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Short Communication

Detection of *Escherichia coli* O157:H7 and *Salmonella* in ground beef by a bead-free quantum dot-facilitated isolation method

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ABSTRACT

The aims of this study were to introduce a new immunological bead-free cell detection method using quantum dots (QDs) as reporter markers for foodborne pathogen detection. QDs are nanosized particles with long-term photostability, high quantum yield, broad absorption spectra, and narrow, symmetric emission and high signal-to-noise ratio. The chemical compound [(1-ethyl-3-3-dimethylaminopropyl) carbodiimide hydrochloride] (EDC) and protein A were used as crosslinkers for manufacturing QD–antibody conjugates. To minimize the inhibition of QD fluorescence by the magnetic beads, the beads were removed after the primary pathogen isolation and before fluorescence measurement. Detection signals were increased four-fold after employing the bead-free isolation method. With a 24-h enrichment, the bead-free QD-facilitated detection method was able to detect 10 CFU/g *Escherichia coli* O157:H7 and *Salmonella* from artificially contaminated ground beef. To our knowledge, this detection method is the first research that combined a new EDC–protein A QD-labeling technique and bead-free fluorescence measurement to detect *E. coli* O157:H7 and *Salmonella* in ground beef.

1. Introduction

In the United States, the latest estimates indicate that one in six or 48 million people are stricken with a foodborne illness each year, resulting in 3000 deaths (CDC, 2011). Although products such as fruits and vegetables are increasingly being implicated in foodborne illnesses related to *Escherichia coli* O157:H7 and *Salmonella*, red meat and poultry remain the predominant products contributing to outbreaks and recalls (Juneja et al., 2009). Accurate and rapid detection of foodborne pathogens is a critical component of the process to reduce risks associated with pathogen-contaminated foods.

Quantum dots (QDs) are a family of nanosized particles comprised of a few thousand atoms, with typical sizes of 1–10 nm in radius. QDs exhibit a size tunable band gap and, hence, fluorescence spectrum, allowing for different colors to be exhibited at one excitation wavelength. Compared with conventional fluorescent dyes, QDs have long-term photostability, high quantum yield, broad absorption spectra, narrow, symmetric emission and high signal-to-noise ratios (Duong and Rhee, 2007; Xue et al., 2009). QDs, as fluorescent markers, have been broadly applied in biological studies, such as cell imaging (Hirschey et al., 2006) and DNA labeling (Wu et al., 2006). Colloidally synthesized CdSe/ZnS (core/shell) QDs are the best understood of all QDs. To use QDs in biological studies, crosslinkers, such as the "biotin-avidin" system, have been used to conjugate them to different biological components.

Immunomagnetic separation (IMS) is a cell isolation/separation method that employs antibody-coated magnetic beads. This method has been applied in combination with detection tools, such as culture-based methods (Wright et al., 1994), ELISA (Cudjoe et al., 1995) and PCR methods (Jeníková et al., 2000; Wang et al., 2007). Successful applications have been shown for detection of *E. coli* O157:H7 pure cultures (Su and Li, 2004), *Salmonella* serovar Typhimurium from chicken carcass wash water (Yang and Li, 2005), and *S.* ser. Typhimurium, *Shigella flexneri* and *E. coli* O157:H7 from apple juice and milk (Zhao et al., 2009). However, a major problem in using immunomagnetic beads for fluorescence immunoassays is that the autofluorescence emitted by the magnetic beads strongly interferes with the target detection signal (Agrawal et al., 2007). Thus, one of the aims of this study was to incorporate a bead removal step after the primary cell isolation and prior to the cell detection step in order to evaluate if the challenges associated with traditional IMS could be overcome.

The avidin-biotin conjugation system is one of the most widely used crosslinking systems applied in biological compound conjugation. The interaction between biotin and avidin is among the strongest non-covalent affinities, with a dissociation constant of approximately 1.3×10^{-15} M. However, a disadvantage of using avidin is that this molecule has the potential to bind nonspecifically to components other than biotin due to its high pI and carbohydrate content. The nonspecific

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interactions can lead to increased background signals (Hermanson, 1996). Thus, to improve the detection sensitivity and avoid background signals, a zero-length crosslinker, EDC, short for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, was used in this study due to its following advantages: (1) amide bonds formed with an EDC crosslinking reaction can provide neutral linkages, (2) excess reagent and crosslinking by-products can be easily removed by washing with water or diluted acid, and (3) EDC is water soluble and the crosslinking reaction can be accomplished in physiological solutions without the addition of organic solvents.

In addition to EDC, protein A was used as a secondary crosslinker to conjugate antibodies in this study. It can bind the Fc region of immunoglobulins through interactions with the heavy chain, while the EDC acts to join the amine group from protein A and the carboxylate group from QDs. A significant advantage of using the EDC–protein A crosslinker instead of the traditional biotin-avidin system is that the former allows conjugation of antibodies on the final antibody–QD conjugates to occur in a uniform format with all antigen binding regions facing the outside of the molecule.

The objectives of this study were to, first, design robust, more cost efficient and specific QD–antibody conjugates by applying EDC and protein A as crosslinkers, and second, to enhance the detection sensitivity of the QD-based system by eliminating bead interference in fluorescence detection.

2. Materials and methods

2.1. Fabrication of water soluble CdSe/ZnS QDs

QDs were fabricated at the Biophotonic Laboratory of the University of Missouri, Columbia. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Four grams of 99% trioctylphosphine oxide (TOPO), 4 g of hexadecylamine, 30 mg of cadmium oxide (CdO) and 0.6 g lauric acid were mixed and heated to 280 °C. Selenium (180 mg in 2 mL trioctylphosphine stock solution) was then injected into the mixture at 240 °C or 300 °C to produce green and orange QD core structures, respectively. To increase the fluorescence and stability of freshly fabricated QDs, shell structures consisting of layers of ZnS were added. The temperature of the three-neck reaction flask was set to 80 °C, and diethylzinc (1.0 M in hexanes) and hexamethyldisilathiane $(TMS)_2S$ stock mixtures were added to form the QD shell layers.

Methods for making the QDs water soluble were adapted from Mattoussi et al. (2000, 2001). QDs dissolved in chloroform were washed with methanol to remove residual TOPO and transferred into a pre-weighed vial. The chloroform was evaporated, leaving the QDs as a dry powder. A mixture of 1 mL dihydrolipoic acid (DHLA) and 0.5 mL methanol was added to 80 mg of dry QDs. The vial was sealed and heated on a hotplate at 80 °C for 8 h with vigorous stirring until the QDs were completely dissolved. An additional 0.5 mL 5 M aqueous sodium and 3 mL dimethylformamide were added to the QD solution and mixed well. Water soluble QDs were collected and purified by centrifugation, re-suspension in sodium phosphate buffer (PBS, pH 7.0) and filtration through a 0.2 μm syringe filter (Whatman, Piscataway, New Jersey, USA).

2.2. Construction of QD–antibody conjugates

The concentration of water soluble QDs was 120 nmol/L based on an instrumental neutron activation analysis (INAA) performed at the Chemistry Department of the University of Missouri, Columbia. To manufacture the QD–antibody conjugates, 10 mg of EDC was first dissolved in 0.5 mL 2-(N-morpholino) ethanesulfonic acid (MES) to make a 20 mg/mL EDC stock solution. The antibody–QD conjugation process started with the addition of various volumes of 20 mg/mL EDC and different amounts of 0.5 mg/mL protein A to 100 μL QDs to form a QD–EDC–protein A complex.

E. coli O157:H7 and *Salmonella* monoclonal antibodies were purchased from Genway (San Diego, California, USA). The final QD–antibody conjugates used for the detection of pathogens were made by adding 25 μL (for green QDs) or 28 μL (for orange QDs) of the 20 mg/mL EDC stock solution and 1.5 mg protein A to each 100 μL of green or orange QDs. The mixture was then incubated at 4 °C for 2 h, after which time, 100 μL *E. coli* O157:H7 antibodies or *Salmonella* antibodies (0.5 mg/mL stock concentration) were added. This QD–EDC–protein A–antibody complex was incubated at 4 °C for an additional 2 h. The structure of the QD–antibody conjugate is shown in Fig. 1 insert.

2.3. Preparation of pure cultures

Two replications of 10-mL overnight *Salmonella* and *E. coli* O157:H7 pure cultures were diluted in 0.1% peptone water to reach concentrations of 10–10^8 CFU/mL. Diluted cultures were plated on tryptic soy agar (Difco Labs., BD Diagnostic Systems, Sparks, Maryland, USA) for enumeration. One milliliter of each dilution was transferred into a 1.5 mL centrifuge tube for further testing.

2.4. Preparation of artificially inoculated ground beef samples

Ground beef samples were obtained from the Meat Laboratory of the University of Missouri, Columbia. Samples were tested by standard culture methods according to the Bacteriological Analytical Manual (FDA, 1995) and confirmed to be free of *E. coli* O157:H7 and *Salmonella* before usage. Two replications of two sets of eight 25-g artificially contaminated ground beef were prepared by inoculating the beef with pure overnight cultures to reach final concentrations of 10^1–10^8 CFU/g of each pathogen among each set of samples. Briefly, overnight bacterial cultures were diluted in 0.1% peptone water to reach concentrations of 10^1–10^9 CFU/mL. For each 25-g of ground beef, 2.5 mL of each dilution of bacterial cultures was added and the meats were massaged for 2 min by hand for evenly distributing the cells.

2.5. Bead-free cell isolation

DSB-X biotin protein labeling kit (D-20655) from Invitrogen (Molecular Probes, Eugene, Oregon, USA) was used to add a DSB-X biotin ligand to *E. coli* O157:H7 or *Salmonella* antibodies. The bead-free cell isolation method started with the addition of 25 μL of these biotin-labeled antibodies to each 1 mL cell or beef suspension. The mixture was incubated at 4 °C for 30 min and 75 μL of FlowComp Dynabeads was added, followed by an additional 30 min of incubation at 4 °C.

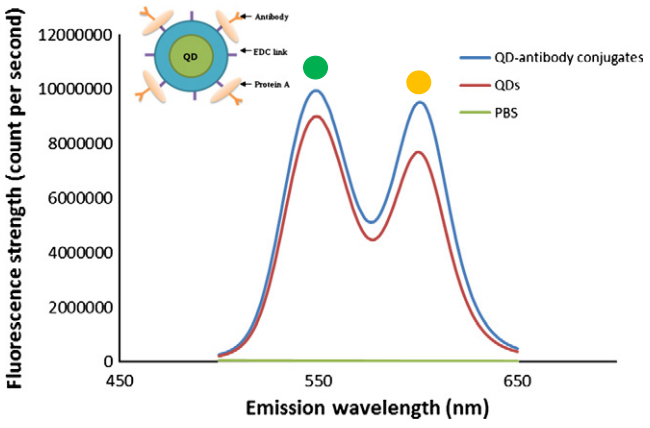

Fig. 1. Fluorescence comparison between QDs and QD–antibody conjugates. Green QDs (green dot) exhibited an emission wavelength of 550 nm while that of orange QDs (orange dot) was 600 nm. (Insert: structure of QD–antibody conjugates with EDC–protein A crosslinker).

Tubes containing cells, DSB-X labeled antibodies and FlowComp Dynabeads were placed on a magnetic particle concentrator (Dynal MPC, Dynal Biotech., Oslo, Norway) for 5 min and the Dynabead-antibody-cell complexes were attracted to the side of the tubes. The complexes were washed once with PBS (pH 7.0) on the magnetic separator. One milliliter of FlowComp release buffer was then added and mixed by rolling and tilting the tube for 20 min at room temperature. Tubes were placed back on the magnetic separator to remove the beads and the suspension containing bead-free cells were recovered and transferred to new tubes.

2.6. Detection of isolated bead-free cells by QD–antibody conjugates

Twenty-five microliters of QD-labeled *E. coli* O157:H7 antibodies or 25 μL QD-labeled *Salmonella* antibodies were added to the bead-free pathogenic cells. Tubes were incubated at 4 °C for 30 min to allow the QD-labeled antibodies to attach to target cells. After 30 min, cells were centrifuged at 12,000 × g for 5 min and washed twice with PBS (pH 7.0). Cell pellets were resuspended in 100 μL PBS and fluorescence was determined using a FluoroMax-3 spectrophotometer (Jobin Yvon, Longjumeau, France) at the excitation wavelength of 350 nm, with a 7 nm slit sitting for excitation light and emission measurement. The integration time was 0.5 s.

2.7. Comparison between the bead-free cell isolation method with IMS cell isolation method

To compare the detection efficiency between the developed bead-free isolation method and traditional IMS method, two separated trials of *E. coli* O157:H7 pure culture samples were prepared and used. In each trial, two tubes of 1 mL 10^8 CFU/mL *E. coli* O157:H7 cells and one tube of water (negative control) were prepared and the cells were washed once with PBS by centrifugation. One tube was processed following the bead-free isolation procedure, while the second tube was processed by the traditional IMS method with the magnetic beads left on the cells. QD-labeled *E. coli* O157:H7 antibodies were then added and fluorescence was measured as described above.

2.8. Isolation and detection of cells from ground beef

To recover the cells from the artificially contaminated ground beef, 250 mL of 0.1% peptone water were first added to each 25 g of artificially contaminated ground beef and the mixtures homogenized by stomaching for 2 min in stomacher. One milliliter suspension was pipetted out and centrifuged at 12,000 × g for 5 min. The pellets were washed twice and resuspended in 1 mL isolation buffer, which was made of Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), 0.1% BSA and 2 mM EDTA. These resuspended samples were then used for cell isolation and detection as described above.

2.9. Enrichment for detecting pathogens in ground beef

Ground beef samples were inoculated with *E. coli* O157:H7 or *Salmonella* at concentrations of 0, 10 and 10^8 CFU/g. Tryptic soy broth (225 mL) was added to each 25-g of artificially contaminated ground beef and the mixtures homogenized for 2 min in a stomacher. Samples were incubated at 35 °C for 24 h before cell isolation.

3. Results

3.1. Antibody–QD conjugation

With confirmation by both gel electrophoresis and microscopy (data not shown), the volume ratio of 1:30 (QD:protein A) was chosen to manufacture the QD–EDC–protein A complexes. Fluorescence emitted by pure QDs and by QD–antibody conjugates was compared and the emission curves examined. The conjugation process did not change the emission efficiency of the QDs and slightly enhanced the emission from QDs (Fig. 1).

3.2. Comparison between the bead-free cell isolation method with IMS cell isolation method

Fluorescence generated from cells isolated by the bead-free method was about four-fold higher than that emitted by cells isolated by traditional IMS (Fig. 2). The bead-free isolation method dramatically increased the fluorescence signal and demonstrated the potential to be able to detect lower pathogen concentrations.

3.3. Detection of bead-free isolated pure pathogen cultures by QD-labeled antibodies

The detection ranges tested in this study were 10^3–10^8 CFU/mL for both *E. coli* O157:H7 (Fig. 3) and *Salmonella* (data not shown). Fluorescence from PBS was read as the baseline. In order to check the specificity of QD-labeled antibodies and the efficiency of the developed bead-free isolation method, negative controls were added in all tests. For example, 10^8 CFU/mL *Salmonella* cells were processed as the negative control when the detection range was examined for *E. coli* O157:H7 (Fig. 3), and vice versa (data not shown). While the bead-free isolation method increased the fluorescence signals, the increased fluorescence detection sensitivity can also cause potential false positive signals from the negative controls. As seen in Fig. 3, *Salmonella* cells (10^8 CFU/mL) emitted a weak signal peak, albeit much lower compared to the signal generated by 10^8 CFU/mL targeted *E. coli* O157:H7 cells.

3.4. Detection of E. coli O157:H7 and Salmonella in artificially contaminated ground beef

When *E. coli* O157:H7 and *Salmonella* antibodies were added simultaneously to artificially contaminated ground beef samples, false negative signals generated by unspecific binding between beef particles and antibodies strongly inhibited the detection sensitivity and range (data not shown). Thus, a 24-h enrichment was incorporated before the cell isolation step to overcome this detection hurdle. The signal generated by the enriched beef samples with 10 CFU/g of *Salmonella* was as high as the signal generated by the beef samples inoculated with 10^8 CFU/g of *Salmonella*. Results with *E. coli* O157:H7 were similar (data not shown). Hence, enrichment allowed this bead-free QD-facilitated method to detect as low as 10 CFU/g of these target pathogens from ground beef (Fig. 4).

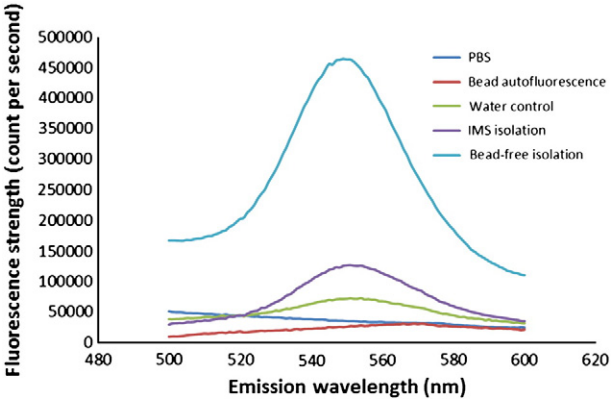

Fig. 2. Comparison of fluorescence generated by 10^8 CFU/ml *E. coli* O157:H7 cells isolated by the bead-free method and the IMS method. Water was used as a negative control to prevent false positive results. Fluorescence from the magnetic beads and PBS solution were also measured to generate the baseline.

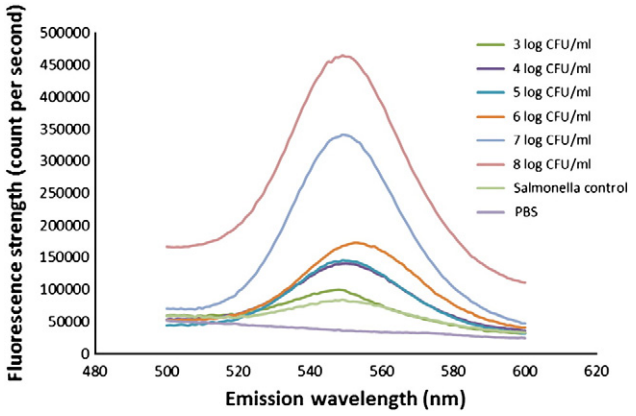

Fig. 3. Detection range of the bead-free green QD-facilitated detection method for *E. coli* O157:H7. The detection range for *E. coli* O157:H7 was from 10^3 to 10^8 CFU/mL. *Salmonella* cells (10^8 CFU) were added as a negative control as well as PBS.

4. Discussion

Quantum dots, due to their size-dependent fluorescence properties, have been suggested to be used as a replacer for traditional dyes. The conjugates made by the EDC and protein A crosslinker in this study showed uniform structures and, with the help of gel electrophoresis, a well controlled QD-EDC/protein A ratio successfully prevented potential self-polymerization.

Compared to a traditional IMS method, the FlowComp Dynabeads and DSB-X biotin protein labeling kit (D-20655) provided bead-free cells that could be used for further analysis. Without inhibition from the magnetic beads, the fluorescence emitted from the QDs was enhanced four-fold. One possible reason for the inhibition of QD fluorescence from magnetic beads is that magnetic beads can generate autofluorescence themselves. In addition, when a blue excitation source was used (λ_{ex}~488 nm), there was a significant spectral overlap between the magnetic bead autofluorescence emission and the QD fluorescence emission. The spectral overlap was reduced when the QD fluorescence emission spectrum was shifted to 525 nm (Agrawal et al., 2007). Coincidentally, Sathe et al. (2006) showed that the excitation light could be strongly absorbed by iron beads and, thus, attenuate the light intensity reaching the QDs. The interference between QDs and magnetic beads can be summarized into two parts: firstly, magnetic beads can inhibit the emission efficiency of QDs due to their autofluorescence and, secondly, magnetic beads have a strong absorbance to both the fluorescence from the excitation light and that from the QDs. Thus, a bead-free isolation method is highly recommended for future reporter dye-related cell detection methods.

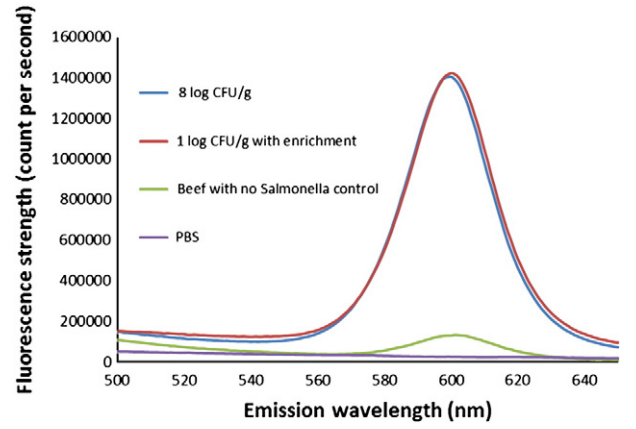

Fig. 4. Detection of ground beef artificially contaminated with 0, 10 and 10^8 CFU/g of *Salmonella* after a 24-h enrichment. Un-inoculated ground beef was used as the negative control. Background signals were much lower than the positive fluorescence generated from 10^8 CFU/g of *Salmonella* and the enriched ground beef with 10 CFU/g of *Salmonella*. *E. coli* O157:H7 data (not shown) was similar.

Initially, different mixtures of *E. coli* O157:H7 and *Salmonella* were inoculated in ground beef and the corresponding antibodies simultaneously added for bacterial isolation, while un-inoculated beef samples served as negative controls. Unfortunately, the un-inoculated beef sample generated very high background signals due to the unspecific binding between beef tissues and antibodies (Fig. 4). Thus, isolating one strain at a time coupled with a 24-h enrichment was found to be the most effective way to achieve high sensitivity while still achieving a fairly short turn-around detection time.

To our knowledge, this study is the first to employ a bead-free isolation method to separate target cells from ground beef samples prior to fluorescence detection. By combining this method with QD-labeled antibodies, this newly designed bead-free QD-facilitated protocol could detect as low as 10 CFU/g *E. coli* O157:H7 or *Salmonella* from artificially contaminated ground beef samples after a 24-h enrichment.

Ongoing research is focusing on further improving the detection sensitivity, decreasing the background interference and applying the method to a wider range of foods. The data from this current study could only be recorded as "positive" or "negative." Bioinformatic tools or additional purification steps could be tested to prevent interferences from background signals and enhance the detection sensitivity of this method.

One concern of using QDs in pathogen detection is the cost of QDs. In this study, the QDs were fabricated in-house, resulting in a cost of less than US$35 for 1 mL of QDs, compared to commercially available pure QDs, which cost around US$350 per 250 μL. The unique EDC–protein A conjugation method employed herein could be applied by others for a cost saving of about US$200 per 250 μL of QDs.

In summary, this bead-free QD-facilitated pathogen detection protocol is fairly fast, requiring less than 3 h (without enrichment step) or less than 27 h (with enrichment) to complete, and highly specific. It, thus, has great potential to be developed for rapid and accurate on-site foodborne pathogen detection efforts.

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