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ScienceDirect
Journal of Microbiological Methods 73 (2008) 7–17
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor

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Received 25 September 2007; received in revised form 3 January 2008; accepted 9 January 2008
Available online 18 January 2008

Abstract

A circulating-flow piezoelectric biosensor, based on an Au nanoparticle amplification and verification method, was used for real-time detection of a foodborne pathogen, *Escherichia coli* O157:H7. A synthesized thiolated probe (Probe 1; 30-mer) specific to *E. coli* O157:H7 *eaeA* gene was immobilized onto the piezoelectric biosensor surface. Hybridization was induced by exposing the immobilized probe to the *E. coli* O157:H7 *eaeA* gene fragment (104-bp) amplified by PCR, resulting in a mass change and a consequent frequency shift of the piezoelectric biosensor. A second thiolated probe (Probe 2), complementary to the target sequence, was conjugated to the Au nanoparticles and used as a “mass enhancer” and “sequence verifier” to amplify the frequency change of the piezoelectric biosensor. The PCR products amplified from concentrations of 1.2×10^2 CFU/ml of *E. coli* O157:H7 were detectable by the piezoelectric biosensor. A linear correlation was found when the *E. coli* O157:H7 detected from 10^2 to 10^6 CFU/ml. The piezoelectric biosensor was able to detect targets from real food samples.
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Keywords: Au nanoparticle; Piezoelectric biosensor; *E. coli* O157:H7

1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 was first recognized as a foodborne pathogen in 1982 during an investigation into an outbreak of hemorrhagic colitis associated with consumption of hamburgers from a fast food chain restaurant (Riley et al., 1983). Since then, numerous cases have been reported around the world including a recent *E. coli* O157:H7 outbreak in spinach (CDC, 2006; FDA, 2006). *E. coli* O157:H7 has become a global public health problem, causing symptoms such as bloody diarrhea, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpurea (Doyle et al., 1997). The US Centers for Disease Control and Prevention reported that an estimated 73,000 cases of *E. coli* O157:H7 infection and 61 resultant deaths occur in the United States each year (CDC, 2006).

Many methods are available for the detection of *E. coli* O157:H7. In the conventional culture method, Sorbitol-MacConkey agar has been used in a direct plating method to isolate *E. coli* O157:H7 from samples (FDA, 2001). The conventional methods are reliable but time-consuming and laborious, further studies are needed to develop rapid and objective methods for foodborne pathogen detection. These methods include antibody-based methods, nucleic acid-based methods, and biochemical and enzymatic methods. Interest in using biosensors to detect foodborne pathogens is on the rise (Hall et al., 2002; Patel, 2006, Rasooly and Herold, 2006). Among these, DNA sensors integrated with a PCR-based DNA system have shown great potential for the specific detection of pathogenic microorganisms. Many techniques are currently available, such as optical DNA detection using fluorescence-labeled oligonucleotides (Heng and Tsui, 2008), application of surface plasmon resonance (Mariotti et al., 2002), direct electrochemical assay (Korri-Youssoufi et al., 1997), and piezoelectric quartz crystal microbalance (QCM) targeting DNA

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doi:10.1016/j.mimet.2008.01.004

(Wu et al., 2007). However, the detection limit of these methods is still needed to be improved before the methods are applied for detecting pathogenic microorganisms in real food or filed samples.

QCM has been extensively investigated as a transducer in hybridization based DNA biosensors for the detection of gene mutation (Su et al., 2004; Tombelli et al., 2000), genetically modified organisms (Mannelli et al., 2003), and foodborne pathogens (Mo et al., 2002; Ryu et al., 2001) including the application of QCM sensors to the detection of *E. coli* O157:H7 (Deisingh and Thompson, 2001; Mao et al., 2006; Wu et al., 2007). To improve the sensitivity of QCM sensors, many methods have been developed including optimizations of probe immobilization (Caruso et al., 1997; Tombelli et al., 2002; Zhou et al., 2001) and various signal amplification strategies using non-specific amplifiers of anti-dsDNA antibodies (Bardea et al., 1999), liposomes (Patolsky et al., 2002), enzymes (Patolsky et al., 2001), and nanoparticles (Mao et al., 2006). Nanoparticles are effective amplifiers used in the QCM DNA detection because they have a relatively large mass compared to the DNA targets. In the QCM DNA detection, the nanoparticles offer substantial improvement in the detection limits with sensitivity from 10^{-12} to 10^{-14} M of DNA (Zhou et al., 2000; Zhao et al., 2001).

One of the "nanogold amplifier" methods utilizes DNA-capped gold nanoparticles for signal amplification (Lin et al., 2000; Liu et al., 2003; Mao et al., 2006; Weizmann et al., 2001; Zhao et al., 2001; Zhou et al., 2000). Coupled with the DNA targets, nanoparticles act as "mass enhancers", extending the limits of QCM DNA detection. A nanoparticle-amplified QCM DNA sensor using the sandwich hybridization of two specific probes has been demonstrated by Mo et al. (2005); however, the method used for foodborne pathogen detection has not previously been described. Therefore, a QCM DNA sensor based on nanoparticle amplification for detection and verification of *E. coli* O157:H7 was designed and reported in the present study. The target DNA was captured by the single-stranded DNA (ssDNA) of Probe 1 which self-assembled on the QCM sensor surface. The signal was then amplified using the sequence specific probe (Probe 2) conjugated to Au (gold) nanoparticles, resulting in highly sensitive detection of *E. coli* O157:H7. The sensitivity and specificity of the QCM system were evaluated. The application of the QCM system was tested in real food samples (apple juice, milk, and ground beef).

2. Materials and methods

2.1. Chemicals

The chemicals, 30% hydrogen peroxide (H_2O_2), sodium phosphate (Na_2HPO_4) and hydrogen chloride (HCl) and 98% sulfuric acid (H_2SO_4) were purchased from Merck (Darmstadt, Germany). The chemicals, sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Oligonucleotide primers, probes and targets

All oligonucleotides were designed using Primer Express software (Applied Biosystems, Foster, CA), and synthesized by Applied Biosystems (Foster, CA) including ssDNA of probes, targets as well as PCR primers (Table 1).

In the detection for *E. coli* O157:H7 gene *eaeA* (Genbank U32312) (Call et al., 2001), the specific Probe 1 oligonucleotides modified with thiol-linkered tag [HS–$(CH_2)_6$] at 3′ end with or without additional of 12 or 24 deoxythymidine 5′-monophosphates (12 dT or 24 dT; /12T or /24T), including P1-30 (30-mer), P1-30/12T (42-mer), and P1-30/24T (54-mer), were designated. These probes were used for immobilization on the Au surface of the QCM sensor to detect target DNA. The second probes (Probe 2) were used to cap the Au nanoparticles (Sigma-Aldrich) and also to detect and verify the *eaeA* sequences of *E. coli* O157:H7. Probe 2 includes P2-30(AS) (30-mer; AS, anti-sense strand, complementary to the target sequences), P2-30/12T (AS) (42-mer), and P2-30(S) (30-mer; S, sense strand, non-complementary to the target sequences).

The synthesized target (T) sequences include complementary target oligonucleotides, T-104(AS) (104-mer) and non-complementary target oligonucleotides, T-104(S) (104-mer).

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

Probe sequences for E. coli O157:H7 eaeA		
P1-30	5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG-3′–$(CH_2)_6$–HS	(30-mer)
P1-30/12T	5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG TTT TTT TTT TTT-3′–$(CH_2)_6$–HS	(42-mer)
P1-30/24T	5′-AGC TCA AGA GTT GCC CAT CCT GCA GCA ATG TTT TTT TTT TTT TTT TTT TTT TTT-3′–$(CH_2)_6$–HS	(54-mer)
P2-30(AS)	HS–$(CH_2)_6$–5′-AAA GTT CAG ATC TTG ATG ACA TTG TAT TTT-3′	(30-mer)
P2-30(S)	HS–$(CH_2)_6$–5′-TTT CAA GTC TAG AAC TAC TGT AAC ATA AAA-3′	(30-mer)
P2-30/12T(AS)	HS–$(CH_2)_6$–5′-TTT TTT TTT TTT AAA GTT CAG ATC TTG ATG ACA TTG TAT TTT-3′	(42-mer)
Target sequences (104 mer) for E. coli O157:H7 eaeA		
T-104(S)	5′-AAA GTT CAG ATC TTG ATG ACA TTG TAT TTT CTC TTA ATT AAA TTT ATA TTT ACA GAA GCT CAA GAG TTG CCC ATC CTG CAG CAA TGT TAT TCC CTG AAA AAT TG-3′	
T-104(AS)	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 region of *E. coli* O157:H7 *eaeA* gene and used to detect the sequence of *E. coli* O157:H7. P1, Probe 1 was used to immobilize on the Au surface of QCM device; P2, Probe 2 was used to cap onto Au nanoparticles. /12T and /24, additional 12 mer and 24 mer of dT oligonucleotides to the probes.
Target (T) sequences include complementary target oligonucleotides, T-104(AS) (104-mer; AS, anti-sense strand to the probe sequence, i.e., complementary target oligonucleotides) and T-104(S) (104-mer; S, sense strand to the probe sequence, i.e., non-complementary target oligonucleotides).
The primer pair was used to amplify the 104-bp DNA fragment of the *eaeA* gene from *E. coli* O157:H7 genomic DNA.

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 designed and used for amplification of the target DNA fragments (104-bp). Sequences of double strands (ds) of the PCR-amplified DNA are the same as the synthesized target sequences, T-104(AS) and T-104(S).

2.3. Culture preparation

E. coli O76:H8 (ATCC 23536), *E. coli* O85:H1 (ATCC 23539), *E. coli* O138:H14 (ATCC 23545), *E. coli* O142:H6 (ATCC 23985), *E. coli* O157:H7 (ATCC 43894), *Listeria monocytogenes* (ATCC 19114), *Salmonella choleraesuis* (ATCC 13311), *Staphylococcus aureus* (ATCC 10832), and *E. coli* K12 (ATCC 15153) were obtained from the American Type Culture Collection (Manassa, VA). *E. coli* O157:H7 and *L. monocytogenes* cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) and the other bacteria were cultured in nutrient agar broth (Difco). Bacterial counts were determined by conventional spread-plating method using tryptic soy agar (Difco).

2.4. Food sample studies

2.4.1. Apple juice

E. coli O157:H7 cells were artificially inoculated into apple juice, and then 1 ml of the mixture was pipetted into a microtube and centrifuged at 12,000 ×*g* for 10 min. The bacteria-containing pellet was suspended in 1 ml of phosphate buffer saline (PBS) and centrifuged again at 12,000 ×*g* for 10 min. The pellet was finally resuspended in 1 ml of PBS and used for genomic DNA extraction (Yamaguchi et al., 2003).

2.4.2. Milk

E. coli O157:H7 cells were artificially inoculated in 1 ml of pasteurized milk. The bacterial cells were then isolated from the milk and used for genomic DNA extraction. The method reported by Yamaguchi et al. (2003) was used to dispose of lipids and proteins from the milk ingredients to isolate *E. coli* O157:H. Firstly, proteinase K (Promega, Madison, WI) and 50 µl of 0.1% Triton X-100 were added to 100 µl of milk, and the milk samples were incubated at 37 °C for 1 h to eliminate protein and lipid components. After incubation, 900 µl of 150 mM NaCl was added and then the mixture was centrifuged at 12,000 ×*g* for 10 min. The pellet containing bacteria cells was collected after the centrifugation. The pellet was suspended in 1 ml of 150 mM NaCl and centrifuged again at 12,000 ×*g*. The pellet was finally resuspended in 150 mM NaCl and used for genomic DNA extraction.

2.4.3. Ground beef

One g of ground beef was artificially inoculated with the cells of *E. coli* O157:H7. After 30 min of inoculation, the sample was added into a tube containing 10 ml of PBS. Samples in the tube was mixed by vortex and centrifuged at 125 ×*g* for 10 min. The middle layer of solution in the tube was removed and pipetted, and then transferred to a microtube. The sample was centrifuged at 12,000 ×*g* for 10 min and the bacteria-containing pellet was resuspended in PBS for genomic DNA extraction (Yamaguchi et al., 2003).

In addition to the food samples, *E. coli* O157:H7 cells were also inoculated in PBS for bacterial detection as positive controls.

2.5. Genomic DNA extraction

Total genomic DNA was extracted by a Wizard™ Genomic DAN Purification Kits following the instructions of the manufacturer (Promega). For each DNA preparation, a pellet contained approximately 1×10^6 CFU/ml of bacteria. For each preparation, a total of 50 µl of DNA was eluted from the DNA purified column. 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., Sunnyvale, CA).

2.6. 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., Waltham, MA) was used with PCR reaction buffer, dNTP (deoxynucleoside triphosphate) concentrated set solution, and Super Taq DNA polymerase (all obtained from HT Biotechnology Ltd, Cambridge, England).

PCR reactions contained 2 µl of genomic DNA, 2 µl forward and reverse primers (final concentration is 1 µM for each primer), 5 µl of 10× PCR buffer (100 mM Tris HCl [pH 9.0], 15 mM $MgCl_2$, 500 mM KCl, 1% [v/v] Triton X-100, and 0.1% [w/v] gelatin), 2 µl of 10 mM dNTP, 1 µl of 0.5 U Super Taq DNA polymerase, and 38 µl distilled water in a total volume of 50 µl. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, and final extension at 72 °C for 3 min. The PCR products were purified in a total volume of 50 µl TE buffer using Wizard™ 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 UV-absorption spectrophotometer at a wavelength of 260 nm.

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

2.7. Probe oligonucleotide–nanoparticle conjugates

Suspensions of Au nanoparticles with an average diameter of 20 nm, which were synthesized by reduction of $HAuCl_4$ solution with sodium citrate, were obtained from Sigma-Aldrich. Preparation of probe oligonucleotide–Au nanoparticle conjugates was carried out as previously described (Li et al., 2006; Goodrich et al.,

2004). Briefly, 50 μl of freshly purified 100 μM thiolated oligonucleotides was added to 800 μl of the Au nanoparticle solution in an Eppendorf tube. The tube was then placed in a heat block at 37 °C for a minimum of 4 h before the addition of 230 μl of H_2O and 120 μl of 1 M NaCl/100 mM phosphate buffer (Na_2HPO_4, pH 7.4). After aging overnight at 37 °C, excess oligonucleotides were extracted by centrifugation at 9500 rpm (7500 ×*g*) for 50 min with the removal of supernatant and resuspension of the Au pellet in 0.1 M NaCl/10 mM phosphate buffer (pH 7.4). These procedures were repeated three times. After the final centrifugation, the Au pellet was resuspended in 0.3 M NaCl/10 mM phosphate buffer (pH 7.4) with a final concentration of 10 nM. This concentration was obtained from UV–vis spectroscopy using an extinction coefficient of 1.94×10^8/M cm, as described previously by Goodrich et al. (2004).

2.8. The circulating-flow QCM system

The piezoelectric quartz crystals which consist of a 9 MHz AT cut quartz crystal slab with a layer of a gold electrode on each side (0.091 cm^2 in area on each side; the detection limit of the QCM instrument in liquid = 1 Hz) were 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 plot the curve of frequency shift in real-time monitoring. The experimental data were analyzed by P-Sensor software. Parameters for the sensor unit were set as follows: resolution: 0.1 Hz; sampling period: 1 s; frequency range: 2–16 MHz; temperature range: 4–60 °C; voltage: 110 V, 50–60 Hz. The reaction cell is one sensor signal channel with 30 μl of reaction cell volume. The circulating-flow system consisted of a temperature controller, sample tube, pipelines, one tubing pump with a flow rate of 10–200 μl min^{-1} and a sample loop volume of 100 μl (Wu et al., 2007).

2.9. Gold-QCM sensor preparations

The Au electrode surface of the QCM sensor 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 (Caruso et al., 1995). 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 remove organic compounds adhering to the gold surface and enhance the efficiency of immobilization when the thiolated DNA probe covalently attaches to the gold surface.

2.10. Immobilization of probe oligonucleotides and hybridization with target sequences

The QCM sensor was inserted into the QCM system. The importing and exporting pipeline ends were placed in the sample tube to create a circulating-flow system. The buffer of 1 M NaCl and 1 mM phosphate (pH 7.4) was flushed through the system at the speed of 50 μl min^{-1}.With the frequency of the sensor steady at frequency change within ±1 Hz/5 min, the solution containing specific probe sequences (3′-thiolated Probe 1: P1-30, P1-30/12T, and P1-30/24T; Table 1) for detecting *E. coli* O157:H7 gene *eaeA* was added to the sample tube (total volume of reactive buffer: 500 μl). The probe oligonucleotides were self-assembly immobilized on the Au electrode surface through the flow-circulation for 30 min. After immobilization of the probes, the probe solution in the sample tube was removed. The circulating pipeline was cleaned by flowing through the buffer for 15 min to remove unbound probes on the Au surface and probe residues in the pipeline. The QCM sensor was then exposed with the sample flow (500 μl) containing target oligonucleotides T-104 (S), T-104(AS), or PCR-amplified DNA (Table 1). The sample flow was circulated for about 30 min with a flow speed of 50 μl min^{-1} allowing hybridization of probes and targets on the QCM sensor. Temperature of the QCM system during DNA hybridization was maintained at 30 °C. Before applying the PCR-amplified DNA, it was heat-denatured at 95 °C for 5 min and then immediately introduced into the sample tube of QCM system for detection. The frequency shift during the hybridization was recorded in a real-time observation.

2.11. Sandwich hybridization by oligonucleotides capped with Au nanoparticles

The PCR-amplified DNA was prepared in the buffer of 1 M NaCl and 1 mM phosphate (pH 7.4), heat-denatured, and then applied onto the Probe 1 immobilized QCM sensor for DNA hybridization. After 30 min of DNA hybridization, frequency of the QCM sensor was steady (frequency change within ±1 Hz/5 min), excess PCR-amplified DNA in the sample tube was removed and the circulating pipeline was cleaned by circulating buffer for 15 min to remove unhybridized DNA in the pipeline. The QCM sensor was then exposed with the sample flow (total volume: 500 μl) containing the 1.0 μM of Probe 2-capped with Au nanoparticles. Sandwich hybridization of the DNA immobilized on the QCM sensor was performed for 20–30 min with a flow speed of 50 μl min^{-1}. The frequency change during the hybridization was recorded in a real-time observation. The temperature of the QCM system was maintained at 30 °C.

In the detection process, the frequency change was observed in a real-time continuous reading and was reported as the difference between the final value and the value before the immobilization or hybridization. The mass increases due to the DNA probe immobilization, the target DNA and probe hybridization, and the gold nanoparticles amplification on the QCM sensor were calculated using the Sauerbrey's equation (Kanazawa et al., 1985),

$$\Delta F = -F_0^{3/2}\left(\frac{\rho_1\eta_1}{\rho_q\mu_q}\right)^{1/2},$$

where ΔF is the measured frequency shift, F_0 is resonant frequency of the unloaded crystal, ρ_l is the density of liquid in

contact with the crystal, η_l is the viscosity of liquid in contact with the crystal, ρ_q is 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 0.391 ng.

2.12. Data analysis

The experimental data (frequency change) were analyzed by P-Sensor software of the ADS system in real time. Each experiment was repeated 3 times using 3 different QCM devices to test the reproducibility of the QCM sensor. All data were presented as the mean ± standard deviation (SD). 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.

3. Results

3.1. The QCM system and detection

Piezoelectric QCM, quartz crystal microbalance, has been extensively applied as mass biosensor for the detection of specific proteins and nuclear acids. In our circulating-flow QCM system, the oscillation frequency of QCM chip decreased when the weight of QCM surface increased, which would be real-time recorded (Fig. 1). The frequency decreased gradually with addition of oligonucleotides, reflecting immobilization of the first *E. coli* O157:H7 specific probes, hybridization of the probes with the *E. coli* O157:H7 targets on the Au surface of QCM, and sandwich hybridization by the second *E. coli* O157:H7 specific probes which were capped onto Au nanoparticles. Fig. 1 shows the typical frequency shift of real-time detection of the QCM system performed in this study. A frequency decrease of approximately 80 Hz was observed after Probe 1, P1-30/12T (1.0 μM), was self-assembly immobilized, which suggested that the Au surface was successfully functionalized. Subsequent association of complementary target oligonucleotides, T-104(AS) (0.5 μM) introduced for the DNA hybridization resulted in an additional frequency decrease of approximately 70 Hz. Additional treatment of the DNA hybridized QCM sensor with Probe 2-capped Au nanoparticles, P2-30/12T(AS), resulted in an amplification and a large frequency shift of approximately 300 Hz.

In our circulating-flow QCM system, the thiolated probes, target sequences and oligonucleotide-functionalized Au nanoparticles were circulated constantly to ensure continuous interaction with the Au surface on QCM sensor thereby enhancing the efficiency of the probe immobilization and DNA hybridizations.

3.2. Immobilization of synthesized probe oligonucleotides

The first step of preparation of the QCM DNA sensor is to immobilize the probe sequences onto the surface of the QCM sensor. The thiol-linkered tag [HS–$(CH_2)_6$] modified probes (1.0 μM), i.e., Probe 1, were applied in the QCM system. The frequency changes of immobilization by P1-30, P1-30/12T and P1-30/24T were 69.5 ± 5.3, 80.4 ± 4.2 and 79.1 ± 7.7 Hz, respectively (Fig. 2A). The increases in mass due to the immobilization of P1-30, P1-30/12T and P1-30/24T on the Au surface of QCM were calculated as approximately 27, 32 and 31 ng, respectively. The thiolated probes with additional 12 dT (P1-30/12T) and 24 dT (P1-30/24T) showed significantly greater frequency change than the probe without additional dT (P1-30) ($P<0.05$).

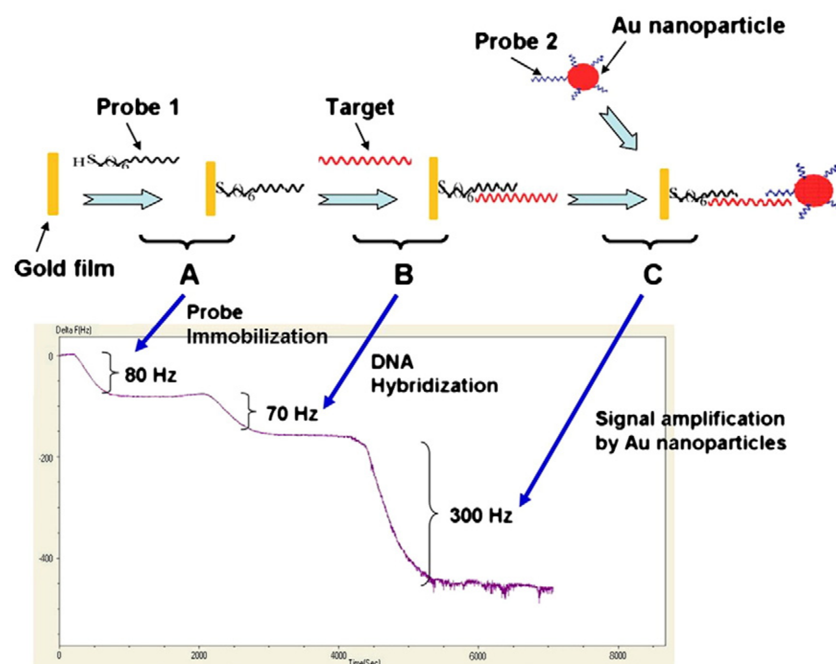

Fig. 1. Time-dependent frequency changes of the circulating-flow QCM sensor. (A) Addition of Probe 1 (1 μM; P1-30/12T) to self-assembly immobilize on the surface of the QCM sensor. (B) The complementary target oligonucleotides [0.5 μM; T-104(AS)] were subsequently introduced for DNA hybridization. (C) Additional treatment of the DNA hybridized QCM with Probe 2 (P2-30/12T)-capped Au nanoparticles. The sequences of Probe 1 and Probe 2 are complementary to the two ends of the analyte DNA (i.e., target sequences).

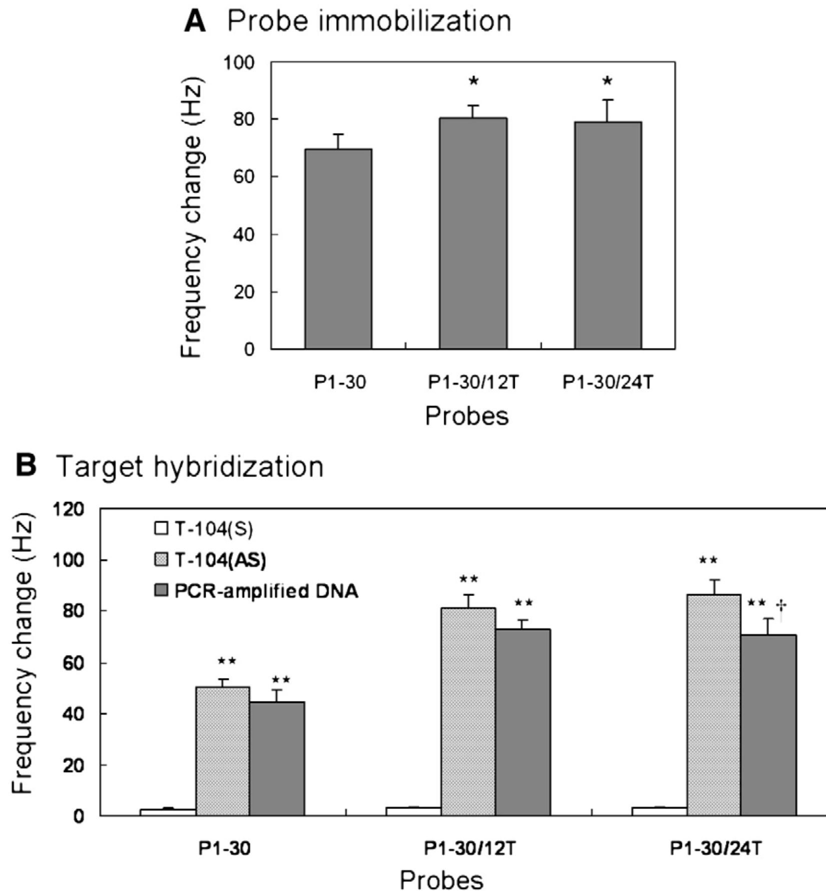

Fig. 2. Immobilization and hybridization efficiencies in the QCM system. (A) The probe oligonucleotides (Probe 1; 1 μM), P1-30, P1-30/12T and P1-30/24T, were immobilized onto the Au surface of QCM sensor and the frequency change of the QCM sensor was measured. * indicates $P<0.05$ *vs.* P1-30 probe. (B) The target oligonucleotides (0.5 μM), T-104(S) (104-mer; a non-complementary strand to the probes immobilized on the QCM sensor), T-104(AS) (104-mer; a complementary strand), and the PCR-amplified DNA (104-bp; 1 μM) were hybridized with the QCM sensor and the frequency changes were measured. ** indicates $P<0.01$ *vs.* T-104(A) and † indicates $P<0.05$ *vs.* T-104(AS). Values derived from 3 independent detections, error bars mean SD.

However, the frequency changes were similar between P1-30/12T and P1-30/24T.

The increases in mass when P1-30/12T or P1-30/24T was applied were larger than when P1-30 was applied because the single molecule weights of P1-30/12T (1.31×10^4 g mol^{-1}) and P1-30/24T (1.67×10^4 g mol^{-1}) are larger than that of P1-30 (9.42×10^3 g mol^{-1}). According to the calculation, the molecule densities of P1-30, P1-30/12T and P1-30/24T immobilized onto the Au surface on QCM sensor were 1.73×10^{12}, 1.47×10^{12} and 1.11×10^{12} (ssDNA cm^{-2}), respectively.

3.3. Hybridization of synthesized target oligonucleotides to probes immobilized QCM sensor

The frequency change of synthesized target oligonucleotides T-104(AS) [104-mer; 30 mer within the sequences is complementary to the probes immobilized on the Au surface of QCM sensor] and T-104(S) [104-mer; non-complementary to the probes] (Table 1) applied in the circulating-flow QCM system were also determined. The frequency change was 50.2±3.5, 81.4±5.2 and 86.6±5.7 Hz when 0.5 μM of T-104(AS) oligonucleotides hybridized with P1-30, P1-30/12T and P1-30/24T, respectively (Fig. 2B). The target oligonucleotide T-104(S) (0.5 μM), as the negative hybridization control in our QCM system, did not yield a measurable frequency change (<3 Hz) when applied to the probe-immobilized QCM sensor. Note that the oligonucleotide T-104(S) is essentially complementary to T-104(AS). Thus, the lack of frequency change upon interaction of the Probe 1 with T-104(S) indicates that non-specific oligonucleotide binding is negligible on the interface. The frequency change due to target DNA hybridization with probes in the P1-30/12T or P1-30/24T immobilized QCM sensors was significantly greater ($P<0.01$) than that in the P1-30 immobilized QCM sensor (Fig. 2B), even though the probe density of P1-30 on the Au surface of the QCM sensor was high. This indicates that the probes with spacer segments (additional 12 dT) influence hybridization with the target sequences.

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

The probe-immobilized QCM sensors were also used for detection of amplified target DNA by PCR. The DNA fragment is 104-bp and located within the respective region of *E. coli*

O157:H7 *eaeA* gene. The amplified DNA fragment was identified by DNA sequencing. The double strand sequences of the PCR-amplified DNA are the same as the sequences of the synthesized 104-mer targets, T-104(AS) and T-104(S) (Table 1).

Before the PCR-amplified DNA was applied to the circulating-flow QCM sensor, the amplified DNA isolated from *E. coli* O157:H7 was denatured and immediately added to the circulating-flow QCM system for DNA hybridization. In each hybridization, 1 μM of PCR-amplified DNA (equivalent to 0.5 μM of the target T-104AS) was used to hybridize with the thiolated probes immobilized the QCM sensor. The results show that the frequency shifts were 44.6±2.7, 72.8±3.9 and 66.8±5.4 Hz when the PCR-amplified DNA hybridized with P1-30, P1-30/12T and P1-30/24T, respectively (Fig. 2B).

Similar to the results of the synthesized target oligonucleotides, the frequency change in the P1-30/12T or P1-30/24T immobilized QCM sensors was significantly greater than that in the P1-30 immobilized QCM sensor. However, the frequency change in the PCR-amplified DNA applied on the three probe-immobilized QCM sensors was less than those of parallel detections in the synthesized target oligonucleotides, T-104(AS).

In comparison of PCR-amplified DNA and synthesized oligonucleotides T-104(AS) as target sequences applied in the QCM system, the frequency change in the PCR-amplified DNA applied on the three probe-immobilized QCM sensors was less than those of parallel detections in the synthesized target oligonucleotides (Fig. 2B). The hybridization results hint that the denatured strands of PCR-amplified DNA can hybridize with complementary strands other than the probe oligonucleotides immobilized on the QCM sensor and/or the coexisted sequences (sense and anti-sense strands) may interfere with the hybridization of complementary targets and probe oligonucleotides.

3.5. Detection of PCR-amplified DNA of *E. coli O157:H7* using oligonucleotide-functionalized Au nanoparticles

As shown in Fig. 1, the detection signal, frequency shift, of the PCR-amplified DNA from *E. coli* O157:H7 genomic DNA was amplified by the specific oligonucleotide-functionalized Au nanoparticles in our QCM system. In the process, the Au nanoparticles were firstly capped with the thiolated probes (designated Probe 2), P2-30(S) (30-mer; a non-complementary oligonucleotide to the target sequences that were hybridized to the Probe 1 immobilized on the QCM sensor), P2-30(AS) (30-mer; a complementary oligonucleotide to the target sequences at the 5′ end), or P2-30/12T(AS) (30-mer with an additional 12 dT). When the Au nanoparticles or P2-30(S)-capped Au nanoparticles were applied to the P1-30/12T-immobilized QCM sensor which was hybridized the target sequences, a negligible frequency change was recorded (<3 Hz). In contrast, the frequency change markedly decreased when the P2-30(AS)- or P2-30/12T(AS)-capped Au nanoparticles were applied (Fig. 3). The value of frequency change in the treatment with P2-30/12T (AS)-capped Au nanoparticles (292±17 Hz) was significantly larger than that with the P2-30(AS)-capped Au nanoparticles (168±13 Hz) ($P<0.01$). The 12 dT spacer segment adapted to Au nanoparticles could enhance hybridization efficiency (Fig. 3).

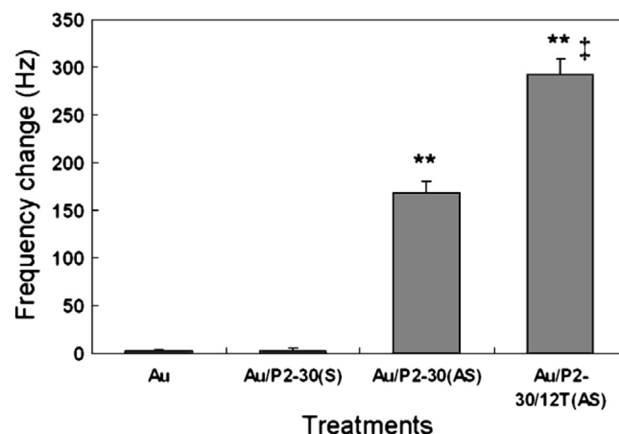

Fig. 3. Sandwich hybridization using the oligonucleotide-functionalized Au nanoparticles. Au nanoparticles and Probe 2-capped Au nanoparticles, including P2-30(S)-, P2-30(AS)- and P2-30/12T(AS)-capped Au nanoparticles, were applied to hybridize the target sequences that were hybridized to the P1-30/12T probe-immobilized QCM sensors. The frequency changes were measured in each treatment. Values derived from 3 independent detections, error bars mean SD. ** indicates $P<0.01$ *vs.* Au nanoparticles and P2-30(S)-capped Au nanoparticles. ‡ indicates $P<0.01$ *vs.* P2-30(AS)-capped Au nanoparticles.

3.6. Specificity of the QCM system in detecting *E. coli O157:H7*

Several bacterial strains, including *E. coli* O157:H7, *L. monocytogenes*, *S. choleraesuis*, *S. aureus* and *E. coli* K12 were applied to the PCR and QCM system for testing the specificity of the method for *E. coli* O157:H7. Except *E. coli* O157:H7, the primer pair (E_{157}eae/F and E_{157}eae/R) was a failure to amplify the DNA fragments from the genomic DNA isolated from bacterial cells of *L. monocytogenes*, *S. choleraesuis*, *S. aureus* and *E. coli* K12 (data not shown). The solutions of PCR reaction from these bacteria were also applied in the QCM detection; however, the results show the frequency changes at background level except *E. coli* O157:H7.

The genomic DNA isolated from the O serotypes of *E. coli*, including O76:H8, O85:H1, O138:H14 and O142:H6, were also used in the PCR and QCM detection. Although the *eaeA* gene presents in these pathogenic strains, the primers which were designed for specifically amplifying the DNA fragment within *E. coli* O157:H7 *eaeA* gene could not successfully amplify the DNA fragments from the *E. coli* O76:H8, O85:H1, O138:H14 and O142:H6 genomic DNA (data not shown). Therefore, none of the PCR-amplified DNA fragments of the *E. coli* O76:H8, O85:H1, O138:H14 and O142:H6 could be detected in the QCM sensor. These results suggest that the QCM system established in this study may be specifically used in the *E. coli* O157:H7 detection.

3.7. Quantitation of the QCM detection of PCR-amplified DNAs

Genomic DNA was extracted from different concentrations of serially diluted *E. coli* O157:H7 (1.2×10^1 to 1.2×10^8 CFU/ml) using the protocol described in the experimental section. PCR was carried out using the DNA extract from each concentration of cells. The gel electrophoresis detection of PCR products is

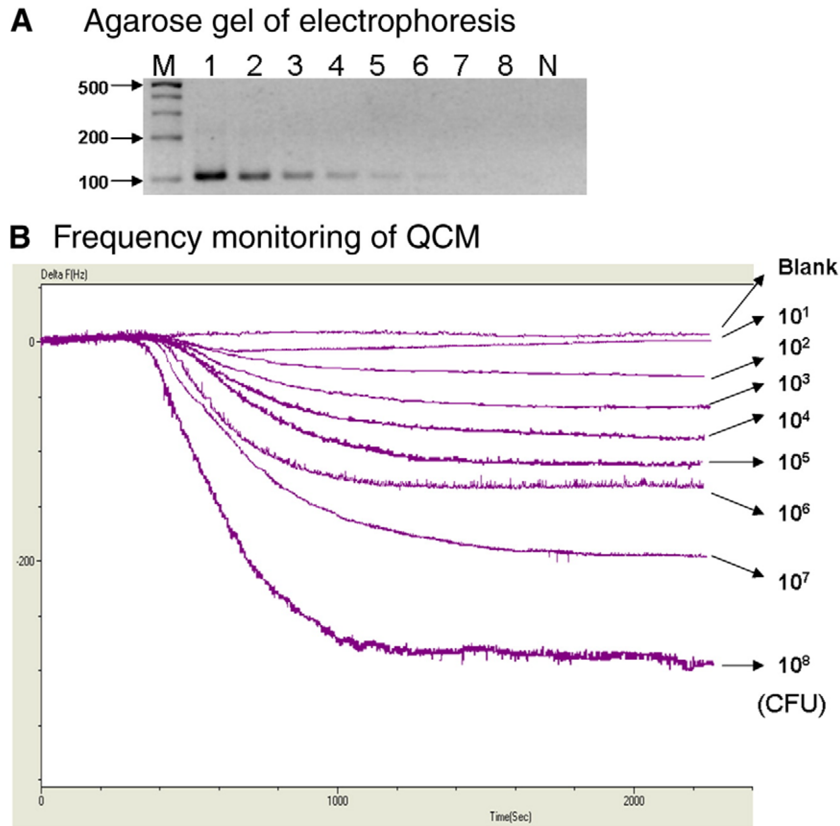

Fig. 4. Gel electrophoresis and QCM detections of PCR-amplified DNA from *E. coli* O157:H7 *eaeA* gene. (A) Gel electrophoresis detection of *E. coli* O157:H7 *eaeA* gene PCR products. M, DNA marker; lanes 1–8, 3 µl of the PCR-amplified products (purified and dissolved in 50 µl of TE buffer after PCR) from *E. coli* O157:H7 cells with concentrations from 1.2×10^8 to 1.2×10^1 CFU/ml; N, PCR blank control (water as PCR template). (B) Frequency shifts of the DNA sensor as a function of time for different PCR samples (3 µl each). The curves of frequency shift from top and in order are blank; 1.2×10^1 to 1.2×10^8 CFU/ml.

shown in Fig. 4A. Electrophoresis confirmed the successful amplification of PCR products with the correct size, 104-bp. The PCR-amplified DNAs for *E. coli* O157:H7 were detected by the P1-30/12T immobilized QCM sensors using P2-30/12T-capped Au nanoparticles as an amplifier and verifier. The time-dependent frequency changes were shown in Fig. 4B. The

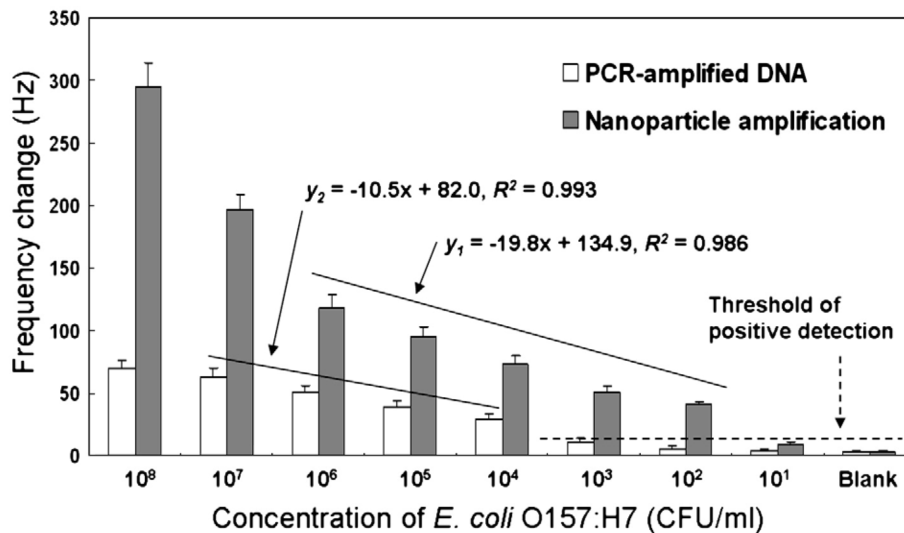

Fig. 5. The responses of the QCM sensor to the PCR-amplified DNAs isolated from different concentrations of *E. coli* O157:H7. In the detection of PCR-amplified DNA with and without P2-30/12T-capped Au nanoparticle amplification, linear relationships were found between the frequency shift *vs.* log (CFU/ml of *E. coli* O157:H7) from 1.2×10^2 to 1.2×10^6 CFU ($y_1=-19.8x+134.9$, $R^2=0.986$) and from 1.2×10^4 to 1.2×10^8 CFU/ml ($y_2=-11.26x+73.8$, $R^2=0.997$), respectively. The threshold for the positive detection was set as signal-to-noise (S/N)=3, and the detection limit was determined as 1.2×10^4 CFU/ml and 1.2×10^2 CFU/ml in the tests without and with Au nanoparticles as the amplifier, respectively. Values derived from 3 independent detections, error bars mean SD.

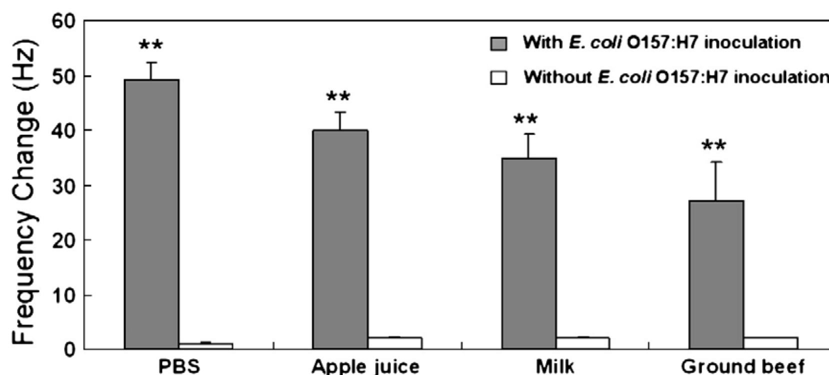

Fig. 6. Detection of *E. coli* O157:H7 in food samples using the circulating-flow QCM sensor with Au nanoparticles for signal amplification. The food samples inoculated with or without 5.3×10^2 CFU/ml (for apple juice and milk) or CFU/g (for ground beef) of *E. coli* O157:H7 were applied to the detection. *E. coli* O157:H7 cells were also inoculated in PBS (5.3×10^2 CFU/ml) as positive control in the detection. Values derived from 3 independent detections, error bars mean SD. ** indicates $P<0.01$ *vs.* the sample without *E. coli* O157:H7 cells inoculation.

frequency change of the sensor was enhanced as the cell concentrations of the PCR products increased. The blank control (PCR products using purified water as the PCR template) was also tested and yielded a frequency change <3 Hz.

The responses of the QCM sensor to differentiate PCR samples with/without amplification by oligonucleotide-functionalized Au nanoparticles are shown in Fig. 5. The measurements were highly reproducible for all concentrations of *E. coli* O157:H7 ($n=3$, RSD<8.3%). In the detection of PCR-amplified DNA with and without probe P2-30/12T-capped Au nanoparticles, linear relationships were found between the frequency change *vs.* log (CFU/ml of *E. coli* O157:H7) from 1.2×10^2 to 1.2×10^6 CFU/ml ($y_1=-19.8x+134.9$, $R^2=0.986$) in the samples treated with P2-30/12T-capped Au nanoparticles and from 1.2×10^4 to 1.2×10^8 CFU/ml ($y_2=-10.5x+82.0$, $R^2=0.993$) in the samples without the Au nanoparticle amplification (Fig. 5). Our results show that the *E. coli* O157 cell concentration was positively relative to the response of the frequency change indicating it is possible to enumerate *E. coli* O157:H7 using the QCM DNA sensor.

The threshold for detection limit of the QCM system is defined by the signal-to-noise (S/N) characteristics as S/N>3; hence, the detection limit of our system was assessed as 1.2×10^2 CFU/ml and 1.2×10^4 CFU/ml in the tests with and without P2-30/12T-capped Au nanoparticles as the amplifier, respectively.

3.8. Detection of E. coli O157:H7 in food samples

For real food detection, the cells of *E. coli* O157:H7 was artificially inoculated in apple juice, milk, or ground beef samples to reach the concentrations of 5.3×10^2 CFU/ml or CFU/g. These mixtures were used for the detection of *E. coli* O157:H7 without pre-enrichment by the QCM circulating system with Au nanoparticles for signal amplification. As shown in Fig. 6, the QCM frequency changes detected in the food samples containing 5.3×10^2 CFU/ml or CFU/g of *E. coli* O157:H7 were significantly larger than those detected in the blank controls ($P<0.01$). However, the detected frequency changes in the food samples were less than those detected in the parallel detection, i.e., PBS containing 5.3×10^2 CFU/ml of *E. coli* O157:H7. We suggested that the less sensitivity of detecting *E. coli* O157:H7 in the food samples compared with that in the PBS may be related to a few bacterial cells being lost when the *E. coli* O157:H7 cells were isolated from the food samples and it can be also related to inhibitors coming from the food (Bhaduri and Cortrell, 2001). The results indicate that the established QCM system in the present study is applicable for the detection of *E. coli* O157:H7 in real food samples.

4. Discussion

For the detection of *E. coli* O157:H7, methods focusing on rapid and accurate have been investigated, including DNA, immunological and biosensor methods. However, the immunological method for detecting *E. coli* O157:H7 needs significant pre-enrichment or relatively high cell numbers to reach sensing ability (Deisingh and Thompson, 2001). For this reason, the researches move toward using multiplex PCR and the methods are gradually becoming a major procedure for the detection of *E. coli* O157:H7 (Mukhopadhyay and Mukhopadhyay, 2007). Among these, DNA sensors integrated with a PCR-based DNA system have shown great potential for the specific detection and easily differentiation of pathogenic microorganisms. In this study, the DNA-based QCM sensor coupled with the PCR not only increases the sensitivity of the systems, but also offers a viable alternative to gel electrophoresis and other traditional DNA sequences detection methods that require labeled probes.

In the present study, we demonstrated a sensitive QCM DNA sensor using the sequence-specifically oligonucleotide-functionalized Au nanoparticles as “mass enhancers” for the detection of *E. coli* O157:H7 (Fig. 1). The method was used to detect the PCR-amplified DNA fragments from the real samples of *E. coli* O157:H7 with a detective limitation as low as 1.2×10^2 CFU/ml and a quantitative relationship was noted between the measured signal and the concentration of *E. coli* O157:H7 cells in a broad range, from 1.2×10^2 to 1.2×10^6 CFU/ml (Figs. 4 and 5). The technique of nanoparticle amplification applied to the DNA sensors resulted in a significant improvement in the detection limit compared with those of immunosensor

methods (Su and Li., 2004; Radke and Alocilja, 2005; Subramanian et al., 2006; Yang et al., 2004). Moreover, the results are comparable to the detection limit of 2.67×10^2 CFU/ml of *E. coli* O157:H7 in a recent report on the QCM DNA sensor based on a nanoparticle amplification method using non-specifically streptavidin-conjugated Fe_3O_4 nanoparticles for the detection of *E. coli* O157:H7 (Mao et al 2006). We reasoned that the PCR-based technique integrated into the DNA sensor system increased the target molecules in the detected samples.

The steric effect on oligonucleotide immobilization onto a solid surface and DNA hybridization in a QCM DNA sensor was improved in this study. The addition of 12 dT to the 5′ end of the probes increases the hybridization efficiency in our circulating-flow QCM system. This confirms the studies that report the poly dT at probes may reduction of steric hindrance in three-dimensional space and the increase of molecule collision caused by the addition of the spacer during DNA hybridization (Mo et al., 2005; Shchepinov et al., 1997). It is also proposed that spacers may reduce steric interference during DNA hybridization by making the probe end closest to the surface of the device more accessible (Shchepinov et al., 1997; Southern et al., 1999). Compared to P1-30/12T, however, the probe P1-30/24T showed similar hybridization efficiency in spite of its longer length (Fig. 2). These results are consistent with the hypothesis that the longer probes behave as flexible, coil-like polymer chains which encounter greater steric hindrance, inhibiting effective hybridization at the sensor/solution interface (Steel et al., 2000; Song et al., 2002).

In this study, we could detect 5.3×10^2 CFU/g of *E. coli* O157:H7 in the food samples without pre-enrichment. With current sufficient data presented, we proposed that our QCM system could be realistic in detecting 1 CFU/g of *E. coli* O157:H7 from a mixture of microorganisms in ground beef or other food, such as milk and juice, if an enrichment procedure is applied. Therefore, it can be expected that two log CFU/g of *E. coli* O157:H7 can be enriched within 2–3 h at initial inoculum of 1 CFU/g when a brief enrichment is used. With the amplification of Au nanoparticles, only a short period of pre-enrichment may be necessary, especially for detection of *E. coli* O157:H7 in food products.

PCR was used for the amplification of target DNA prior to the application of the QCM circulating system, but no enrichment was used in our present study. The total detection time of our current assay on prepared samples is within 3 h (DNA extraction for 0.5 h, PCR for 1.5 h, and real-time QCM detection for 1 h). Considering the total preparation time from food sampling to results including an enrichment procedure, with the current sensitivity of our assay, the total detection time is approximately 6 h for ground beef samples using the standard enrichment with our QCM system (3 h for enrichment at initial inoculum of 1 CFU/g and 3 h for QCM detection including PCR application). Our method is compatible to the current commercial PCR-based techniques.

5. Conclusions

We have demonstrated the use of oligonucleotide-functionalized Au-nanoparticles as amplifying and confirming probes for the microgravimetric QCM DNA-sensing method. By this method, probe immobilization, target hybridization and sandwich hybridization with the oligonucleotide-functionalized Au nanoparticles were successfully completed in a circulating-flow QCM system with real-time monitoring of frequency change.

In the present study, we demonstrated a sensitive QCM DNA sensor using oligonucleotide-functionalized Au nanoparticles as “mass enhancers” and “verifiers” for the detection of *E. coli* O157:H7 in food samples. This use of nanoparticles effectively amplified the signals in frequency change due to their relatively large mass of the nanoparticles compared to DNA targets, and yield increasingly sensitive detection limits for *E. coli* O157:H7 (1.2×10^2 CFU/ml for PCR products) without enrichment of the culture and a quantitative relationship was noted between the measured signal and the concentration of *E. coli* O157:H7 cells in a broad range, from 1.2×10^2 to 1.2×10^6 CFU/ml. Our results suggest that the DNA piezoelectric sensor with specifically oligonucleotide-functionalized Au nanoparticles as amplifiers has potential for additional applications in detecting *E. coli* O157:H7 or 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 systems for testing food that may be contaminated with pathogenic organisms.

Acknowledgments

This work was supported by grants NSC 93-2313-B-009-001, NSC 94-2313-B-009-002 and NSC 95-2918-I-009-006, from the National Science Council of Taiwan, and grants of MOE 96W808 and ATU Programs from the Taiwan Department of Education. This work was also supported in part by the Maine Agricultural and Forest Experiment Station at the University of Maine with external publication number 2993 and Main Wild Blueberry Research Advisory Committee.

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