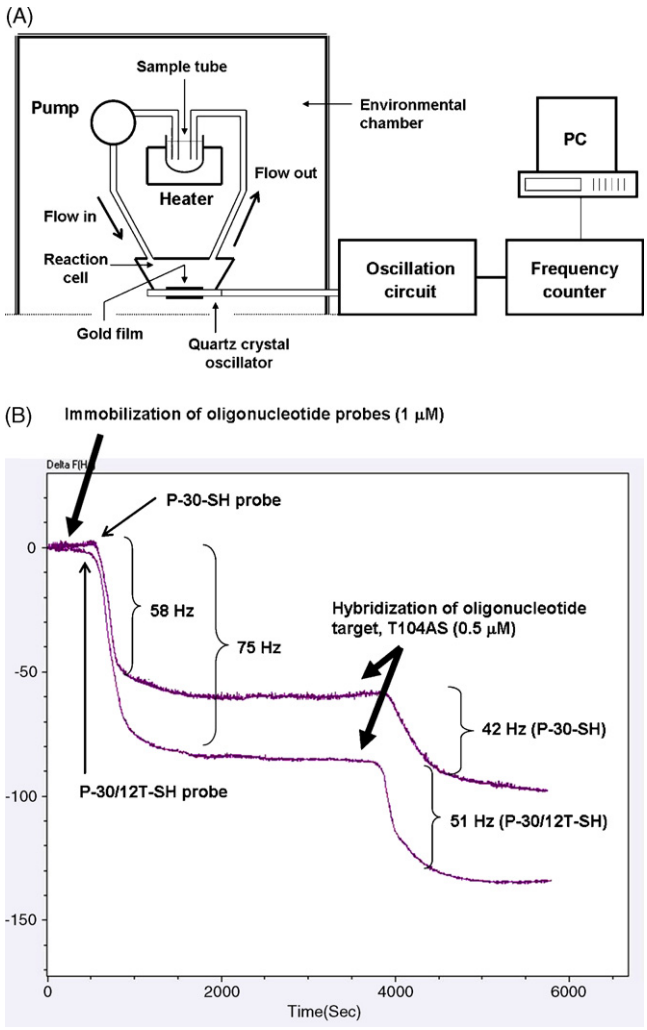


Fig. 1. The real-time and circulating-flow QCM system. (A) A schematic illustration of the real-time and circulating-flow QCM system. The gold films on the quartz crystal oscillator are 3.4 mm in diameter and 0.2 cm^2 in the area of both sides. The volume of the sample tube is 1.5 ml. The volume of the reaction cell is 30 μl and the total volume of the pipeline loop, including flow in and flow out, is 100 μl. (B) An example of real-time detection of *Escherichia coli* O157:H7 sequences performed in the study using the circulating-flow QCM system.

in the sample tube to create a circulating-flow system (Fig. 1A). The phosphate buffer (1 M NaCl and 1 mM Na_2HPO_4, pH 7.4) was flushed through the system at the speed of 50 μl/min. With the frequency of the chip steady at 300 s, ΔF within ±1 Hz/100 s, the solution containing specific probe sequences (5′-thiolated: P-30-SH, P-30/12T-SH or non-thiolated: P-30, P-30/12T; Table 1) for detecting the *E. coli* O157:H7 gene *eaeA* was added to the sample tube (total volume: 500 μl). The probes were self-assembly immobilized on the gold electrode through the flow-circulation for 30 min. After immobilization of the probes, the sample tube containing the probes was removed. The circulating pipeline was cleaned by flowing through the phosphate buffer for 15 min to remove unbound probes on the gold surface and probe residues in the pipeline. The gold-QCM device was then exposed with the sample flow (total volume: 500 μl) containing target sequence (T-30S, T-30AS, T-104S or T-104AS; Table 1). Hybridization of probes and targets in the QCM device was at a flow speed of 50 μl/min for 30 min. The temperature of the QCM system was maintained at 30 °C except in the study of temperature effects on the hybridization. The ΔF during the hybridization were recorded in a real-time observation.

In these experiments, different concentrations of the probe oligonucleotides (0, 0.25, 0.5, 1.0 and 2.0 μM) were evaluated for the efficiency of immobilization on the gold surface of the QCM device. The influence of varying concentrations of the target oligonucleotids on ΔF and hybridization efficiency (H%) were also compared. The ΔF was observed in a real-time continuous reading and reported as the difference between the final value and the value before the hybridization or immobilization. The H% was calculated by dividing the hybridized target coverage by the immobilized probe coverage (Su et al., 2005). The mass of immobilized probes as well as hybrid DNA of probes and targets on the QCM device was calculated using Sauerbrey's equation (Kanazawa and Gordon II, 1985)

$$\Delta F = -F_0^{3/2}\left(\frac{\rho_l \eta_l}{\pi \rho_q \mu_q}\right)^{1/2} \quad (1)$$

where ΔF is the measured frequency shift, F_0 a resonant frequency of the unloaded crystal, ρ_l the density of liquid in contact with the crystal, η_l the viscosity of liquid in contact with the crystal, ρ_q the density of quartz (2.648 g cm^{-3}) and μ_q is the shear modulus of quartz, 2.947×10^{11} g cm^{-1} s^{-2}. The frequency change of 1 Hz corresponds to a mass change of 0.883 ng.

For detection of PCR-amplified DNA of *E. coli* O157:H7 gene *eaeA*, the thiolated probe, P-30/12T-SH (1 μM), was used. The double-strand sequences of the PCR-amplified DNA are the same as the synthetic sequences of 104 mer targets, T-104AS and T-104S (Table 1). Before the PCR-amplified DNA was applied into the circulating-flow QCM system, the DNA was treated in a denaturing solution (TE and 0.5 M NaCl) at 90 °C for 5 min. The denatured DNA was then applied to the circulating-flow QCM system following the same procedure used for synthetic target oligonucleotides. Temperature effects (20, 30, 40, 50 and 60 °C) on the DNA hybridization (hybridization buffer: 1.0 mM Na_2HPO_4 and 1.0 M NaCl) during the circulating-flow detection were evaluated. In each hybridization, 1 μM of PCR-amplified DNA (equivalent to 1 μM of the target T-104AS) was used to hybridize with the thiolated probes immobilized on the QCM device.

2.8. Data analysis

The experimental data were analyzed by P-Sensor software of the ADS system in real time. Each experiment was repeated five times using five different QCM devices to test the reproducibility of the QCM sensor. All data were presented as the mean ± standard deviation (S.D.). Differences between groups were evaluated by a two-tailed Student's t-test. P-values less than 0.05 were considered to be statistically significant differences.

3. Results and discussion

3.1. The QCM system and detection

In the circulating-flow QCM system, real-time frequency shift was recorded. The frequency decreased gradually with addition of oligonucleotides, reflecting immobilization of the probes or hybridization of probes and targets on the gold surface of QCM. Fig. 1B shows an example of the real-time detection of the QCM system performed in our study. There was a 58 Hz decrease in series resonant frequency for immobilization of probes P-30-SH (30 mer), while probes P-30/12T-SH (42 mer) yielded a decrease of 75 Hz. There were decreases in series resonant frequency of approximately 42 and 51 Hz when complementary strands (T-30AS) were introduced and hybridized with P-30-SH and P-30/12T-SH, respectively.

In our circulating-flow QCM system, the thiolated probes were circulated constantly to ensure continuous interaction with the gold surface on QCM device thereby enhancing the efficiency of probe immobilization and the hybridization of probes and targets. The flow circulation also limits non-specific binding, recirculating unattached probes for immobilization.

3.2. Immobilization of synthesized oligonucleotide probes

In fabricating a DNA sensor, maximizing the immobilization of DNA on the sensor's surface is crucial. Therefore, various concentrations (0, 0.25, 0.5, 1.0 and 2.0 μM) of thiolated probes, with or without the addition of 12-dT [P-30-SH (30 mer) and P-30/12T-SH (42 mer)], were used to evaluate immobilization efficiency on the gold surface of QCM devices. The results showed that ΔF increased almost linearly with the increase of probe concentrations up to concentrations of 1.0 and 2.0 μM. Probe concentrations of 1.0 and 2.0 μM yielded the greatest covalent attachment to the gold surface compared with other concentrations ($P < 0.01$) (Fig. 2A). There was no significant difference between ΔF at concentrations of 1.0 and 2.0 μM ($P > 0.05$), indicating saturation of the immobilization sites on the gold surface of the QCM device. Therefore, the probe concentration of 1.0 μM was selected for the following experiments.

In general, the thiolated probes with additional 12-dT (P-30/12T-SH) showed greater ΔF ($P < 0.05$) than thiolated probes without additional 12T (P-30-SH) among the various concentrations tested (Fig. 2A). This was because the weight of P-30/12T-SH per single molecule (1.31×10^4 g mol^{-1}) is larger than that of P-30-SH (9.42×10^3 g mol^{-1}). According to the calculation by Sauerbrey's equation, the molecule densities of P-30-SH and P-30/12T-SH immobilized onto the gold surface of the QCM device were 1.7 ± 0.1 and 2.1 ± 0.2 (ssDNA/10 nm^2), respectively, when 1.0 μM of probes was used in the immobilization. These molecule density values show no significant differences ($P > 0.05$). Therefore, the efficiency of immobilization of P-30-SH and P-30/12T-SH onto the gold surface of the QCM was similar.

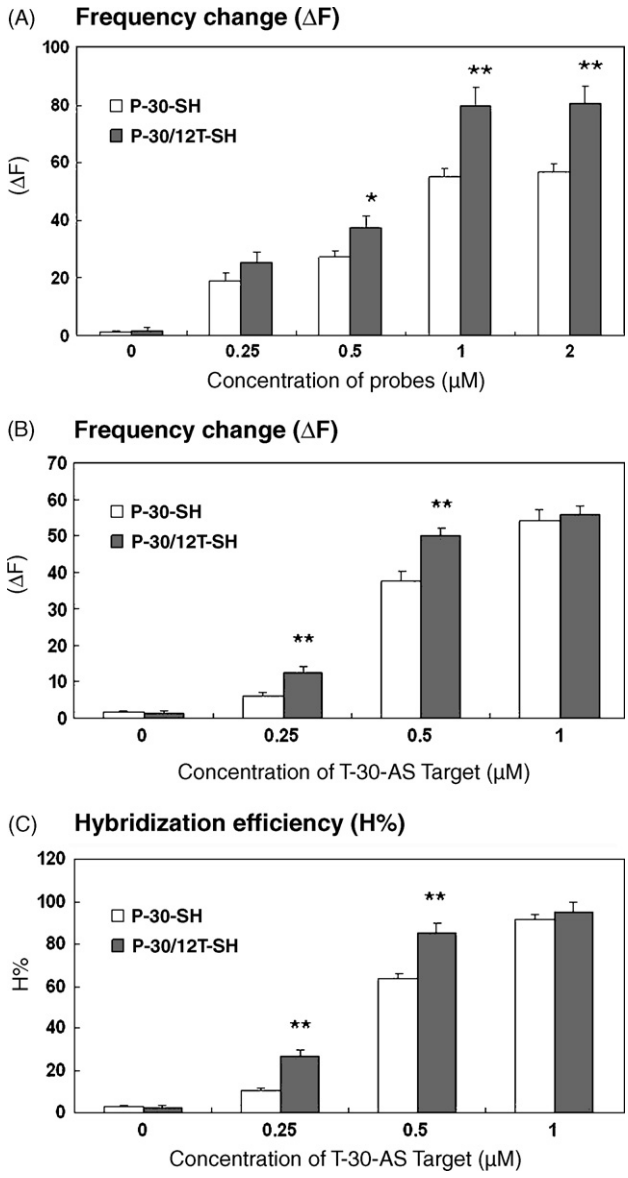


Fig. 2. Immobilization efficiency of oligonucleotide probes on the gold surface of the QCM device and detection of the short target oligonucleotides, T-30-AS, hybridized with the thiolated probes (1.0 μM) immobilized onto the gold surface of the QCM device. Frequency change (ΔF) after immobilization (A), frequency change (B) and hybridization efficiency (C) after hybridization were measured and calculated. Values derived from five independent detections, error bars mean standard deviation (S.D.). Asterisk (*) and (**) indicate $P < 0.05$ and $P < 0.01$, respectively, vs. P-30-SH.

3.3. Detection of the short (30 mer) synthesized target oligonucleotides

Different concentrations (0, 0.25, 0.5 and 1.0 μM) of the short synthesized target oligonucleotides (T-30AS, 30 mer) complementary to the probes immobilized on the gold surface of QCM device were compared for the ΔF and $H\%$ in the circulating-flow QCM system. The ΔF due to hybridization increased with increasing concentrations of the targets up to 0.5 μM ($P < 0.01$) (Fig. 2B). At concentrations ≥1 μM, the frequency shift was less sensitive, indicating the saturation of the probe hybridization sites (Fig. 2B and data not shown). The $H\%$ also increased

with increasing concentrations of the targets (Fig. 2C). The *H%* of target T-30AS at 0.25 and 0.5 μM hybridized with the probe P-30/12T-SH were significantly higher ($P < 0.01$) than those of the target hybridized with probe P-30-SH. Results indicate the addition of 12-dT to the probes increased the *H%* in the QCM system.

According to the ΔF results, the probes were able to hybridize with their complementary sequences. The probes with the spacer (12-dT) influenced the hybridization with the target oligonucleotides (Shchepinov et al., 1997), and increased the hybridization efficiency in our circulating-flow QCM system. During DNA hybridization, spacers have been shown to reduce steric interference, making the probe end closet to the surface of the device more accessible (Shchepinov et al., 1997; Southern et al., 1999). Spacers, such as 12-dT also reduce steric hindrance in three-dimensional space and increase of molecule collision to increase *H%*. The use of spacers, such as poly dT or dA were reported for other biosensor development (Bassil et al., 2003; Wolf et al., 2004; Mukumoto et al., 2006).

3.4. Specificity of the QCM detection

We evaluated the DNA hybridizations among four probes (P-30-SH, P-30/12T-SH, P-30 and P-30/12T; 1 μM) and two targets (T-30AS and T-30S; 0.5 μM) (Table 1). We compared the probes with or without thiol-linkered tag modification on the 5′-end for influencing the efficiency of probe immobilization on the gold surface of the QCM device and subsequent hybridization. The results indicate that non-thiolated probes (P-30 and P-30/12T) fail to attach covalently to the gold surface ($\Delta F < 2$ Hz for 30 min), preventing the subsequent hybridization of probes and targets (Fig. 3A). Non-specific binding was investigated using T-30S (a sense strand to the probe sequences, non-complimentary strand). The thiolated probes (P-30-SH, P-30/12T-SH) were covalently immobilized on the gold surface and specifically hybridized to the complementary targets (T-30AS), but not to the non-complementary targets (T-30S) (Fig. 3). The target T-30S, as the negative control of hybridization in our QCM system did not yield a measurable frequency shift when applied to the probe-immobilized QCM device. Hybridization of surface-bound ssDNA is dependent on surface coverage and materials. The thiolated ssDNA has a more profound effect on surface coverage, such as with the gold in our system, than non-thiolated ssDNA (Herne and Tarlov, 1997; Levicky et al., 1998). The hybridization of P-30/12T-SH and T-30AS showed greater ΔF ($P < 0.01$) (Fig. 3A) and higher *H%* (Fig. 3B) than the hybridization of P-30/SH and T-30AS, reflecting the reduction of steric hindrance in the three-dimensional space and the increase in molecule collisions caused by the additional 12-dT.

3.5. Detection of the long (104 mer) synthesized target oligonucleotides

The 104 mer synthesized targets (T-104AS and T-104S) were applied in the QCM system and the ΔF and *H%* were evaluated. Within the target of T-104AS, only the 30 mer sequences are complementary to the probes, P-30-SH and P-30/12T-SH

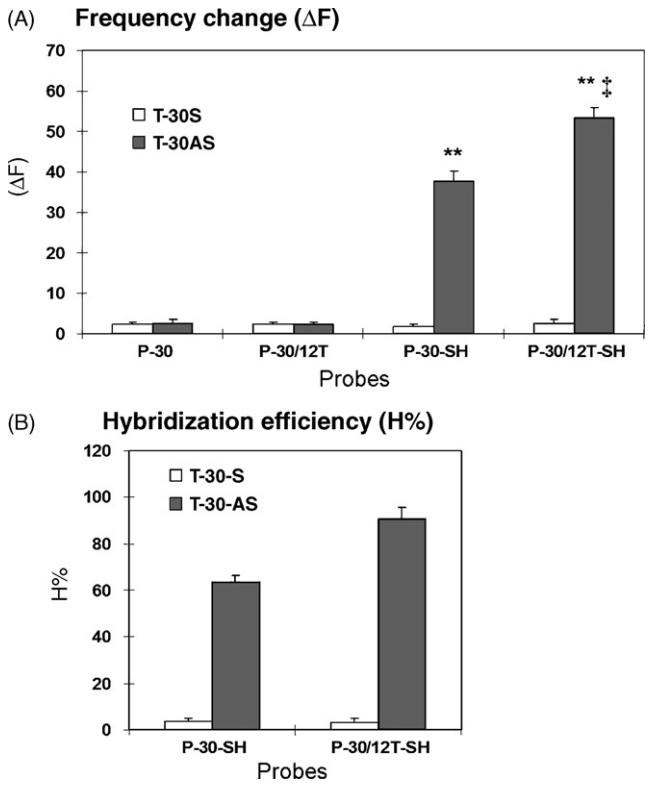


Fig. 3. Detection specificity of the circulating-flow QCM system. Four probes (P-30-SH, P-30/12T-SH, P-30 and P-30/12T; 1 μM) and two targets (T-30AS and T-30S; 0.5 μM) were applied to test the efficiencies of probe immobilization and hybridization of the target to the probe immobilized QCM device. In each treatment, frequency change (A) and hybridization efficiency (B) were measured and calculated. Values derived from five independent detections, error bars mean S.D. Asterisk (**) indicates $P < 0.01$ vs. T-30S and ‡ indicates $P < 0.01$ vs. P-30-SH.

(Table 1). As shown previously, no measurable frequency shift was detected when the targets were applied to the immobilized probes without thiol-linkered tag modification. Using T-104S, a non-complementary strand to the probes, non-specific binding was investigated. No measurable ΔF was detected when T-104S was applied to the probe-immobilized QCM. The ΔF (due to the hybridization), and the *H%* significantly increased ($P < 0.05$ or $P < 0.01$) with increasing concentrations (0.5 and 1 μM) of the target T-104AS (Fig. 4A and B). However, the frequency shift decreased when a relatively high concentration of T-104AS (2 μM) was applied for the hybridization with P-30/12T-SH, indicating the upper limit of hybridization and saturation of the probe hybridization sites.

In Fig. 2, the results show that the 12-dT spacer enhanced the ΔF and *H%* by approximately 1.4-fold when the probes hybridized with the 30 mer target T-30AS (0.5 μM). In Fig. 4, the ΔF had a two-fold increase when the 104 mer target T-104AS hybridized to the probe P-30/12T-SH instead of to the probe P-30-SH ($P < 0.01$). This phenomenon indicates that the 12-dT spacer had greater effects on the 104 mer targets than on the 30 mer targets in reducing steric interference during DNA hybridization. This result is confirmed by previous reports, which showed that the addition of spacers to probes has a greater increase in hybridization efficacy when the probes hybridized to

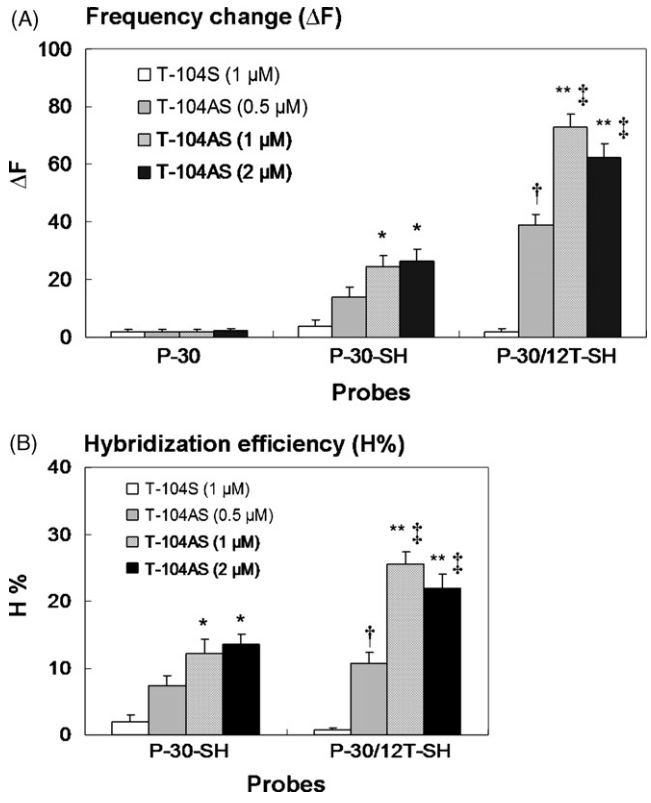


Fig. 4. Detection of the long oligonucleotide targets, T-104AS and T-104S, hybridized with the probes (1.0 μM) immobilized onto the gold surface of the QCM device. Frequency change (A) and hybridization efficiency (B) were measured and calculated. Values derived from five independent detections, error bars mean S.D. Asterisk (*) and (**) indicate $P < 0.05$ and $P < 0.01$, respectively, vs. target T-104AS (0.5 μM). † and ‡ indicate $P < 0.05$ and $P < 0.01$, respectively, vs. probe P-30-SH.

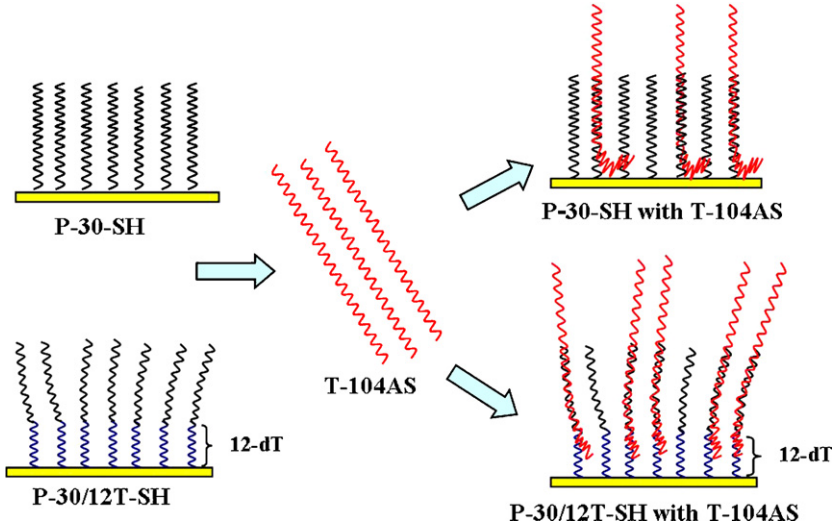
a longer rather than shorter target sequence (Herne and Tarlov, 1997; Levicky et al., 1998). We infer that it is easier to form a bent sequence and generate surface inhibition on the gold surface of the QCM device when the target T-104AS molecules hybridize to the probe P-30-SH than to the probe P-30/12T-SH. This occurrence may obstruct entrance of other target sequences for hybridization (Scheme 1).

Interestingly, the values of *H*% (20–25%) in the hybridization of the probe P-30/12T-SH with the 104 mer targets were significantly lower ($P < 0.05$) than those in the hybridization of the 30 mer targets at optimal conditions (85–95%) (Figs. 2C and 4B). This indicates that free fragments extending from the complementary region of the target obstructed the hybridization of the target sequences to their probes. In addition, long target oligonucleotides may produce the secondary structures due to hairpin formation in the hybridization conditions we used. Even though *H*% was reduced to 20–25% in the hybridization of the probe P-30/12T-SH and the 104 mer targets, the detectable ΔF reached 70–80 Hz, indicating that the sensitivity of our QCM system is sufficient to detect sequences, such as the 104 mer targets that hybridized to the 30 mer probes.

3.6. Detection of PCR-amplified DNA of *E. coli* O157:H7 gene *eaeA*

The PCR-amplified DNA fragment is 104 bp and is located within the respective region of *E. coli* O157:H7 *eaeA* gene (Fig. 5A). The PCR products were detected in real-time by the circulating-flow QCM system. The temperature effects on the hybridization of P-30/12T-SH and PCR-amplified DNA are indicated in Fig. 5B. The results show that the ΔF equalled 70 ± 4 Hz when the system temperature was maintained at 30 °C and was significantly higher ($P < 0.01$) than when the system was maintained at 20, 40, 50 and 60 °C. The frequency shift of the QCM system for detecting PCR-amplified products was equivalent to that for detecting the synthetic target oligonucleotides, T-104AS.

The piezoelectric biosensor detected the presence of *E. coli* O157:H7 when the DNA strand was complementary to the immobilized probes with synthetic oligonucleotides. The system will be further applied in the detection of field samples or other



Scheme 1. Schematic representation of steric hindrance of the probe and target DNA hybridization on the QCM device. The target T-104AS molecules hybridized to the probe P-30-SH easily to form a bent sequences adjacent to the surface of gold film in contrast to the targets hybridized to the probe P-30/12T-SH. This phenomenon may obstruct the entrance of other target sequences for hybridization.

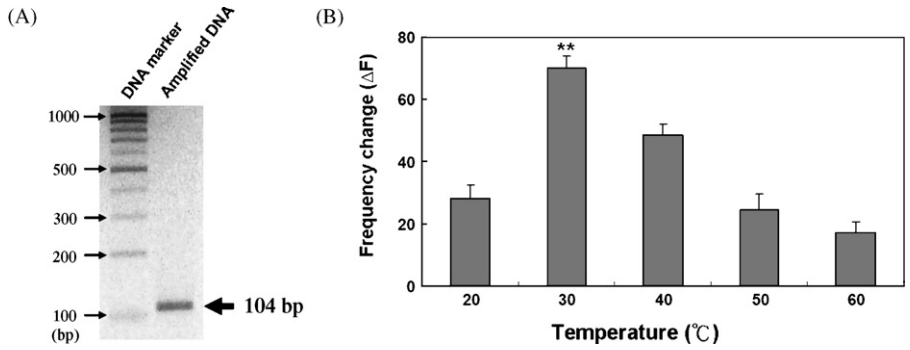


Fig. 5. Detection of PCR-amplified DNA. (A) The 104 bp DNA fragment amplified by PCR from the *eaeA* gene of *E. coli* O157:H7. The amplified sequence was also identified by DNA sequencing and the sequence is "5′-CAA TTT TTC AGG GAA TAA CAT TGC TGC AGG ATG GGC AAC TCT TGA GCT TCT GTA AAT ATA AAT TTA ATT AAG AGA AAA TAC AAT GTC ATC AAG ATC TGA ACT TT-3′" (this is the sequence of one strand). (B) Temperature effect on the hybridization of the PCR-amplified DNA fragment from the *eaeA* gene of *E. coli* O157:H7 to the probe P-30/12T-SH. In the hybridization, 1 μM of PCR-amplified DNA (equivalent to 1 μM of target T-104AS) was used to hybridize the thiolated probes immobilized on QCM device. Asterisk (**) indicates $P < 0.01$ vs. other temperature treatments.

sequences by employing various probes to be immobilized on the gold surface of the crystal. In conjunction with PCR application, the QCM sensor labeled with DNA probes can be used as a quantitative and highly sensitive assay. The optimal conditions of our QCM system used to detect the PCR-amplified DNA from the real sample had relative standard deviation values (%R.S.D.) as low as 5.3%, which are considered acceptable among established analytical techniques (Skoog et al., 1998). Our QCM device may be used to detect a single sequence, a single organism or an entire community of environmental and food microorganisms with selectivity controlled by the choice of primers and primer annealing temperature for the PCR procedures.

4. Conclusions

We developed a DNA piezoelectric sensor for the real-time detection of *E. coli* O157:H7. Synthetic probe oligonucleotides were self-assembly immobilized on the sensor surface of the QCM device and the hybridization between the immobilized probes and the complementary sequences of the targets in solution was monitored in real-time. A spacer (12-dT) linked to the probes enhanced the detection signals because the spacer molecules reduced the steric interference of the support on the hybridization behavior of the immobilized oligonucleotides. The QCM system was also used to detect the PCR-amplified DNA from real samples. Our results suggest that the DNA piezoelectric sensor has potential for further applications in detecting *E. coli* O157:H7 as well as other microorganisms in food, water and clinical samples. This approach lays the groundwork for incorporating the method into an integrated system for rapid PCR-based DNA analysis.

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