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Rapid, Sensitive, and Specific Detection of *Salmonella* Enteritidis in Contaminated Dairy Foods using Quantum Dot Biolabeling Coupled with Immunomagnetic Separation

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Abstract

Colloidal semiconductor CdSe-ZnS core-shell nanocrystal quantum dot (Qdot) are luminescent inorganic fluorophores that show potential to overcome some of the functional limitations encountered with organic dyes in fluorescence labeling applications. *Salmonella* Enteritidis has emerged as a major cause of human salmonellosis worldwide since the 1980s. A rapid, specific, and sensitive method for the detection of *Salmonella* Enteritidis was developed using Qdot as a fluorescence marker coupled with immunomagnetic separation. Magnetic beads coated with anti-*Salmonella* Enteritidis antibodies were employed to selectively capture the target bacteria, and biotin-conjugated anti-*Salmonella* antibodies were added to form sandwich immune complexes. After magnetic separation, the immune complexes were labeled with Qdot via biotin-streptavidin conjugation, and fluorescence measurement was carried out using a fluorescence measurement system. The detection limit of the Qdot method was a *Salmonella* Enteritidis concentration of 10^3 colony-forming units (CFU)/mL, whereas the conventional fluorescein isothiocyanate-based method required over 10^5 CFU/mL. The total detection time was within 2 h. In addition to the potential for general nanotechnology development, these results suggest a new rapid detection method of various pathogenic bacteria from a complex food matrix.

Keywords: *Salmonella* Enteritidis, nanotechnology, Quantum dot, FITC, photostability

INTRODUCTION

Nanotechnology has exploded onto the scientific scene in the last few years and has impacted nearly every area of scientific research (Wang *et al*., 2015). Quantum dot (Qdot) had received a great deal of attention for their high fluorescence efficiency, lack of photobleaching, and long fluorescence (decay) lifetimes, which leaded to ultrasensitivity (Gerion *et al*., 2001; Yang and Li, 2005 and 2006). Colloidal semiconductor CdSe-ZnS core-shell nanocrystals quantum dots were luminescent inorganic fluorophores that had the potential to overcome some of the functional limitations encountered by organic dyes in fluorescence labeling applications (Kloepfer *et al*., 2003; Howarth *et al*., 2005). Qdot had absorption spectra that increase dramatically to the blue of the emission, and although the absorption was broad, the emission was narrow, symmetric, and independent of the excitation wavelength (Lee *et al*., 2004; Wang *et al*., 2004). Therefore, the optical systems could be simplified if Qdot substituted for the conventional dyes as a fluorescent label (Gerion *et al*., 2001; Su and Li, 2004). Also Qdot had an advantageous over fluorescent dyes because of their long-term photostability and high quantum yield (Yang and Li, 2005; Wang *et al*., 2007). Qdot could apply to analyze DNA detection, cell staining, cell surface receptor targeting, immuno-

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assays of IgG, and so on, because of its extraordinary properties (Yang and Li, 2005 and 2006). Especially, immunomagnetic separation was a specific, efficient, rapid method that could be used to isolate target bacteria from original samples without any need for filtration or centrifugation (Yang and Li, 2005; Yang and Li, 2007). Generally, when magnetic separation was done, the bacteria captured by antibody-coated magnetic beads were determined by conventional plating, but it was time consuming procedure to take one day. Recently, several researchers reported that the detection of *Escherichia coli* O157:H7 (Su and Li, 2004) and *Salmonella typhimurium* (Su and Li, 2005) using Qdot, and And Zhu *et al.* (2004) published the comparison between *Cryptosporidium parvum* and *Giardia lamblia* using Qdot as a Novel Immunofluorescent Detection System. Recently, with increasing development of the nanotechnology (Wang *et al*., 2015), Qdot could be used as the newly rapid detection method for several pathogenic bacteria from various dairy foods.

Especially, consistent with the need to provide safe feeding for all infants, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly convened an expert meeting on *Salmonella* spp., *Cronobacter* spp. (former *Enterobacter sakazakii*) and other microorganisms in powdered infant formula (FAO/WHO, 2004). After reviewing the available scientific information, the expert meeting concluded that intrinsic contamination of powdered infant formula with *Salmonella* spp. and *Cronobacter* spp. (former *Enterobacter sakazakii*) as category A had been a cause of infection and illness in infants, including severe disease which could lead to serious developmental sequelae and death (FAO/WHO, 2004).

Therefore, this study was applied to detect immunomagnetic-bead-captured *Salmonella* Enteritidis, which was a cause of human salmonellosis as category A, using Qdot as a fluorescent marker and spectrometer for the fluorescence measurement, and also to have the basic platform as validation methods to detect the pathogenic bacteria from various dairy foods.

MATERIALS AND METHODS

1. Preparation of stain and counting method

Salmonella Enteritidis was obtained from FDA (Food and Drug Administration, MD, USA). The pure culture of *Salmonella* Enteritidis was grown in Tryptic soy broth at 37℃ for 24 h before experiment. The culture was serially diluted to 10^{-8} with phosphate buffer saline (PBS) solution, and the viable cell number was determined by conventional plate counting. *Salmonella* Enteritidis dilution was plated on the Brilliant Green agar at 37℃ for 24 hour and the colonies formed were counted to determine the number of colony- forming units per mL (CFU/mL).

2. Chemical

The 525 nm CdSe-ZnS quantum dot-streptavidin conjugates was obtained from Quantum Dot Corp. (Hayward, CA, USA). Superparamagnetic, polystyrene microscopic beads covalently coated with affinity-purified polyclonal anti-*Salmonella* antibody was purchased from Dynal Biotech Inc. (Lake Success, NY, USA). Biotin-conjugated anti- *Salmonella* antibody was supplied by Biodesign International (Saco, ME, USA). Fluorescein isothiocyanate (FITC)-labeled affinity-purified anti-*Salmonella* antibody were manufactured by Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Phosphate buffered saline with bovine serum albumin (pH 7.4) were received from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ultrapure water (18 M cm) produced by a Millipore Milli-Q system was used.

3. Fluorescence measurement system

Fluorescence measurements were performed on a laptop-controlled portable system (Ocean Optics Inc.; Dunedin, FL) (Fig. 1). The system consisted of a USB2000 miniature fiber-optic spectrometer, a USB-LS-475 LED light source module, QP600-025-UV-vis optical fiber, and CUV-ALL-UV (cuvette holder) from Ocean Optics Inc. (Dunedin, FL, USA) (Fig. 1).

4. Sample preparation

This sample preparation was followed by the instruction of Seo *et al.* (1998), Su and Li (2004), and Yang and Li (2005

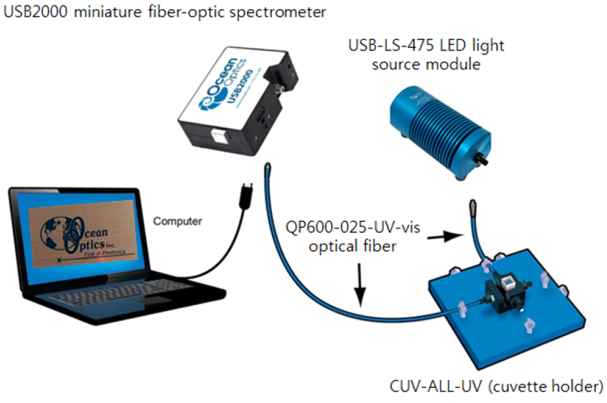

Fig. 1. Schematic diagram of the fluorescence measurement

and 2005).

The first step was immuno incubation and IMS. One milliliter of *Salmonella* Enteritidis cultures diluted in PBS containing 1% bovine serum albumin (PBS-BSA) was pipetted into 1.5 mL standard micro test tubes. IMB (20 µL) specific to *Salmonella* were added to these tubes and incubated at room temperature for 1 h with continuous rotation of 10 rpm. After incubation, the beads were recovered form the cell suspension with a Magnetic Particle Concentrator (MPC-M) and then washed twice consecutively in PBS containing 0.1% Tween 20 (PBST). The MPC-M was used to collect the washed beads. Biotin-conjugated anti-*Salmonella* antibodies (100 µL of 500 µg/mL concentration) were added to these tubes and incubated at room temperature for 1 h with continuous rotation of 10 rpm. After incubation, the microcentrifuge tubes were washed by same procedure as described above.

The second step was Qdot or Fluoresceinisothiocyanate (FITC)-labeled affinity-purified anti-*Salmonella* antibodies labeling. A total of 300 µL of 10 nM Qdot-streptavidin conjugates or 160 µL (1:200 dilution) FITC were added to the above microtubes containing the rinsed sandwich complexes, separately. After vortexing, the mixtures were incubated at room temperature for 30 min with gentle rotation at 10 rpm, and then washed twice successively in PBST. After the last washing, the complexes were resuspended in 300 µL of PBS and transferred into cuvettes to measure the fluorescence emission spectra. The blank (PBS) was subjected to the same treatment as that of the samples. After subtracting the background signal of the blank, the intensities were correlated to the cell concentrations of *Salmonella* Enteritidis.

5. Photostability test

The samples prepared were incubated at room temperature to compare fluorescence intensity between Quantum dot and FITC, according to the instruction of Ness *et al.* (2003) and Zhu *et al.* (2004).

6. Statistical analysis

All tests were replicated three times. Data were analyzed using GraphPad (InStat version 3.10, San Diego, CA, USA) and Duncan's test was used to determine significant difference between Qdot or FITC and blank ($P \leq 0.05$).

RESULTS AND DISCUSSION

To detect *Salmonella* Enteritidis, the immuno-complexes of immunomagnetic bead and biotin-conjugated antibodies obtained with IMS were labeled with Qdot via the strong biotin streptavidin bond. Then, this complex was followed by a fluorescence measurement using a simple-easy-handle spectrometer made by Ocean Optics Inc. (Fig. 1).

The peak intensity of the fluorescence emission spectra of 525 nm Qdot and FITC was well separated respectively (data not shown), hence Qdot could apply to detect *Salmonella* Enteritidis-target bacteria using by this new improved method.

Fig. 2 and Fig. 3 showed the successful attachment of the Qdot to the IMB-captured *Salmonella* Enteritidis cells. To eliminate the influence of background reflection/backscattering, the blank spectrum was subtracted from the sample spectrum. Fig. 2 shows the Qdot-fluorescence spectra of 0 to 10^7 CFU/mL *Salmonella*

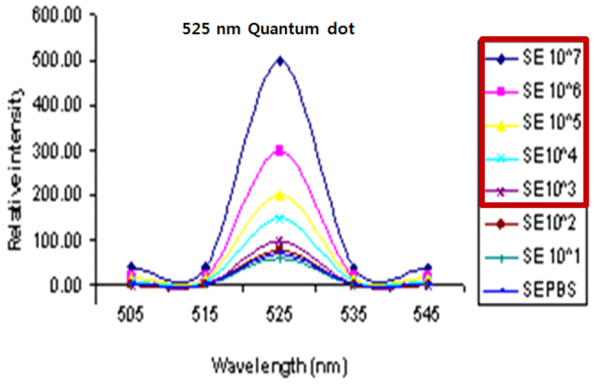

Fig. 2. Fluorescence spectra obtained for samples with different *Salmonella* Enteritis numbers in PBS at levels of 0 to 10^7 CFU/mL using by 525 nm Quantum dot

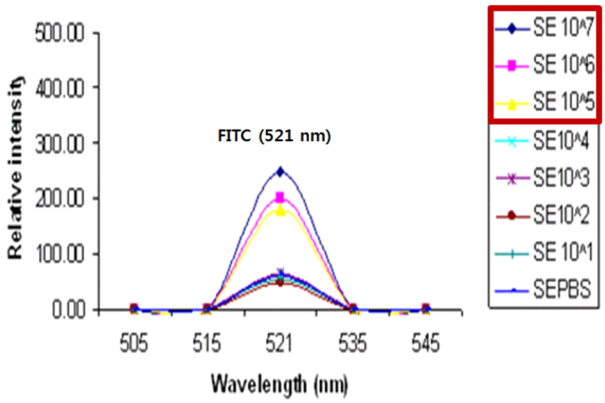

Fig. 3. Fluorescence spectra obtained for samples with different *Salmonella* Enteritis numbers in PBS at levels of 0 to 10^7 CFU/mL using by FITC (521 nm)

Enteritidis, and Fig. 3 shows the FITC-fluorescence spectra of 0 to 10^7 CFU/mL *Salmonella* Enteritidis, respectively. According to the result of Fig. 2, the Qdot-spectra of 10^3 – 10^7 CFU/mL all have a peak emission around 525 nm, but those of 0 – 10^2 CFU/mL do not. While Fig. 3 shows the FITC-fluorescence spectra of 0 to 10^7 CFU/mL *Salmonella* Enteritidis. Comparison of detection limitation between Qdot and FITC, the FITC-labeled complexes were detectable at a sample concentration of up to 10^5 CFU/mL (Fig. 2 and 3). Hence, the Qdot labeling-based method was at least 100 times more sensitive than the FITC method in detecting *Salmonella* Enteritidis ($P \leq 0.05$) (Fig. 2 and 3), and total detection time was less than from 1.5 h to 2 h. The result of this study showed the similar pattern from previous studies (Su and Li, 2004; Yang and Li, 2005 and 2006; Varshney *et al.*, 2007).

Next, no photobleaching phenomenon was observed after the Qdot-labeled immune-complexes were exposed to room light for 24 h (data not shown). Qdot were high-intensity photostable fluorophores that had several distinct advantages over standard organic fluorescent dyes (Ness *et al.*, 2003). Zhu *et al.* (2004) reported Qdot labeling exhibited better photostability and higher brightness than the two most commonly used commercial staining kits - A100DF AquaGlo Dual Fluorochrome Kit and KR1 Crypto-Cel IF test kit. And Yang and Li (2005 and 2006) reported the Zine sulfide-capped cadmium selendie quantume dots are 20 times as bright, 100 times as stable potobleaching, and one-third as wide in spectral linewidth compared with the organic dye rhodamine. The result of this study showed the similar pattern (data not shown). Hence, Qdot had the strong resistant against photobleaching than FITC in rate of fluorescence intensity at the storage of ambient temperature.

Until now, many scientists reported the usability of combination both Qdot and IMB to detect target-bacteria rapidly, easily, and accurately (Su and Li, 2004; Yang and Li, 2005 and 2006; Varshney *et al.*, 2007). Especially, they focused at low concentration of cell population, Seo *et al.* (1998) suggested one important finding that an excess of IMB would decrease the sensitivity of the final fluorescence detection. Hence, for improving the detection limit using by the IMS-Qdot method, it should be optimized (1) the concentrations of IMB, (2) biotin-conjugated antibodies, (3) Qdot, (4) incubation time so as to enhance the signal-to-background ratios at lower cell concentrations of less than 10^3 CFU/mL.

The IMS technique could easy-efficiently isolate cells from cell culture or bodily fluids (Seo *et al.*, 1998 and Su and Li, 2004). Furthermore, it could be used as a method for quantification of target-pathogens in food samples. Owing to this newly technique, antibodies coasted with paramagnetic beads could bind to antigens presenting on the surface cells. The beads strongly binding to target-pathogens could be recollected by passing them through an immunomagnetic separation unit (Su and Li, 2004). A new technique as IMS that could be used to separate target-pathogens or microorganisms, will reflect that initial concentration of each microorganisms present in food samples (Want *et al.*, 2007). Irrespective of size and composition, all of Qdot were observed to be easily conjugated with biomolecules by using a universal approach, and the emission properties of Qdot further facilitated the multicolor imaging of one cell labeled with different Qdot or different target cells labeled with different Qdot (Zhu *et al.*, 2004). It could be possible for multiplexing detection simultaneously using Qdot.

Hence, it is urgently need a further study that (1) could be modified for simultaneous detection of multiple pathogens using multicolor Qdot and multi-antibody-coated MB (ex. both *Cronobacter* spp. and *Salmonella* spp. from dried infant formula or dairy foods) and (2) could IMS to broaden the application of the real-time PCR in the detection of foodborne pathogens including *Cronobacter* spp. (former *Enterobacter sakazakii*) on various dairy foods. Also, Su and Li (2004) reported that the excitation source used was a blue LED; it excited the Qdot conjugates effectively and did not interfere with the fluorescence measurement, and also blue light did not kill cells. Hence, this system could be reasonable for the research of live cells. This method could be replaced the traditional way (ex. Ethidium bromide (EtBr), etc) to count live cell, therefore, further research is needed to distinguish live cell from death cell.

CONCLUSION

This study demonstrated a sensitive, specific, and rapid method for the detection of *Salmonella* Enteritidis based on the combination of Qdot biolabeling with IMS. The IMS-Qdot fluorescence method could detect 10^3 – 10^7 CFU/mL of *Salmonella* Enteritidis within 2 hours, and almost 100 times lower than the FITC method. The concept described could be modified for simultaneous detection of multiple pathogens using multicolor Qdot and multi-antibody-coated MB from various dairy foods. The photostability of Qdot was stronger than one of FITC. Furthermore,

this technique could be developed as a promising method for a simultaneous in situ monitoring of various proteins with specific antibodies labeled with fluorescence probes of different colors. Hence, it needs to develop the new rapid method for detecting single or multiplex food-borne pathogenic live bacteria using Qdot on various dairy foods or complex food matrix.

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