

## Research Note

# Quantum Dots as Fluorescent Labels for Quantitative Detection of *Salmonella* Typhimurium in Chicken Carcass Wash Water

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### ABSTRACT

Fluorescent semiconductor quantum dots have recently emerged as a novel and promising class of fluorescent labels for biological detection. In this study, quantum dots were used as fluorescent labels in immunoassays for quantitative detection of foodborne pathogenic bacteria. *Salmonella* Typhimurium cells were separated from chicken carcass wash water using anti-*Salmonella* antibody coated magnetic beads and reacted to secondary biotin-labeled anti-*Salmonella* antibody. Quantum dots coated with streptavidin were added to react with biotin on the secondary antibody. Measurement of the intensity of fluorescence produced by quantum dots provided a quantitative method for microbial detection. A linear relationship between *Salmonella* Typhimurium cell number ( $\log N$ ) in the samples of chicken carcass wash water and the fluorescence intensity (FI) was found for the cell numbers ranging from  $10^3$  to  $10^7$  CFU/ml. The regression model can be expressed as  $FI = 198.6 \log N - 639.03$  with  $R^2 = 0.96$ . The detection limit of this method was  $10^3$  CFU/ml.

The development of rapid and sensitive methods for detection of foodborne pathogenic bacteria remains a challenging and important issue for ensuring food safety and security. Immunoassays are one class of rapid methods that have been extensively investigated for detection of bacteria in food, environmental and clinic samples. Most immunological methods are based on optical detection of fluorescent label molecules (6, 17) or enzyme-linked absorbent and luminescent products (11, 18). Typical fluorescence dyes used in immunoassays are susceptible to photobleaching and have broad excitation and emission spectra. Most methods based on fluorescence detection usually involve image analysis and are difficult to quantify.

Fluorescent semiconductor quantum dots (QDs) have recently emerged as a novel and promising class of fluorescent labels for biological detection. QDs have several advantages over conventional fluorophores, including resistance to photodegradation, improved brightness, and size-dependent, narrow-emission spectra (3, 13). To make QDs suitable for biological detection, water-soluble and biomolecule-conjugated QDs have been pursued. Desirable biological molecules can be coupled to QDs and retain their biological activities, which leads to the development of molecular conjugates of QDs that are biocompatible and suitable for use in cell biology and immunoassays. At present, most QD applications for biological detection are limited to cellular imaging and staining (2, 7, 10, 13, 19). For example, Bruchez et al. (2) demonstrated the use of QDs as fluorescent labels in a dual-emission, single-excitation la-

beling experiment on mouse fibroblasts, indicating that QDs are complementary and, in some cases, might be superior to existing fluorophores. Dubertret et al. (7) and Larson et al. (10) reported the use of QDs for in vivo imaging of tissues. However, the application of QDs for microorganism detection is rarely reported. Zhu et al. (21) reported a dual color imaging method that used two different QDs as labels for two species of microbial cells, *Cryptosporidium parvum* and *Giardia lamblia*. Our recent study demonstrated that the use of QDs as fluorescent labels for detection of *Escherichia coli* O157:H7 achieved a detection limit of  $10^3$  CFU/ml, which was 100 times lower than that obtained with fluorescein isothiocyanate (FITC) as the fluorescent label (16).

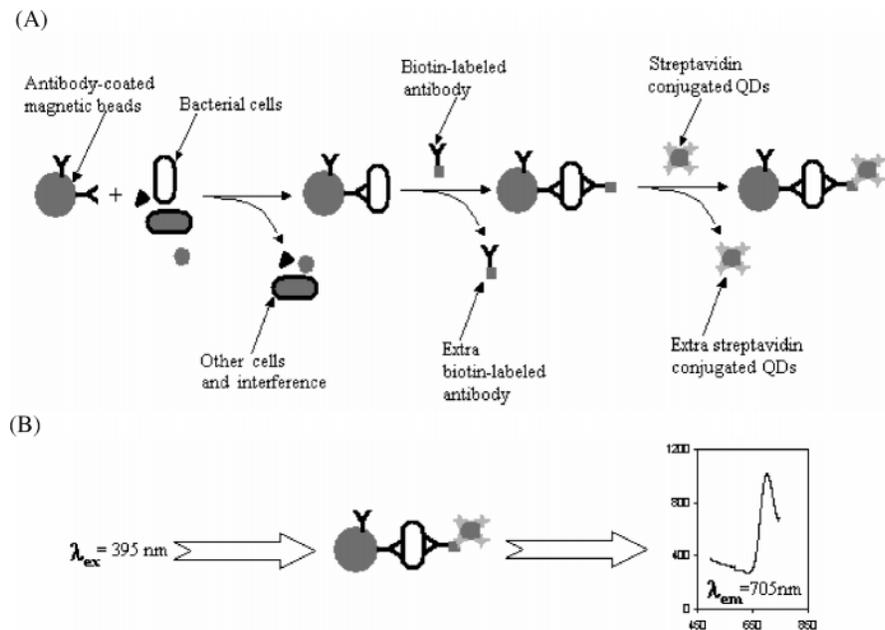
In this study, semiconductor QDs were used as a fluorescent label in immunoassays for quantitative detection of foodborne pathogenic bacteria. *Salmonella* Typhimurium was used as an example of target bacteria. *Salmonella* Typhimurium cells were separated from the samples of chicken carcass wash water with anti-*Salmonella* antibody-coated magnetic beads and reacted to secondary biotin-labeled anti-*Salmonella* antibody. Streptavidin-conjugated QDs were added to react with biotin on the secondary antibody. Measurement of the intensity of fluorescence produced by QDs provided a quantitative method for microbial detection. Detection of *Salmonella* in food samples of chicken wash water was also investigated.

### MATERIALS AND METHODS

**Chemicals and biochemicals.** Phosphate-buffered saline (PBS; 0.01 M, pH 7.4) was purchased from Sigma-Aldrich (St.

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FIGURE 1. (A) Schematic diagram of the detection procedure. Anti-Salmonella antibody-coated magnetic beads were used to capture Salmonella cells from the samples. The biotin-conjugated secondary anti-Salmonella antibodies were added to react with the captured Salmonella cells. Streptavidin-conjugated QDs were used as fluorescence labels through the reaction between biotin and streptavidin. (B) The bacterial cell numbers were determined by the measurement of fluorescence intensity produced by bound QDs.



Louis, Mo.). Biotinylated rabbit anti-*Salmonella* antibody (4 to 5 mg/ml) was obtained from Biodesign International (Saco, Maine). A 1:10 dilution of anti-*Salmonella* antibody was prepared with PBS (pH 7.4) for further use. Magnetic beads coated with rabbit anti-*Salmonella* antibody were from Dynal, Inc. (Lake Success, N.Y.). Qdot 705 streptavidin conjugate (2  $\mu\text{M}$ ) was purchased from Quantum Dot Corporation (Hayward, Calif.). All solutions were prepared with deionized water from Millipore (Milli-Q, 18.2 M $\Omega$ -cm, Bedford, Mass.), except when otherwise stated.

**Bacterial cultures and surface plating methods.** Stock cultures of *Salmonella* Typhimurium (ATCC 14028) and *E. coli* O157:H7 (ATCC 43888) were obtained from American Type Culture Collection (Manassas, Va.). *Listeria monocytogenes* (FDA, 101M 4B) was from U.S. Food and Drug Administration (FDA). Cultures were grown for 18 to 20 h at 37°C in brain heart infusion broth (Remel, Lenexa, Kans.). Serial 10-fold dilutions were made in physiological saline solution. The viable cell numbers of *Salmonella* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* were determined by surface plating 0.1 ml of the appropriate dilutions onto XLT4 agar (Remel), MacConkey sorbitol agar (Remel), and Oxford listeria selective agar (Oxoid, Basingstoke, Hampshire, UK), respectively. Colonies were counted after incubation at 37°C for 24 or 48 h (for *Listeria*). The cultures were killed by boiling them in a water bath for 20 min for further use. Pure culture samples were made by diluting *Salmonella* culture in PBS (pH 7.4) to obtain serial 1:10 dilutions with different concentrations.

**Chicken carcass wash water samples.** Chicken carcasses (24) were obtained from a poultry processing plant. Each carcass was put into a Whirl-plastic bag (Nasco, Fort Atkinson, Wis.) containing 100 ml of 0.1% buffered peptone water (Difco, Kansas City, Mo.) and mechanically shaken for 2 min. Carcass wash water from each bird was mixed together for the experiment. The carcass wash water was inoculated with *Salmonella* Typhimurium at different concentrations to make serial samples with the same volume of 5 ml. Each sample was filtered with a syringe filter (pore size, 5  $\mu\text{m}$ ; MSI, Westboro, Mass.) to remove solid components. Then 1 ml of the filtered solution was centrifuged by VSMC-13 minicentrifuge (Shelton Scientific, Shelton, Conn.) at 10,000  $\times g$  for 15 min to separate *Salmonella* cells from other

components, such as protein, plasma, etc. After removing the supernatant, 1 ml of PBS (pH 7.4) was added to the tube to resuspend *Salmonella* cells.

**Assay procedure.** The entire assay procedure is outlined in Figure 1. First, *Salmonella* cells were captured by magnetic beads coated with rabbit anti-*Salmonella* antibody and separated from the sample matrix. In this step, 20  $\mu\text{l}$  of immunomagnetic beads were added to 1 ml of *Salmonella* sample in a microcentrifuge tube. The mixture was shaken on a RKVSD 10101 mixer (ART Inc., Laurel, Md.) at a speed of 10 rpm for 1 h at room temperature. The bead-cell conjugates were separated from the solution by putting the tube on a magnetic separator for 2 min. Liquid was removed with a syringe. The conjugates were resuspended with 0.3 ml PBS (pH 7.4) after they were washed with 0.5 ml PBS (pH 7.4). Second, biotinylated secondary anti-*Salmonella* antibodies were linked to the above bead-cell conjugates to form bead-cell-biotinylated antibody “sandwich” conjugates. To perform this step, 30  $\mu\text{l}$  of 0.4 to 0.5 mg/ml biotinylated anti-*Salmonella* antibody in PBS was added to the conjugates. After 1 h of reaction time, the sandwich conjugates were separated from the solution with the magnetic separator again. Liquid was removed by a syringe. The sandwich conjugates were washed with 0.5 ml of PBS (pH 7.4) twice. Finally, 200  $\mu\text{l}$  of 10-nm streptavidin-coated Qdot 705 was added to the tube and incubated at room temperature for 30 min. Qdot 705 attached to the bacterial cell through the reaction between streptavidin on the QDs and biotin on the secondary antibodies. After removing excess QD solution, the final conjugates were washed with PBS (pH 7.4) twice and resuspended with 150  $\mu\text{l}$  of PBS (pH 7.4). The fluorescence intensity produced by these QDs was measured.

**Fluorescence measurement.** The fluorescence measurement was performed with a laptop-controlled portable system that included a USB2000 miniature fiber optic spectrometer, an LS-450 LED light source module, an R400-7 UV-vis optical probe (Ocean Optics Inc., Dunedin, Fla.), and a probe and cuvette holder housed in a dark box. The spectrometer contained a low-cost 2,048-element linear CCD array detector with a working range from 360 to 900 nm. The LED module contained a 395-nm wavelength VIS LED in place of the 470-nm bulb in the LS-450. The optical probe comprised a tight bundle of seven optical fibers (400  $\mu\text{m}$  in di-

TABLE 1. Binding efficiency of anti-Salmonella antibody-coated magnetic beads to *Salmonella Typhimurium* cells in PBS, pH 7.4

Sample	<i>Salmonella</i> cell number (CFU/ml) <sup>a</sup>		Binding efficacy (%)
	Supernatant		
$3.04 \times 10^3$	$2.07 \times 10^3$		31.9
$3.04 \times 10^4$	$2.31 \times 10^4$		24.0
$3.04 \times 10^4$	$2.30 \times 10^5$		24.3
$3.04 \times 10^6$	$2.20 \times 10^6$		27.6
$3.35 \times 10^4$ (with $1.95 \times 10^3$ <i>E. coli</i> O157:H7 and $2.64 \times 10^4$ <i>L. monocytogenes</i> )	$2.40 \times 10^4$		28.4

<sup>a</sup> Cell numbers were the average of colony numbers from three plates.

ameter for each fiber) in a stainless steel ferrule with six illumination fibers around one reading fiber.

**Microscopy.** QDs were linked with antibodies to form antibody-conjugated QDs. In detail, 100  $\mu$ l of QDs (1:10 dilution) were first incubated with 10  $\mu$ l of biotinylated anti-*Salmonella* antibody (1:10 dilution) for 30 min. Five microliters of *Salmonella Typhimurium* culture ( $10^9$  CFU/ml) was spotted onto a glass slide and heat dried. The fixed cells were washed with water and dried with air. They were then incubated with 10  $\mu$ l of antibody-conjugated QDs for 30 min at room temperature. After washing with water, the slide was observed under a Zeiss Axioplan II imaging microscope (Carl Zeiss, Jena, Germany) equipped with an ORCA-ER digital camera (Compix Inc., Cranberry Township, Pa.).

## RESULTS AND DISCUSSION

**Capture efficiency of magnetic beads for *Salmonella Typhimurium*.** Immunomagnetic separation has been recognized as an effective method of separating target pathogens from food samples and competing organisms and has been coupled with conventional plating (15), flow cytometry (14), immunoassay (1), electrochemical (4, 9, 20), chemiluminescence (18), and conductance (12) methods for rapid detection of bacteria in food samples. To assess the binding efficiency of the magnetic beads to target bacteria, samples with different cell numbers of *Salmonella* were tested. Table 1 shows the binding efficiency of immunomagnetic beads for separation of viable *Salmonella Typhimurium* in pure and mixed culture. Percent binding efficiency data were based on loss to the supernatant. The results indicated that the binding efficiency of immunomagnetic beads was between 24 and 31.9% in bacterial cell numbers of  $10^3$  to  $10^6$  CFU/ml for the separation of *Salmonella* in pure culture.

The binding efficiency of anti-*Salmonella* antibody-coated immunomagnetic beads for a mixed culture of  $3.35 \times 10^4$  CFU/ml *Salmonella Typhimurium* with  $1.95 \times 10^3$  CFU/ml *E. coli* O157:H7 and  $2.64 \times 10^4$  CFU/ml of *L. monocytogenes* was 28.4%, which is close to the binding efficiency for pure *Salmonella* culture. This result shows that *E. coli* O157:H7 and *L. monocytogenes* cells did not interfere with the capture of *Salmonella* cells by anti-*Salmonella* antibody-coated immunomagnetic beads, suggesting this magnetic separation can be used for capture of *Sal-*



FIGURE 2. The image of Qdot 705-labeled *Salmonella Typhimurium* cells.

*monella Typhimurium* from a sample containing multiple bacterial species.

The capture efficiency in this study (24 to 32%) was lower compared with other studies (86% for  $10^3$  CFU/ml *Salmonella* (8)), but it was very stable for different concentrations of bacterial cells. This stable binding efficiency is critical to obtain a linear correlation of cell number with fluorescence intensity. However, this is inconsistent with other reports stating that the capture efficiency (86 to 15.7%) decreased significantly with increasing cell concentrations ( $10^3$  to  $10^8$  CFU/ml (8)), probably because of the increased cell-to-bead ratio. In this study, percent binding efficiency was calculated on the basis of loss of cells to the supernatant, which should be more accurate considering that plating of cell-bead complexes could result in clumps of cells that grow as a single CFU on the plate.

**Detection of *Salmonella Typhimurium* in pure culture.** Figure 2 displays the fluorescent image of Qdot 705-labeled *Salmonella Typhimurium* cells. This image indicates that Qdot 705-labeled cells produced red fluorescence under an excitation source at 545 nm. This image also demonstrates that streptavidin-conjugated QDs could be evenly and completely attached to the bacterial cells through the use of biotin-labeled secondary antibodies while retaining their fluorescence efficacy.

Figure 3 shows the results of one experiment to enumerate cell numbers of *Salmonella Typhimurium* in pure culture. The background gave a fluorescent peak at 705 nm with an intensity of 166 counts. With  $2.9 \times 10^3$  CFU/ml *Salmonella* cells, the sample produced a fluorescent peak with an intensity of 224 counts, which is significantly different from the background signal. It also can be seen that the fluorescence intensity increased with increasing cell number of *Salmonella* from  $2.9 \times 10^3$  to  $2.9 \times 10^7$  CFU/ml. This result demonstrated that the more bacterial cells in the sample, the greater the amount of QDs they could bind and thus the stronger the fluorescence they could produce.

It is interesting to know the loading of QDs per bacterial cell. The number of QDs bound to one bacterial cell could be estimated on the basis of the measurement of fluorescence intensity. One hundred fifty microliters of 0.5 nM QD solution produced a fluorescence intensity of 299 counts, which is close to the fluorescence signal (291 counts) produced by a sample with  $2.9 \times 10^4$  CFU/ml *Sal-*

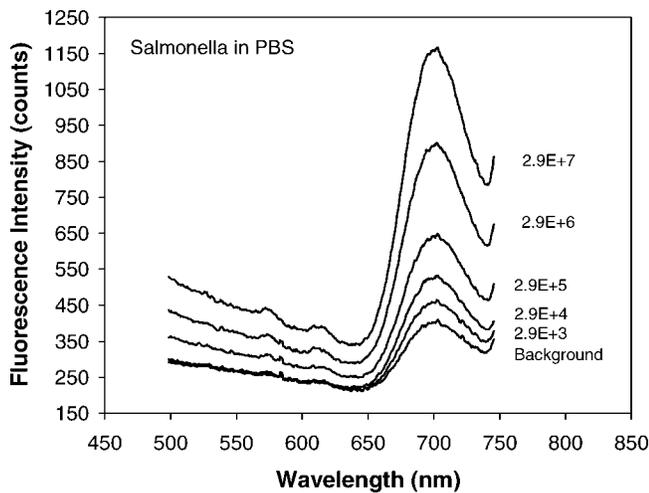


FIGURE 3. Representative fluorescence spectra obtained for samples with different *Salmonella* numbers in PBS at levels of  $2.9 \times 10^3$ ,  $2.9 \times 10^4$ ,  $2.9 \times 10^5$ ,  $2.9 \times 10^6$ , and  $2.9 \times 10^7$  CFU/ml.

*monella* cells. Taking 25% as the binding efficiency of magnetic beads, the final cell number captured by the beads is  $7.25 \times 10^3$  cells. The number of QDs bound to this number of bacterial cells is the same as the number of QDs in 150  $\mu$ l of 0.5 nM QD solution, which is  $4.5 \times 10^{10}$ . Therefore, each bacterial cell is estimated to accept  $6.2 \times 10^6$  QDs.

A linear relationship between *Salmonella* Typhimurium cell number ( $N$ ) in the samples and the fluorescence intensity (FI) was found for cell concentrations ranging from  $10^4$  to  $10^7$  CFU/ml (Fig. 4). The regression model can be expressed as  $FI = 166.07 \log N - 447.81$ , with  $R^2 = 0.99$ . Three measurements of the background signal gave an average at 163 counts with a standard deviation of 7.6 counts. When the background signal plus three times its standard deviation was taken as the threshold of the signal, the detection limit of this method was  $10^3$  CFU/ml.

**Detection of *Salmonella* in chicken carcass wash water.** Figure 5 shows the relationship between fluorescence intensity and *Salmonella* Typhimurium cell numbers in the samples of chicken carcass wash water with and without the presence of *L. monocytogenes* and *E. coli* O157:H7. For the samples containing *Salmonella* only, the fluorescence intensity increased with increasing *Salmonella* cell number in the range from  $2.9 \times 10^3$  to  $2.9 \times 10^7$  CFU/ml. The regression model can be expressed as:  $FI = 198.6 \log N - 639.03$ , with  $R^2 = 0.96$ . This trend is similar to the signal responses to *Salmonella* in PBS solution. The blank sample containing no *Salmonella* produced an average signal of 63 counts with a standard deviation of 18, which was lower than the background signal of the blank sample in PBS (163 counts). This low background signal could be caused by some proteins in the chicken carcass wash water that might reduce nonspecific bindings between antibodies on magnetic beads and secondary antibodies in the solution. The sample containing  $2.3 \times 10^4$  CFU/ml of *L. monocytogenes* and  $3.5 \times 10^3$  CFU/ml of *E. coli* O157:H7 produced a fluorescent signal of 45 counts, which was close to the background signal of 63 counts, indicating that the presence

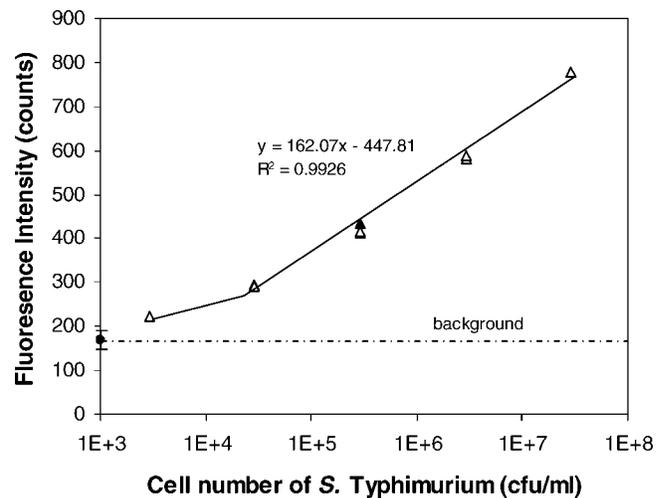


FIGURE 4. The linear relationship between the logarithmic values of *Salmonella* cell numbers in PBS and the fluorescence intensity in the cell number, ranging from  $2.9 \times 10^3$  to  $2.9 \times 10^7$  CFU/ml. Error bar on background line is  $\pm 3$  SD ( $n = 3$ ).

of these nontarget bacteria did not increase nonspecific binding. Samples containing the same numbers of *L. monocytogenes* and *E. coli* O157:H7 but with a different cell number of *Salmonella* Typhimurium presented increasing fluorescence signal with increasing cell number of *Salmonella*. At each level of *Salmonella* cell number ( $2.9 \times 10^3$ ,  $2.9 \times 10^4$ ,  $2.9 \times 10^5$ ,  $2.9 \times 10^6$ , and  $2.9 \times 10^7$  CFU/ml), samples with and without *L. monocytogenes* and *E. coli* O157:H7 produced a similar fluorescence intensity, indicating that nontarget bacteria did not interfere with the detection of *Salmonella* Typhimurium in chicken carcass wash water.

Although QDs have been reported as a novel class of fluorescent markers that have the potential to revolutionize biological imaging, little is reported on the use of QDs as

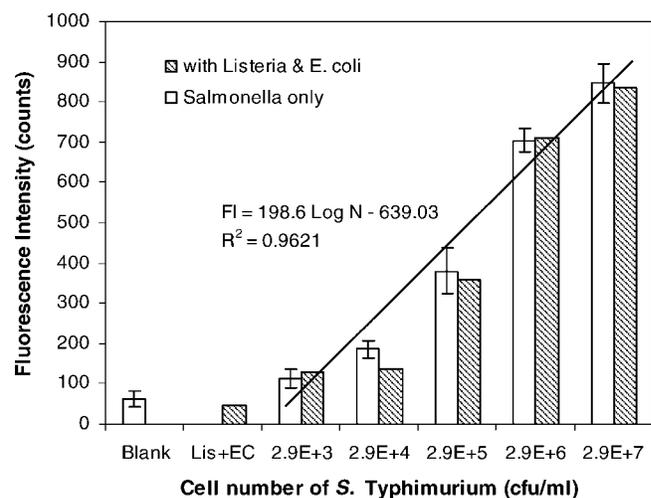


FIGURE 5. Relationship between the logarithmic values of *Salmonella* cell numbers in chicken carcass wash water and fluorescence intensity in the cell number, ranging from  $2.9 \times 10^3$  to  $2.9 \times 10^7$  CFU/ml with and without the presence of  $3.5 \times 10^3$  CFU/ml of *E. coli* O157:H7 and  $2.3 \times 10^4$  CFU/ml *L. monocytogenes*. Error bars indicate  $\pm$ SD ( $n = 3$ ).

labels for quantitative analysis of bacteria. Our results indicated that the optical properties of QDs remained unchanged after they were attached to bacteria cells with biotin-labeled secondary antibodies, and their strong fluorescence intensity makes the quantitative detection of bacterial cells possible by fluorescence measurement. This study demonstrated the use of QDs as fluorescence labels for detection of *Salmonella* in pure culture and chicken carcass wash water.

The detection limit of  $10^3$  CFU/ml in this study is comparable to most rapid methods that use magnetic separation investigated in recent years. For example, methods based on immunomagnetic separation coupled with electrochemical enzyme electrodes (5, 10) and enzyme-linked immunomagnetic electrochemical detection (9) achieved detection limits of  $10^3$  CFU/ml for *Salmonella* Typhimurium. The use of QDs as labels also simplified the detection procedure by avoiding the use of substrate and the enzymatic reaction compared with the enzyme-linked methods. The performance of this method could be improved with the use of immunomagnetic beads with high capture efficiency and by optimizing the concentrations of biotin-labeled antibodies and QDs and incubation time.

The principle of the use of QDs as fluorescent labels could be applied to the detection of any other foodborne pathogenic bacteria by conjugating QDs with desirable antibodies that are specific to target bacterial cells. In addition, QDs with different sizes can be excited with a single wavelength of light, resulting in different emission peaks that can be detected simultaneously. This property could lead to a method for detecting multiple bacteria in one sample with the use of different QDs as fluorescent labels to each species of bacterial cell.

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