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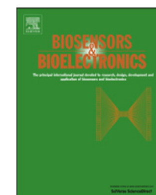

A piezoelectric immunosensor for specific capture and enrichment of viable pathogens by quartz crystal microbalance sensor, followed by detection with antibody-functionalized gold nanoparticles

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ABSTRACT

A sensitive bacteria enrichment and detection system for viable *Escherichia coli* O157:H7 was developed using a piezoelectric biosensor-quartz crystal microbalance (QCM) with antibody-functionalized gold nanoparticles (AuNPs) used as detection verifiers and amplifiers. In the circulating-flow QCM system, capture antibodies for *E. coli* O157:H7 were first immobilized onto the QCM chip. The sample containing *E. coli* O157:H7 was circulated through the system in the presence of 10 ml of brain heart infusion (BHI) broth for 18 h. The cells of *E. coli* O157:H7 specifically captured and enriched on the chip surface of the QCM were identified by QCM frequency changes. *Listeria monocytogenes* and *Salmonella* Typhimurium were used as negative controls. After bacterial enrichment, detection antibody-functionalized AuNPs were added to enhance the changes in detection signal. The use of BHI enrichment further enhanced the sensitivity of the developed system, achieving a detection limit of 0–1 log CFU/ml or g. The real-time monitoring method for viable *E. coli* O157:H7 developed in this study can be used to enrich and detect viable cells simultaneously within 24 h. The unique advantages of the system developed offer great potential in the microbial analysis of food samples in routine settings.
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1. Introduction

Traditional detection of *E. coli* O157:H7 is labor-intensive and time consuming (Tu et al., 2009). Alternative methods for detecting foodborne pathogens have focused on the detection of immunological (protein-based) and genetic (DNA-based) targets (Fu et al., 2005; Girones et al., 2010; Velusamy et al., 2010). These methods do not require isolation or biochemical tests and are thus more convenient and rapid than the traditional method. The detection limit of enzyme-linked immunosorbent assay (ELISA) is usually 3–5 log CFU (Park et al., 2008; Wang et al., 2007), while the detection limit of traditional agar plating methods is 1 log CFU (FDA, 2009a). The polymerase chain reaction (PCR) method can provide more sensitive results; with detection limits usually in the 1–3 log CFU range (Bai et al., 2010; Elizaquivel and Aznar, 2008). However, PCR inhibitors from complex food and environment samples can limit the efficiency of PCR amplification (Lee and Levin, 2011). False positive results due to the detection of DNA from dead cells can also influence the accuracy of this method (Wolffs et al., 2005).

Currently established biosensors can provide a detection limit for pathogens as low as 1–2 log CFU (Chen et al., 2008; Setterington and Alocilja, 2011; Waswa et al., 2007). Though the detection limit has been improved using biosensors, even lower detection limits (0–1 log CFU) are still desired for bacterial analysis in food samples since the infection dose of *E. coli* O157:H7 is as low as ten cells (FDA, 2009b). In addition, unlike the traditional method, most biosensor detection systems that are based on the recognition of DNA/RNA from the target cells using specific DNA-probes (Baeumner et al., 2003; Chen et al., 2008) or the recognition of protein on target cell membranes using specific antibodies (Lin et al., 2008; Rodriguez-Emmenegger et al., 2011; Varshney and Li, 2007) can cause false positive results when they encounter dead cells. Current biosensor detection systems still cannot differentiate between viable and dead pathogenic cells. Moreover, in order to eliminate background noise and contamination during the process, most of the current biosensor detection systems use a small sample size (10–100 μl) (Chen et al., 2008; Lin et al., 2008) that is less than the sample size applied in the standard bacteriological analytical manual (FDA, 2009a,b). This disadvantage restricts the application of biosensor detection

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systems in practical situations, where an additional concentration procedure on the sample preparation must often be performed (Wang et al., 2007).

Quartz crystal microbalances (QCMs), piezoelectric biosensors that detect the resonance frequency change that results from mass changes upon the chip surface, can be used in pathogen detection (Wu et al., 2007). The positive signals due to mass changes can be detected if the target cells or DNA/RNA from the target cells are captured by the immobilized specific antibodies or DNA probes on the crystal chip surface (Wu et al., 2007). Magnetic beads (Liu et al., 2007; Shen et al., 2011) and nanoparticles (Chen et al., 2008, 2009; Mao et al., 2006; Shen et al., 2011) have been used as signal amplifiers by conjugating them with antibodies or DNA probes in QCM systems. However, such QCM systems' pathogenic detection levels still cannot reach 0–1 log CFU. The lowest reported detection limit is 1–2 log CFU (Chen et al., 2008; Mao et al., 2006; Hao et al., 2011; Shen et al., 2011). Also, like most modern foodborne pathogen detection methods, the viability of the detected cells cannot be confirmed through recognition by antibodies and DNA probes. To our knowledge, the idea of incorporating the enrichment and detection of foodborne pathogens simultaneously in a QCM system to verify the viability of the target and increase the sensitivity of the system has never been reported.

In this study, we developed a simultaneous enrichment and detection QCM biosensing system to enrich and detect viable *E. coli* O157:H7 by real time monitoring within 24 h using a comparably large sample size of 10 ml, and applied the established QCM biosensing system to the detection of *E. coli* O157:H7 in real food samples.

2. Material and methods

2.1. Culture preparation

Foodborne pathogens, *Escherichia coli* O157:H7 (ATCC 35150, 12900, and 700594), *Listeria monocytogenes* (ATCC 49594 and 19115), and *Salmonella* Typhimurium (ATCC 6962 and 072209) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Fresh cultures for the experiments were made by transferring stocking cultures twice into BHI (Acumedia, Lansing, MI, USA) broth with incubation at 37 °C for 18–24 h. Bacteria samples were purified by centrifugation at 12,000 × *g* for 10 min at 4 °C twice before they were introduced to the QCM system. MacConkey sorbitol agar (MSA; Acumedia), oxford medium (OX; Acumedia) and xylose lysine deoxycholate (XLD; Acumedia) agar were used for enumeration of *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium, respectively.

2.2. Chemicals

Sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glutaraldehyde (GA) and bovine serum albumin (BSA) were purchased from Acros Organics (Morris Plains, NJ, USA). Gold chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) and sodium citrate ($C_6H_5Na_3O_7$) were purchased from RICCA Chemical (Arlington, TX, USA). Polyethyleneimine (PEI) was purchased from Sigma (St. Louis, MO, USA). Polyclonal antibodies for *E. coli* O157:H7 were purchased from Meridian Life Science (Saco, ME, USA). Acridine orange was purchased from AnaSpec Inc. (San Jose, *CA*, USA).

2.3. QCM system

The QCM 200 Digital Controller (5 MHz), O100R × 3 Crystal, and Gold QCM chips (2.54 cm reaction area diameter) used in this study were purchased from Stanford Research Systems (SRS, Sunnyvale, *CA*, USA). Five main components of the system (Fig. 1) include a QCM25 Crystal oscillator, a QCM200 QCM controller, a crystal holder with an axial flow cell (150 μl of reaction volume), a circulating-flow system (ISMATEC, Vernon Hills, IL, USA) with a flow rate of 186 μl/min in a 37 ± 0.05 °C water bath and a computer to demonstrate the curve of frequency in real time. The experimental data were real-time recorded per 1 s by SRSQCM200 software. Charts of the QCM signals were made using R software (R Foundation for Statistical Computing, Vienna, Austria). The frequency change (ΔF) was calculated by the difference of the measured QCM frequencies before and after the dAb-functionalized AuNPs were applied.

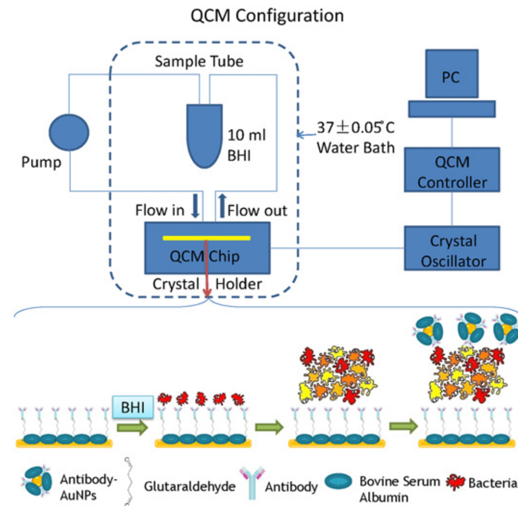

Fig. 1. Schematic illustration of the developed simultaneous enrichment and detection nanoparticle-functionalized piezoelectric biosensor-QCM system.

2.4. Preparation of detection antibody-functionalized AuNPs (dAb-functionalized AuNPs)

Thirteen nm AuNPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ solution with sodium citrate, with minor modifications of the procedures as described by Lin et al. (2008). The conditions for making dAb-functionalized AuNPs described in Pasqua et al. (2009) were followed, with some modifications. Twenty μl of 55.6 μg/ml polyclonal detection antibodies for *E. coli* O157:H7 diluted in 0.1 M phosphate buffer (PB) were added into 970 μl of AuNPs and incubated overnight at 4 °C with periodic manual inversion. Then, 10 μl of 1 mg/ml BSA diluted in 0.1 M PB were added into the dAb-functionalized AuNPs solution and incubated overnight again at 4 °C with periodic manual inversion. The solution was kept in the refrigerator (4 °C) for future use.

2.5. Application of dAb-functionalized AuNPs in QCM

The self-assembled monolayer (SAM) technique was used to establish the QCM immunosensor in this study. The QCM crystal chip was soaked in a piranha solution comprised of H_2SO_4 and H_2O_2 (30%) in a 3:1 ratio for 1 min. The cleaned gold-coated chip was then treated with 20 μl of 1% (w/v) PEI solution for 1 min. The pre-treated QCM chip was placed into the QCM chip holder and 1 × PBS buffer was flowed in. PBS was used as a washing buffer between each step in order to obtain a baseline for the next step. Once the baseline was obtained, 1 ml of 2.5% (v/v) GA solution was added into the system and circulated for 30 min in order to facilitate immobilization of the capture antibodies for *E. coli* O157:H7 on the chip surface. One ml of 55.6 μg/ml capture

antibodies for *E. coli* O157:H7 was then circulated through the QCM system for 30 min. The capture antibody-immobilized QCM crystal chip was then treated with 1 ml of 10 mg/ml BSA for 1 h in order to block the nonspecific binding sites on the chip surface. One ml of *E. coli* O157:H7 in the studied concentrations (3–6 log CFU/ml) was circulated into the QCM system for 2 h, where the bacteria were captured by the capture antibodies for *E. coli* O157:H7. Next, 1 ml of the detection antibodies for *E. coli* O157:H7 conjugated with AuNPs (dAb-functionalized AuNPs) were added into the system. The frequency decrease after the addition of the dAb-functionalized AuNPs was recorded in signal form.

2.6. Bacteria enrichment and detection by the QCM biosensor

In the detection of viable *E. coli* O157:H7, BHI broth was circulated before the addition of the bacteria (0–1 log CFU/ml) (Fig. 1) and the bacteria were enriched in the system with 10 ml BHI broth for 18 h. In this simultaneous enrichment and detection AuNPs-functionalized QCM biosensor system, the bacteria sample of *E. coli* O157:H7 was mixed from three different strains of *E. coli* O157:H7. The sample size was scaled up to 10 ml by adding 10 ml of BHI in a 25 ml sample tube in order to overcome the weakness of the small sample size used in most current biosensor applications. The mixture of detected sample solution and BHI medium could be flowed and circulated into the pipeline and chip's chamber of QCM system. The frequency change of the QCM was monitored during the enrichment. After 18 h enrichment, the system was stopped to record data. The sample tube with enriched bacteria was taken away. The QCM system was then circulated with 1 × PBS for 2 min in order to rinse any bacteria residue remained in the system. After the PBS washing, the QCM chip was taken out, washed with ddH_2O and dried in the air. The chip was then put back into the QCM chip holder and the QCM system was re-started by flowing 1 × PBS. One ml of dAb-functionalized AuNPs was circulated into the system for the next 30 min and relative frequency changes were recorded. The system signal was collected by resonance frequency change of the QCM chip. The conjugation of the AuNPs with antibody, *i.e.* dAb-functionalized AuNPs, could provide additional mass change on the chip surface and thus increase the frequency change. The final concentration and the purity of the post-enrichment sample were confirmed by plating the sample on selective media and TSA plates. *L. monocytogenes* and *S.* Typhimurium were used as negative controls. In order to confirm capture of bacteria on the QCM chip surface, chips were stained with 0.1% acridine orange for 1 min after the experiment and were rinsed with ddH_2O then dried using an air gun. The stained QCM chips were examined using a motorized inverted microscope at a magnification of 800 × (Olympus IX81, Center Valley, PA, USA).

2.7. Food sample preparation

Wild blueberries were chosen as the food sample to test the developed QCM system. Twenty five grams of wild blueberries (Hancock County, ME, USA) were cleaned and inoculated with bacteria by dipping blueberries into a 3 log CFU/ml mixture of *E. coli* O157:H7 for 2 min. The inoculated blueberries were dried on two glass rods in a fume hood for 1–2 h until the surface of the blueberries was completely dry. Another group of blueberries not inoculated with *E. coli* O157:H7 but cleaned was used as negative control. When both inoculated and non-inoculated blueberries were dry, each sample was put into a Whirl-Pak filter bag (Nasco, Fort Atkinson, WI, USA) along with 25 ml of 0.1% peptone water. The bag was stomached in a BagMixer (Interscience, Weymouth, MA, USA) for 2 min. The final blueberry sample containing 1 log CFU/g mixture of *E. coli* O157:H7 was filtered by 5 μm aseptic syringe filter. The initial concentration of *E. coli* O157:H7 on blueberry samples applied to the QCM system was enumerated on both MSA and TSA plates. Instead of adding 1 ml of pure bacteria samples, 1 ml of inoculated homogenous blueberries was added into the QCM system containing 10 ml of BHI broth following the procedure mentioned above. Mixtures of *L. monocytogenes* and *S.* Typhimurium inoculated on blueberry samples were used to test the specificity of the developed QCM system.

2.8. Statistical analysis

The experiments were repeated three times. Bacterial populations were reported as log CFU/ml or g. Analysis of variance (ANOVA) was performed using SYSTAT 12 software (Systat Software Inc., Chicago, IL, USA). Significance of difference was defined as $p < 0.01$.

3. Results

3.1. Fabrication of dAb-functionalized AuNPs

The AuNPs solution mixed with different concentrations of detection antibodies for *E. coli* O157:H7 was tested through both color observation (Fig. 2A) and the spectrum absorbance (400–700 nm) (Fig. 2B). It was found that at the concentration of 1 μg/ml, dAb-functionalized AuNPs presented the least aggregation while having the highest quantity of antibodies.

3.2. Signal enhancement with dAb-functionalized AuNPs in the QCM system

The additional mass enhancement of the dAb-functionalized AuNPs for *E. coli* O157:H7 was used to amplify the signal in the QCM system. The application of the dAb-functionalized AuNPs was first examined by adding it after the 2 h circulation of the

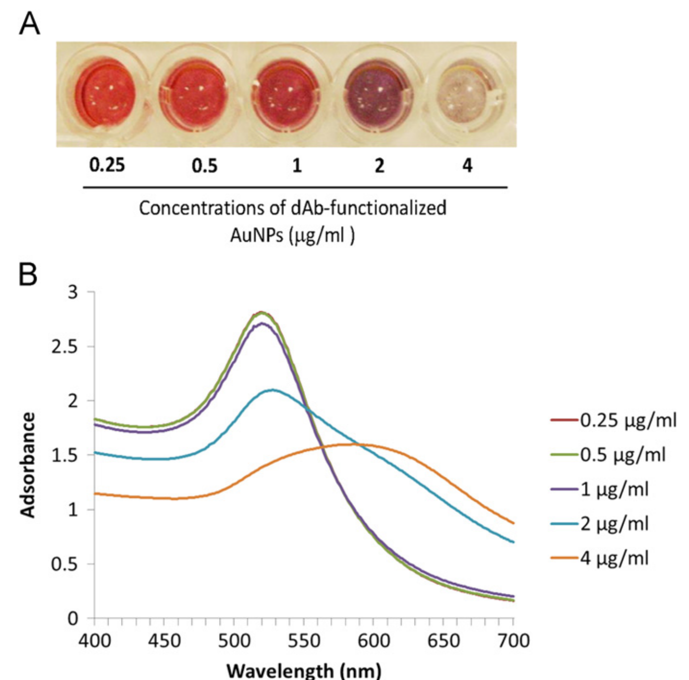

Fig. 2. Concentration effect of detection antibodies used to conjugate with AuNPs. (A) Results of differing concentrations of antibody (0.25, 0.5, 1, 2, and 4 μg/ml) applied into AuNPs to prepare dAb-functionalized AuNPs are shown. (B) The spectrum profiles were monitored for each dAb-functionalized AuNPs preparation from 400 nm to 700 nm.

bacteria in the QCM system, where no BHI enrichment was involved. With 6 log CFU/ml of *E. coli* O157:H7 present, frequency change (ΔF) without the addition of the dAb-functionalized AuNPs was only 6 Hz. However, when the dAb-functionalized AuNPs were applied, ΔF was enhanced to 95 Hz (Fig. 3A). As a final development, the dAb-functionalized AuNPs were applied after the 18 h BHI enrichment, producing a simultaneous enrichment and detection system for viable *E. coli* O157:H7 in a nanoparticle-functionalized piezoelectric biosensor-QCM.

The specificity of the modified QCM chips for the detection of *E. coli* O157:H7 was studied by comparing ΔF when applying the same concentration (6 log CFU/ml) of *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium, separately to the system. The results were significantly different ($p<0.01$): while *E. coli* O157:H7 produced a change of 95 Hz, the other three produced only ΔF < 20 Hz (data not shown). Bacterial capture was confirmed by the use of a motorized inverted microscope at a magnification of 800 × (Fig. 3B). As shown, the chip used to capture *E. coli* O157:H7 had much more bacteria on the chip surface than the chips that encountered other species.

The sensitivity of dAb-functionalized AuNPs used in the QCM system was examined by adding 3–6 log CFU/ml of *E. coli* O157:H7. The results show that a linear relationship was found between the frequency change *vs.* log (CFU/ml of *E. coli* O157:H7) from 4–6 log CFU/ml ($y=-42.917x+148.89$, $R^2=0.9962$). The frequency change of 3 log CFU/ml of *E. coli* O157:H7 did not meet the detection limit threshold, which was defined by the signal-to-noise (S/N) characteristics as S/N > 3, where the target bacteria could provide a signal at least three times greater than the signal from non-bacteria sample (Chen et al., 2008; Lin et al., 2010; Waswa et al., 2007); hence, the detection limit of dAb-functionalized AuNPs in this experiment was assessed as 4 log CFU/ml (Fig. 4).

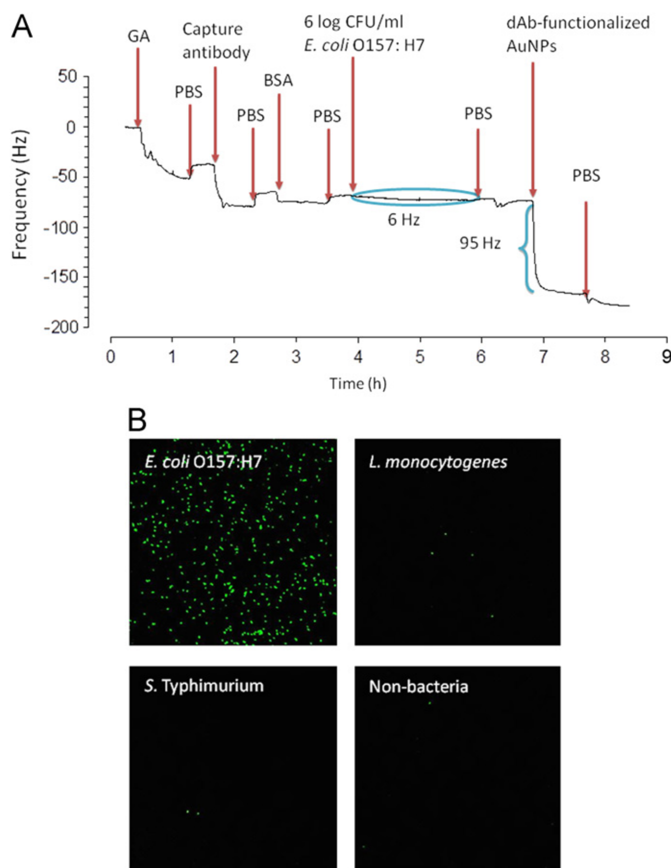

Fig. 3. Representative changes in QCM as a result of *E. coli* O157:H7 detected by the QCM system with dAb-functionalized AuNPs amplification without enrichment. (A) *E. coli* O157:H7 cells were captured by the capture antibodies on the QCM chip surface. The dAb-functionalized AuNPs were added into the QCM system after a 2 h circulation of bacteria. (B) Images of the QCM chip surfaces resulting from the application of different bacteria (6 log CFU/ml) to the QCM system as observed by a motorized inverted microscope at a magnification of 800 ×.

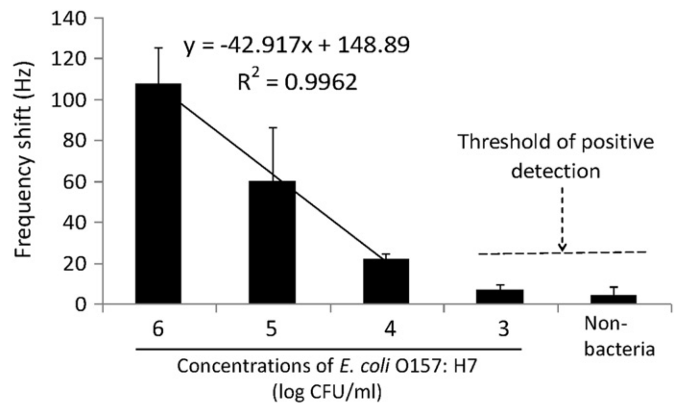

Fig. 4. Frequency shifts observed in the detection of *E. coli* O157:H7 at various concentrations using QCM with dAb-functionalized AuNPs without enrichment. *E. coli* O157:H7 cells were captured on the chip surface via the immobilization of the capture antibodies. The dAb-functionalized AuNPs were added after a 2 h circulation of bacteria through the detection system. Relative frequency decreases were observed after adding dAb-functionalized AuNPs. The threshold of the detection limit was defined by the signal-to-noise (S/N) characteristics as S/N > 3. The results were averaged from three independent determinations and error bars mean standard error (S.E.).

3.3. Simultaneous enrichment and detection of viable E. coli O157:H7

To grow bacteria on the QCM chip surface and also in the circulated bacteria enrichment medium used in the circulating-flow QCM system, BHI broth was introduced into the QCM system in order to enrich the target bacterium and establish simultaneous enrichment and detection. The BHI broth and low concentration (0–1 log CFU/ml) of viable *E. coli* O157:H7 were circulated in the QCM system for 18 h. The bacteria were enriched from 0–1 log ($1.5\times10^{\circ}-2.6\times10^{1}$) to 8 log CFU/ml after 18 h circulation in the QCM system, hence more bacteria were captured on the chip surface. The dAb-functionalized AuNPs were then introduced into the QCM system, resulting in significant frequency decreases. The signal pattern from the detection of 1 log CFU/ml *E. coli* O157:H7 using the simultaneous enrichment and detection nanoparticle-functionalized piezoelectric biosensor-QCM system is shown in Fig. 5A and the pattern of the blank control is shown in Fig. 5B. The frequency changes after the addition of dAb-functionalized AuNPs in the developed QCM system are shown in Fig. 5C. The results show that after the addition of the dAb-functionalized AuNPs, frequency decreases of 125 ± 13 Hz and 110 ± 35 Hz were observed from initial concentrations of *E. coli* O157:H7 at 1 and 0 log CFU/ml respectively, while the frequency decrease observed from the non-bacteria sample was only 18 ± 10 Hz.

The specificity of the QCM system's simultaneous enrichment and detection was evaluated using mixtures of different bacterial strains (1 log CFU/ml). The results show that a frequency decrease of 125 ± 13 Hz was observed from the mixture of *E. coli* O157:H7 (including ATCC 35150, 12900, and 700594) after the addition of the dAb-functionalized AuNPs, while frequency decreases of 25 ± 13, 27 ± 12, 14 ± 8 and 18 ± 10 Hz were observed from the mixture of *L. monocytogenes* (including ATCC 49594 and 19115), the mixture of *S.* Typhimurium (including ATCC 6962 and 072209), and the non-bacteria samples respectively. The frequency change caused by applying the mixture of *E. coli* O157:H7 into the QCM

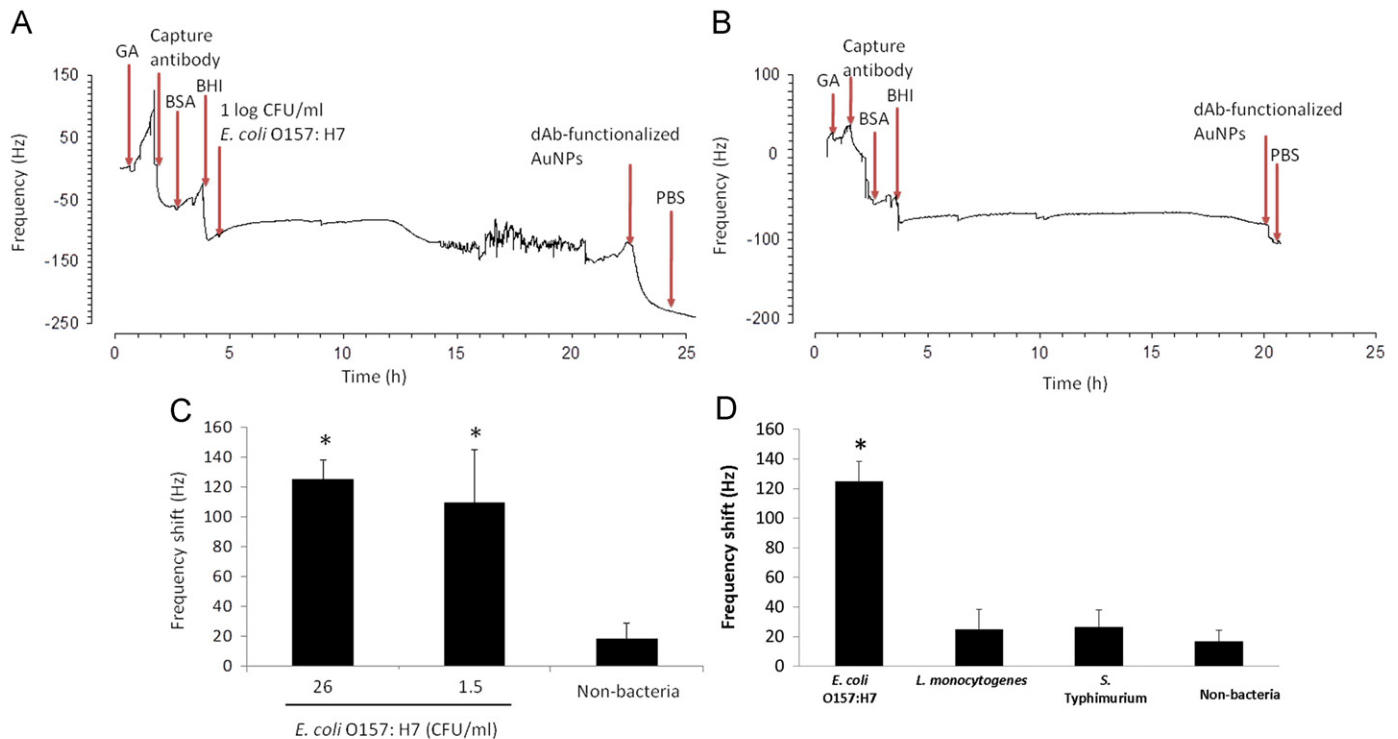

Fig. 5. Detection of viable *E. coli* O157:H7 in the simultaneous bacterial capture and enrichment QCM system, followed by detection with dAb-functionalized AuNPs. (A) Frequency change was observed from a low concentration (1 log CFU/ml) of viable *E. coli* O157:H7 enriched in the QCM system in the presence of 10 ml BHI broth. (B) Little frequency shift was observed from non-bacteria. (C) After the BHI enrichment, the dAb-functionalized AuNPs were added as a signal amplifier and detection verifier. Relative frequency decreases were observed, where * indicates $p < 0.01$, *vs.* non-bacteria. (D) Detection specificity of the QCM system/dAb-functionalized AuNPs system, where mixtures of *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium at 1 log CFU/ml were used. * indicates $p < 0.01$ *vs.* mixtures of *L. monocytogenes* and *S.* Typhimurium, and non-bacteria.

system was significant different ($p < 0.01$) from the frequency changes caused by the other bacteria (Fig. 5D).

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

Wild blueberry samples were inoculated with viable *E. coli* O157:H7 at a final concentration of 1 log CFU/g. The results of signal patterns indicated that in the presence of viable *E. coli* O157:H7, frequency changes were observed during the BHI enrichment, whereas no frequency changes were observed when the viable cells were absent (data not shown). After the addition of dAb-functionalized AuNPs, significant frequency change ($p < 0.01$) was observed using the blueberry samples containing initial concentration of 1 log CFU/g mixture of *E. coli* O157:H7 when compared with the frequency changes from the blueberry samples containing initial concentrations of 1 log CFU/g mixture of *L. monocytogenes*, *S.* Typhimurium and non-bacteria (Fig. 6). A frequency change of 93 ± 24 Hz was observed from the blueberry samples containing 1 log CFU/g mixture of *E. coli* O157:H7, while the frequency decreases of 8 ± 4, 15 ± 14, and 7 ± 3 Hz were observed from the blueberry samples consisting of the mixtures of *L. monocytogenes* and *S.* Typhimurium, and non-bacteria. The results indicate that the QCM system developed in the present study is practical for the simultaneous enrichment and detection of viable *E. coli* O157:H7 in real food samples.

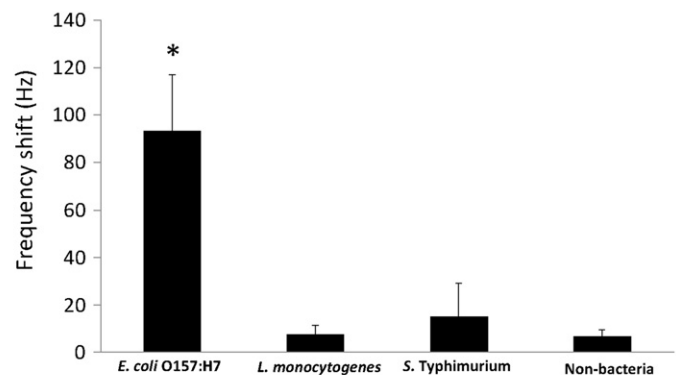

Fig. 6. Specific detection of *E. coli* O157:H7 inoculated on blueberry samples by the QCM/dAb-functionalized AuNPs system. Mixtures of *E. coli* O157:H7, *L. monocytogenes* and *S.* Typhimurium at 1 log CFU/g on blueberries were introduced into the QCM/dAb-functionalized AuNPs system. The blueberry samples with viable bacteria were enriched in the QCM system in the presence of 10 ml BHI broth for 18 h. * indicates $p < 0.01$, *vs.* blueberries with *L. monocytogenes*, blueberries with *S.* Typhimurium, and blueberries without bacteria inoculated.

4. Discussion

AuNPs have previously been conjugated with antibodies in order to amplify signals in biosensors (Guo and Wang, 2007; Han et al., 2011; Shen et al., 2011). In the QCM immunosensing system, the conjugation of antibodies and AuNPs can be used as the detection complex, enhancing the mass change on the chip surface and verifying the target captured. Ideally, if more antibodies could be immobilized on the AuNPs surface, more targets could be recognized by the dAb-functionalized AuNPs. However, antibodies that have positive charges can also lead to the aggregation of AuNPs (negatively charged) if their concentrations are too high. The best ratio between antibodies and AuNPs is defined as the lowest level of aggregation possible with the highest quantity of antibodies. The results show that at the concentration of 1 μg/ml or less, the AuNPs remained dark-red color. In the sake of maximizing the efficiency of dAb-functionalized AuNPs, 1 μg/ml of antibodies was chosen.

The sensitivity of the QCM system was greatly improved using dAb-functionalized AuNPs. A 15-fold improvement was found in the sample of 6 log CFU/ml *E. coli* O157:H7. The detection limit with the addition of the dAb-functionalized AuNPs was 4 log CFU/ml (without simultaneous enrichment). Compared to the other studies that did not apply nanoparticle enhancement, the detection limit in a 5-MHz QCM system was only 5 log CFU/ml (Poitras and Tufenkji, 2009). In further experiments of the present study, the dAb-functionalized AuNPs were added after the BHI enrichment, when more bacteria could be captured on the QCM chip surface via the capture antibody and then be recognized by the dAb-functionalized AuNPs, resulting in further improvement for the detection limit of the system (0–10 log CFU/ml).

In the detection of foodborne pathogens, it is preferred to detect viable bacteria since only viable cells are hazardous. Traditionally, agar plating methods were used to achieve the goal. Recently, a variety of more rapid conventional methods were developed. Most of them use DNA genes or proteins on the cell membrane as the target (Chen et al., 2008; Lin et al., 2008; Varshney and Li, 2007). The signals generated through recognition of these targets are considered indirect signals because the whole cell of the target bacteria has not been detected. Both DNA-based and protein-based assays fail to determine viability of the target cells (Lazcka et al., 2007). Even though some studies have been done using viable cells, there is limited evidence to show that the viability of the cells in real food samples can be determined. In order to solve this problem, BHI enrichment was introduced into the QCM system to establish a simultaneous enrichment and detection mechanism for the nanoparticle-functionalized piezoelectric biosensor-QCM system in this study. The procedure involved the immobilization of the capture antibodies for *E. coli* O157:H7, BHI enrichment of the viable target cells, and the addition of dAb-functionalized AuNPs for signal enhancement and target verification. In this detection setting, food sample with low pathogen contamination (0–1 log CFU/ml) can be directly added into the QCM system without pre-process of sample concentration and enrichment. Viable cells of the bacteria can be detected by the system while being enriched with BHI in the system.

BHI broth was applied into the QCM system before the addition of the bacterial samples. The detection sample size was scaled up to 10 ml, an improvement upon the small sizes of the samples used in many other biosensor studies (Buchatip et al., 2010; Hao et al., 2011; Hong et al., 2009). Also, more nutrients could be added into the system since the sample size was increased. Low concentrations (<10 CFU/ml) of *E. coli* O157:H7 were studied in the system. Although the signal pattern obtained from the QCM system did not represent the real growth curve of the cells, it was found that after 8–10 h enrichment, a noticeable frequency decrease was observed, showing the growth of the bacteria and confirming the viability of the bacterial cells. After 18 h enrichment, the bacteria concentration increased to 8 log CFU/ml in the BHI medium as confirmed by plating the final enriched and captured samples on agar plates. The viscosity and the density of the liquid solution increased during the enrichment, causing a relative frequency change. Since more bacterial cells were captured onto the QCM chip surface during the enrichment, the addition of the dAb-functionalized AuNPs after the BHI enrichment could further verify the captured bacteria on the chip surface through antibody-antigen reaction processes. More mass change would be sensed by the QCM system due to the additional enhancement of the AuNPs, resulting in further frequency decreases from the QCM.

Another question issue is that some types of bacteria (*i.e. L. monocytogenes*) can form biofilm on a solid surface with given nutrients (Reipa et al., 2006). It is believed that the long-term enrichment of bacterial samples in QCM systems may cause biofilm to be formed by other non-target bacteria on the chip surface. However, the literature indicates that biofilms are formed in a 24–48 h growth period (Reipa et al., 2006). Since the BHI enrichment applied in the developed QCM system took place for 18 h, a shorter period of time than that necessary for biofilm formation, the system could eliminate false positive frequency decreases resulting from biofilm formed by non-bacteria samples. Furthermore, the specificity of the system also indicated that even if some non-target bacteria remained on the chip surface as a result of non-specific binding, they could not be recognized by the detection antibodies for *E. coli* O157:H7 conjugated with AuNPs. According to the results shown in Fig. 5, the frequency decrease in the *E. coli* O157:H7 containing sample after the addition of dAb-functionalized AuNPs was significant ($p<0.01$) compared with that of the other non-target bacteria.

The BHI enrichment procedure applied in this study successfully improved the sensitivity of the system from 1×10^4 to 1.5×10^0 CFU/ml. The real-time growth of the bacteria that was observed during the enrichment also confirmed the viability of the cells. This is reported as the first time that a QCM biosensor system can detect and differentiate viable cells with such a low detection limit.

This is the first time a QCM system has been developed as a simultaneous enrichment and detection device to detect and differentiate viable *E. coli* O157:H7 at the detection limit of <10 CFU/ml in real food samples. Other QCM and SPR studies of application detecting foodborne pathogens in real foods been done (Chen et al., 2008; Mao et al., 2006; Poitras and Tufenkji, 2009; Rodriguez-Emmenegger et al., 2011; Shen et al., 2011). However, their detection limits were not as low as the present study. Also, the other studies could not differentiate between viable and non-viable cells.

To our knowledge, this is the first time that BHI broth was used as the nutrient to enrich and confirm the viability of bacteria samples for the detection of *E. coli* O157:H7 in a QCM system. Compared with the traditional cell counting, the QCM biosensing platform developed in this study has several advantages, including improvement of the detection limit and time. In the method of cell counting, the detection limit is approximate 10 cells/ml and needs the steps of bacteria enrichment and confirmation with total time up to 3–5 days. In the QCM system with dAb-functionalized AuNPs used for signal enhancement and target verification, live cells of bacteria can be enriched in the flow and circulating QCM system. The detection limit is <10 cells/ml and the detection can be completed within one day. In previous studies of pathogen detection using QCM systems, the viability of the bacteria was not confirmed (Liu et al., 2007). The sensitivity of the reported QCM systems varied from 2–3 log CFU/ml (Buchatip et al., 2010; Chen et al., 2008). The total detection time including the pre-treatment of the samples could be 2–3 days (Shen et al., 2007; Su and Li, 2005). On the other hand, the QCM system developed in the present study showed that concentrations as low as one single viable target cell can be detected within 24 h, and the viability of the target bacteria was confirmed by frequency change during the enrichment.

5. Conclusion

In this study, a simultaneous enrichment and detection nanoparticle-functionalized piezoelectric biosensor-QCM system was developed. The sensor utilized BHI broth to enrich the target samples in the system, hence confirming the viability. AuNPs serving as further detectors and signal amplifiers were used to improve the sensitivity of the sensor, thereby improving the

detection limit of the QCM system. The detection limit was improved from 4 log to 0–1 log CFU/ml when the detection antibodies for *E. coli* O157:H7 conjugated-AuNPs were applied after the BHI enrichment. Moreover, the whole detection can be completed in one day without additional enrichment outside of the system. This study reports, for the first time, the enrichment and detection of viable bacterial cells in a nanoparticle-functionalized piezoelectric biosensor-QCM system, provides a more sensitive and specific immunosensor than those reported previously. By strategically combining culture methods, immunology, nanotechnology, and QCM sensing technology, QCM biosensors can be made more sensitive and specific than those previously reported, providing for future practical application in food safety inspections. The new trend of high-throughput multiple pathogen detection may also be archived by further studies with multiple antibody coating using QCM array.

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Appendix A. supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.05.024.

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