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A microfluidic nano-biosensor for the detection of pathogenic *Salmonella*

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ABSTRACT

Rapid detection of pathogenic *Salmonella* in food products is extremely important for protecting the public from salmonellosis. The objective of the present study was to explore the feasibility of using a microfluidic nano-biosensor to rapidly detect pathogenic *Salmonella*. Quantum dot nanoparticles were used to detect *Salmonella* cells. For selective detection of *Salmonella*, anti-*Salmonella* polyclonal antibodies were covalently immobilized onto the quantum dot surface. To separate and concentrate the cells from the sample, superparamagnetic particles and a microfluidic chip were used. A portable fluorometer was developed to measure the fluorescence signal from the quantum dot nanoparticles attached to *Salmonella* in the samples. The sensitivity for detection of pathogenic *Salmonella* was evaluated using serially diluted *Salmonella* Typhimurium in borate buffer and chicken extract. The fluorescence response of the nano-biosensor increased with increasing cell concentration. The detection limit of the sensor was 10^3 CFU/mL *Salmonella* in both borate buffer and food extract.

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1. Introduction

Contamination of food with pathogens poses a significant threat to public health. Recent foodborne pathogen outbreaks from various food sources have increased public awareness of these pathogens. *Salmonella* is a major foodborne pathogen and *Salmonella* Typhimurium is the one of the most commonly reported serotypes. *S.* Typhimurium is a gram-negative rod that causes self-limiting gastroenteritis in humans, and infection is associated with symptoms of fever, abdominal pain, nausea and vomiting, diarrhea, dehydration, weakness, and loss of appetite. The symptoms may appear 12–72 h after consumption of a contaminated food or beverage. The pathogen is associated with raw or undercooked eggs, poultry, beef, and unwashed fruit. *S.* Typhimurium outbreaks continue to occur, and outbreaks from various food sources have increased the need for simple, rapid, and sensitive methods to detect foodborne pathogens.

Conventional methods for *Salmonella* detection and identification involve multiple, prolonged enrichment steps. Although some immunological rapid assays are available, these assays still require enrichment steps and give results in 18–48 h. Enzyme-linked immunoassay (ELISA) and polymerase chain reaction (PCR) are robust but require several lengthy steps, expensive laboratory instruments, and experienced operators (Hart et al., 2011; Hossain et al., 2012). Biosensing methods, such as optical (Kramer et al., 2007), impedimetric (Radke and Alocilja, 2004; Varshney and Li, 2007; Yang et al., 2004), electrochemical (Setterington and Alocilja, 2012), and piezoelectric (Campbell and Mutharasan, 2008) methods, have shown great potential for rapid detection of foodborne pathogens.

Among biosensing methods, fluorescent dye-based optical immunoassays have been widely used because they can be highly sensitive and simple to operate (Geng et al., 2004; Kim et al., 2007; Kramer and Lim, 2004). However, methods that rely on organic fluorescent dyes are limited by their sensitivity to photobleaching, which limits long-term analysis. They also have a narrow excitation bandwidth and overlapping emission profiles from different fluorophores, which limits multiplexed applications (Kampani et al., 2007). Recently developed nanotechnology has the potential to overcome the limitations of organic fluorophores. Fluorescent nanoparticles and quantum dots (QDs) have several advantages over conventional organic dyes, such as high quantum yield and brightness, photostability, and resistance to chemical degradation. Semiconductor QDs exhibit size- and composition-dependent fluorescence properties that are suitable for multi-target and highly sensitive imaging using single excitation wavelengths (Tallury et al., 2010). The water solubility of QDs makes them suitable for biological applications, including imaging, detection, and biomolecular conjugation. Several groups have reported the application of QDs for detection of microorganisms, including *Escherichia coli* O157:H7 (Su and Li, 2004; Wang et al., 2012; Zhu et al., 2012), *Campylobacter jejuni* (Bruno et al., 2009), *Listeria*

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monocytogenes (Wang et al., 2007), and avian influenza viruses (Chou and Huang, 2012).

To increase the sensitivity of detection, many researchers have used immunomagnetic separation (IMS) methods. In this technique, antibodies conjugated to superparamagnetic beads capture analytes and are collected from the sample solution to concentrate the target analytes. Rotariu et al. (2005) designed a flow-through immunomagnetic separator that consists of a ferromagnetic wire and a silicone tube to improve the recovery of *E. coli* O157 from large-volume samples. Yang and Li (2006) separated *E. coli* O157: H7 and *S.* Typhimurium from samples by using specific antibody-coated magnetic beads. Quantum dots were used as fluorescence labels in immunoassays for simultaneous detection of two species of bacteria.

Several studies also coupled immunomagnetic separation with QD labeling (Dudak and Boyaci, 2008; Liandris et al., 2011; Zhao et al., 2009). The IMS method effectively separates target analytes from the sample; however, many steps (such as reaction, washing, collection with external magnetic force, and dispersion in buffer solution) are involved in the separation process, and analytes may be lost between these steps.

An increasing research effort has focused on the use of microfluidic devices to further improve the sensitivity of detection. Microfluidic devices are mainly used to enhance analytical performance by miniaturization. Reduction of the size of the devices provides additional advantages, such as reduced consumption of reagents and the ability to integrate several analytical steps together (Mairhofer et al., 2009). Microfluidic systems are able to efficiently and rapidly obtain measurements from small volumes of complex fluids, and they can be used to concentrate pathogens into a small volume and remove interfering foreign materials from the sample (Ramadan and Gijs, 2012). Many reports have indicated that microfluidic devices enhance the sensitivity of bacterial detection. Liu et al. (2005) improved the minimal detectable concentration of a virus by using antibody-coated microbeads and a pillar-type filter-equipped microfluidic system. Zaytseva et al. (2005) collected dengue virus RNA by using superparamagnetic beads inside a microfluidic device. Chen et al. (2010) generated a highly enriched solution of viral products concentrated 40–80-fold relative to the initial sample by using microfluidic immunomagnetic separation. Beyor et al. (2008) developed a microfluidic concentration device based on a polydimethyl siloxane (PDMS) valve membrane, a glass microfluidic wafer, and a glass manifold for immunomagnetic *E. coli* isolation from a dilute sample.

In this study, *S.* Typhimurium was detected using a microfluidic device to demonstrate the advantages of using magnetic beads and QDs as a fluorescent label. *S.* Typhimurium cells were separated and concentrated into small-volume samples by using anti-*Salmonella* antibody-coated magnetic beads. The cells were labeled with antibody-conjugated QDs to form "sandwich" complexes. The fluorescence signals from the complexes were measured using a custom-made fluorometer to provide a quantitative detection method for *S.* Typhimurium.

2. Materials and methods

2.1. Bacteria and media

S. Typhimurium (ATCC 14028) and *E. coli* (ATCC 25922) were obtained from American Type Culture Collection (Manassas, VA, USA). Fresh cultures of *S.* Typhimurium and *E. coli* were prepared by incubation in brain heart infusion (BHI; Difco Laboratories, USA) broth at 37 °C for 14 h. The cell-containing buffer was changed to 50 mM borate buffer (pH 7.4) by the following procedure: 1 mL of the enriched cell suspensions was centrifuged for 10 min at 5000*g*, and the supernatant was discarded. The collected cell pellet was resuspended in 1 mL of borate buffer and washed two more times with the same procedure. The cells were diluted to appropriate numbers (10^3–10^6 CFU/mL) with borate buffer for the experiment. Borate buffer without inoculation of *S.* Typhimurium cells was used as a control. The enriched *S.* Typhimurium was enumerated using the standard plate count (SPC) method.

Food samples were prepared by inoculating 100 μL of the cell suspension into homogenized food extracts. The cells were also diluted to appropriate numbers (10^3–10^6 CFU/mL) with the food extract. Borate buffer or plain food extract that did not contain *Salmonella* cells was used as a negative control. For food sample preparation, packages of chicken breast were purchased from a local grocery store.

2.2. Reagents and antibodies

BacTrace affinity-purified goat anti-*Salmonella* antibodies were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Anti-*Salmonella* antibody-conjugated superparamagnetic beads (Dynabeads® anti-*Salmonella*, 2.8 μm in diameter) and CdSe/ZnS core/shell QDs (Qdot® 605 antibody conjugation kit, 20 nm in diameter) were purchased from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from Pierce (Rockford, IL, USA), and borate buffer was purchased from Sigma (St. Louis, MO, USA). The Sylgard® 184 silicone elastomer kit containing PDMS prepolymer and catalyst was purchased from Dow Corning Corp. (Midland, MI, USA). Syringes were purchased from BD Biosciences (San Jose, CA, USA), and tubing was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.3. Conjugation of antibodies to QDs

Antibodies were conjugated to QDs prior to the assay. Goat anti-*Salmonella* antibodies were coupled directly to the QD surface using the Qdot antibody conjugation kit. Antibody conjugation with QDs was performed according to the manufacturer's instructions. Briefly, 10 mM succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC) was first added to the QD nanocrystals and incubated at room temperature for 1 h to activate the QDs. Second, 1 M dithiothreitol (DTT) was added to the purified antibody (1 mg/mL) and incubated at room temperature for 30 min to reduce it. Then, the activated QDs and the reduced antibodies were mixed and incubated at room temperature for 1 h. After the conjugation reaction, the antibody-QD conjugates were concentrated via ultrafiltration (centrifugation at 7000 rpm for 15 min). Finally, the concentrated QD-antibody conjugate was purified using size exclusion chromatography and dispersed in 200 μL of 50 mM borate buffer, pH 7.4, containing 1% BSA.

2.4. Fabrication of microfluidic channels

A silicon microfluidic channel design (width, 400 μm; height, 50 μm) was created using a CFD program (Coventorware 2010, Coventor Inc., Cary, NC, USA). The microfluidic design is shown in Fig. 1 and consists of a serpentine channel and detection well. A silicon wafer master mold containing the microfluidic channel design was fabricated by a third-party service company (Buysemi Inc., Suwon, Korea) by standard photolithography techniques. The surface of the master mold was passivated with fluorosilane to prevent it from permanently adhering to the PDMS during the molding. To create a PDMS mold, a 10:1 mixture of PDMS prepolymer and coring agent was stirred thoroughly and then

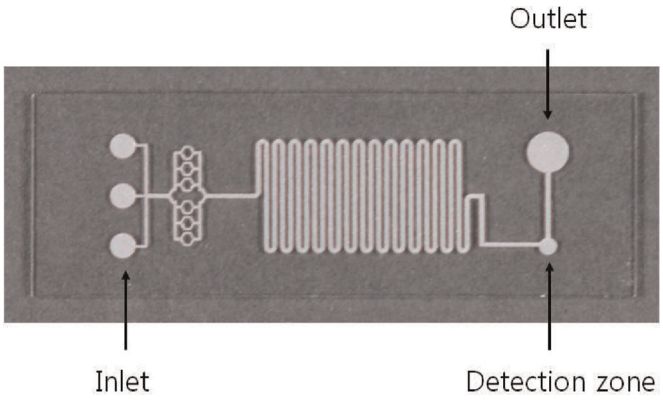

Fig. 1. Layout of the microfluidic channel. The well adjacent to the outlet hole is the detection zone.

degassed under vacuum. The prepolymer-coring agent mixture was poured onto the silicon master, which was placed inside a container formed by an aluminum foil. The PDMS mold was cured in an oven for 2 h at 65 °C. After curing, the PDMS mold was peeled from the master. The final thickness of the PDMS mold was ~3 mm. To provide inlets and outlets, connecting holes to the channels were fabricated in the PDMS mold using a puncher. A wide-gauge (23 G) needle with a blunt end was inserted into each hole to connect the tubing. The microfluidic channels contained two inlet ports and one outlet port (Fig. 1). Inlet port 1 was used for injections of magnetic beads mixed with sample solutions, followed by a rinse buffer to wash away unbound components. An antibody-conjugated QD-containing solution was injected through inlet port 2. The patterned PDMS mold was bonded to a glass slide by air plasma treatment to form closed channels and wells.

2.5. Portable fluorometer

Fluorescence signals from QD-labeled *S.* Typhimurium cells were measured using a custom-built portable fluorometer developed in a previous study (Kim et al., 2013). As shown in Fig. 2, the fluorometer used a LED unit as the excitation light source and highly sensitive silicon PMT (PCDmini, SenSL, Santa Clara, CA, USA) as a detector for signals with very low fluorescence. The light source unit comprised a 415 nm LED module connected to a fiber coupler module. The excitation light was delivered to the sampling unit via a bifurcated fiber optic bundle that was connected to the fiber coupler module of the light source unit. The fiber probe was placed at the bottom of the sampling unit to reduce the signal variation caused by gravity-induced falling of target cells. The sampling unit contained a tray to mount a microfluidic chip containing the concentrated sample liquid and a housing to block ambient light. The collected fluorescence signal entered the detection unit from the collection fibers to the fiber coupler module, which collimated the light and transmitted it to the C-mount adaptor module. In the C-mount adaptor module, an edge filter with a 515 nm cut-on wavelength was installed to pass only the fluorescence signal from the QDs. Finally, the fluorescence signals acquired by PMT were digitized and transferred to a PC for storage by the digital to analog converter of the silicon PMT.

2.6. Detection procedure

Bacteria were detected using a sandwich assay. First, 20 μL of the antibody-coated magnetic beads were mixed with 1 mL of sample solution containing 10^3–10^6 CFU/mL of *S.* Typhimurium and vortexed on a Genie-2 mixer (Daigger, Vernon Hills, IL, USA) for several seconds. The mixtures were incubated at room temperature for 30 min on an RKVSD mixer at a speed of 10 rpm. The magnetic bead–cell conjugates were separated from the solution by loading into MPC-S magnetic particle concentrators (Dynal Biotech) for 3 min. The liquid portion was carefully removed using a pipette. The bead–cell conjugates were rinsed with 0.5 mL of 1% PBS–BSA, followed by immunomagnetic separation, which was repeated twice. Then, the captured and washed cells were dispersed in 200 μL of 50 mM borate buffer, pH 7.4, containing 1% BSA. Finally, the captured cells and antibody-conjugated QDs were introduced to inlet ports 1 and 2 of the microfluidic chip, respectively.

The solutions containing the captured cells and the antibody-conjugated QDs were withdrawn by negative pressure created by a peristaltic pump at a flow rate of 40 μL/min. The two solutions were mixed in the meandering channel, and the cells were labeled with the antibody-conjugated QDs. The QD-labeled cells were captured in the detection zone using an external magnetic field from a permanent magnet placed under the detection zone (Fig. S1). Immediately after the sample introduction, 0.5 mL of borate buffer was introduced into the two inlet ports to remove the

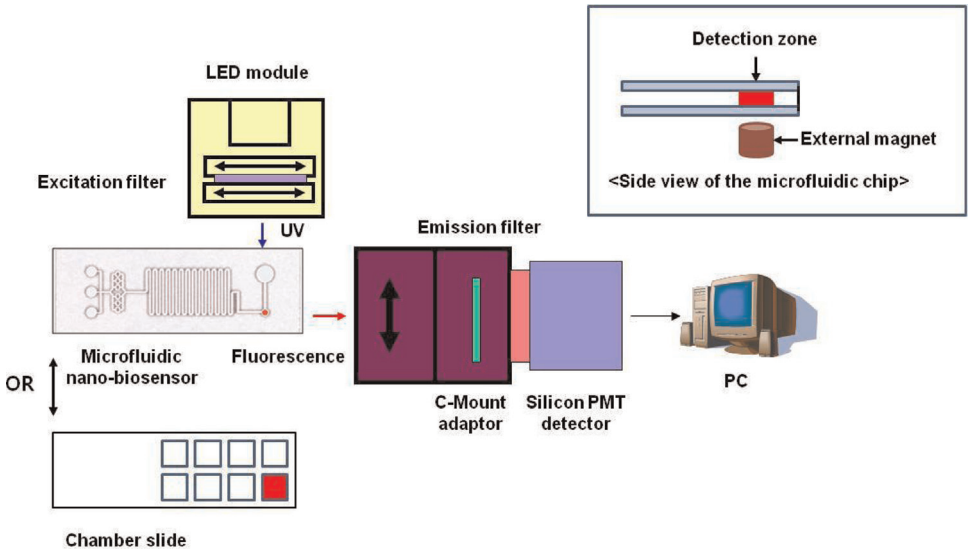

Fig. 2. Schematic diagram of the portable fluorometer.

unbound QDs. The microfluidic chip was then inserted into the portable fluorometer for measurement.

In addition to labeling with QDs and concentration of the cells inside the microfluidic device, a typical QD labeling method was used for comparison with the microfluidic concentration method. The cells captured with the antibody-coated magnetic beads were incubated with 200 μL of 10 μg/mL antibody-QD conjugates at room temperature for 30 min. The magnetic bead–cell–QD conjugates were separated from the solution by loading into the magnetic particle concentrators for 3 min. The liquid portion was carefully removed, and the remaining bead–cell–QD conjugates were rinsed three times with 0.5 mL of borate buffer containing 1% BSA. The captured and washed bead–cell–QD conjugates were then dispersed in 200 μL of borate buffer containing 1% BSA. Finally, the bead–cell–QD conjugate solution was loaded into an 8-well chamber slide (Lab-Tek II, Thermo Fisher Scientific Inc., MA, USA) and inserted into the fluorometer for measurement.

The fluorescence signal was recorded for 5 s. For each experiment, the standard deviation (SD) for signals from 3 samples was calculated. The error bars in each graph indicate $\pm$ SD. The limit of detection was designated as the number of cells producing fluorescence values higher than three times the SD plus the mean of the control signals. A sample was considered to test positive if the signal difference was higher than the limit of detection.

3. Results and discussion

The feasibility of detecting *S.* Typhimurium using a microfluidic-based nano-biosensor for immunomagnetic separation and QD labeling was investigated. The responses of the microfluidic-based nano-biosensor to increasing concentrations of *S.* Typhimurium spiked into borate buffer are shown in Fig. 3, which shows that the intensity of the fluorescence signals linearly increasing with increasing cell numbers. When the concentration of *S.* Typhimurium was increased from 0 to 10^6 CFU/mL, the fluorescence intensity increased from 686,689 counts to 1,177,415 counts. The sandwich assay with the microfluidic nano-biosensor produced positive results at a *S.* Typhimurium concentration of 10^3 CFU/mL in the borate buffer sample within 30 min.

The detection performance of the microfluidic nano-biosensor was compared to that of a typical QD labeling method in which bead–cell–QD conjugates were separated and applied to an 8-well chamber slide for signal measurement. The responses of the 8-well chamber slide biosensor to increasing concentrations of *S.* Typhimurium spiked into borate buffer are shown in Fig. 4. The intensity of the fluorescence signals also increased with increasing

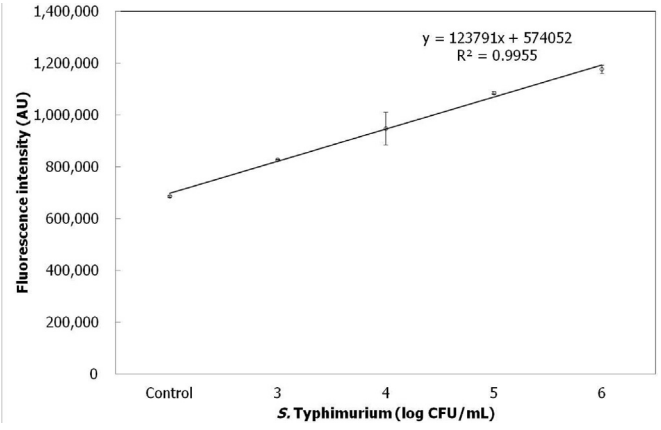

Fig. 3. Response of the microfluidic nano-biosensor for different concentrations of *S.* Typhimurium in borate buffer.

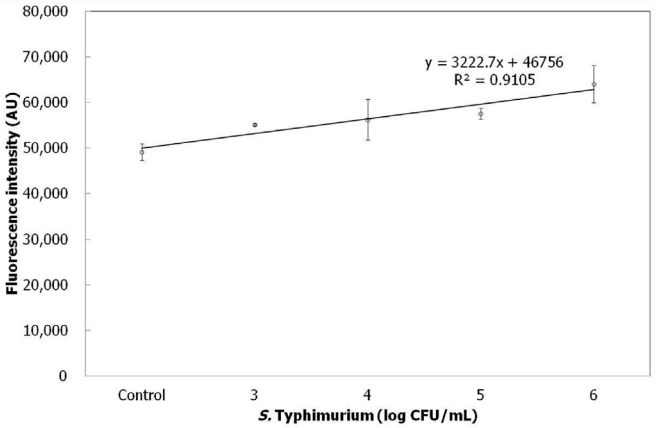

Fig. 4. Response of a nano-biosensor in an 8-well chamber slide for different concentrations of *S.* Typhimurium in borate buffer.

cell numbers. When the concentration of *S.* Typhimurium increased from 0 to 10^6 CFU/mL, the fluorescence intensity increased from 49,117 counts to 64,022 counts. The 8-well chamber slide method produced a positive result at an *S.* Typhimurium concentration of 4×10^3 CFU/mL of in the borate buffer sample. The signal increase from 0 to 10^6 CFU/mL for the microfluidic nano-biosensor, 490,926, was more than 33 times larger than that for the 8-well chamber slide, 14,905. This enhanced signal increase could be explained by the concentration effect of the reduced sample volume, as the sample volume of the detection well in the microfluidic chip, 0.7 μL, was 142 times lower than that of the 8-well chamber slide, 100 μL. In addition to the concentration effect, the signal increase may have resulted from reduced loss of the analyte. Ramadan and Gijs (2012) suggested that sample preparation should be performed inside a single confined channel to minimize analyte loss.

The microfluidic nano-biosensor specificity for *S.* Typhimurium was evaluated with *E. coli*. Fig. S4 shows fluorescence signals obtained from samples containing 10^3–10^6 CFU/mL *E. coli* and 10^3 CFU/mL *S.* Typhimurium in borate buffer. The sample consisting of 10^3 CFU/mL *S.* Typhimurium produced 17,983 counts more than the 10^6 CFU/mL *E. coli* sample. The results indicate that the anti-*Salmonella* antibody conjugated QD and magnetic bead selectively bind to *S.* Typhimurium and produce a specific signal for *S.* Typhimurium.

The effectiveness of *S.* Typhimurium detection using the microfluidic-based nano-biosensor was also tested in complex food samples. Chicken extracts were used as a sample matrix, and *S.* Typhimurium cells were inoculated to prepare sample sets. The responses of the nano-biosensors to increasing concentrations of *S.* Typhimurium spiked into chicken extracts are shown in Fig. 5. The intensity of the fluorescence signals increased with increasing cell numbers, as observed for the test with the borate buffer sample. When the concentration of *S.* Typhimurium increased from 0 to 10^6 CFU/mL, the fluorescence intensity increased from 755,485 counts to 1,184,996 counts. The detection limit of the QD-based nano-biosensor was also 10^3 CFU/mL *S.* Typhimurium in the food sample.

The microfluidic nano-biosensor had good linearity with the number of *S.* Typhimurium cells at concentrations of 0–10^6 CFU/mL for both borate and chicken extract samples. The standard curve showed a linear relationship between the cell concentrations (C) and the fluorescence intensity (FI) values of the microfluidic nano-biosensor. The regression model can be expressed as FI = 1,23,791C + 574,052 with R^2 = 0.995 and FI = 103,114C + 625,349 with R^2 = 0.949 for the borate buffer and chicken extract, respectively.

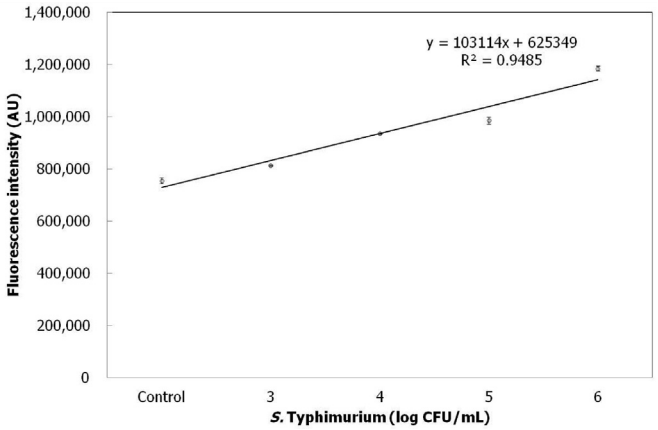

Fig. 5. Response of the microfluidic nano-biosensor for different concentrations of *S.* Typhimurium in chicken extracts.

The detection limit of this method is comparable to or better than that of other studies: 10^4 CFU/mL (Yang and Li, 2006) and 6×10^3 CFU/mL (Zhao et al., 2009). Similarly, a lateral flow strip was only able to detect 10^6 CFU/mL *Salmonella* cells in 30 min (Kim et al., 2011). PCR-based analyses (real-time PCR or real-time RT-PCR) were able to increase the detection limit from 10^3 CFU/mL to 10 CFU/mL, but required approximately 24 h (Miller et al., 2011). When using an interdigitated microelectrode-based impedance biosensor, Liébana et al. (2009) detected 7.5×10^3 CFU/mL *Salmonella* cells in milk using a magneto-electrode. However, they pre-concentrated the cells prior to the measurement and required an additional electrochemical signal enhancement method. For *E. coli* O157:H7, the limit of detection was 8×10^5 CFU/mL in beef samples when using a similar magnetic enhancement strategy with an impedimetric biosensor.

At least 10^5 *Salmonella* cells are required to cause salmonellosis in healthy adults (Kothary and Babup); thus, the detection limit of our method should be suitable for outbreak prevention.

4. Conclusions

Conventional methods for pathogen detection and identification are labor-intensive and take days to complete. Some rapid immunological assays have been developed, but these assays require several lengthy steps, expensive laboratory instruments, and experienced operators. Recently developed nano-biosensors provide the potential for rapid detection of foodborne pathogens. This study showed that a microfluidic nano-biosensor enables sensitive detection of *S.* Typhimurium in borate buffer and chicken samples. For selective detection, an anti-*Salmonella* polyclonal antibody was used to capture and label *Salmonella*. Semiconductive fluorescent QDs were attached to *Salmonella* in the samples and produced fluorescent light. Magnetic separation and a microfluidic chip were used to separate and concentrate the cells from the sample. The fluorescence response of the nano-biosensor was measured using a custom-built fluorometer. The sensitivity of the sensor was 10^3 CFU/mL *Salmonella* in both borate buffer and food extracts.

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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.2014.08.023.

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