A simple quantum dot-based fluoroimmunoassay method for selective capturing and rapid detection of *Salmonella* Enteritidis on eggs

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**A B S T R A C T**

The aim of this paper was to demonstrate a rapid and selective fluorescence-linked immunoassay method based on quantum dots as the fluorescent marker for the detection of *Salmonella* Enteritidis on eggshell. Highly-fluorescent and water-soluble CdTe quantum dots were prepared by using thioglycolic acid and 1-thioglycerol as ligands and were then conjugated with anti-*Salmonella* antibodies. As a result of specific interaction, *Salmonella* Enteritidis were specifically captured by bioconjugated CdTe quantum dots which led to the detection of a fluorescent signal. The bacterial cell images were obtained using fluorescence microscopy. Under optimal conditions, the quenched fluorescence intensity increased linearly with the log total count of *Salmonella* Enteritidis ranging from $10^2$ to $10^7$ CFU/mL within 1--2 h. The low detection limit was $1 \times 10^2$ CFU/mL without sample enrichment. This method was satisfactorily applied to the analysis of egg samples, which was demonstrated as a simple scheme for quick and selective detection of *Salmonella* Enteritidis on eggshell.

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1. Introduction

Food contamination with *Salmonella* is an important public health concern in the whole world. Among all the *Salmonella* serotypes, *Salmonella* Enteritidis is identified as the main cause of human salmonellosis infection, thus infecting thousands of people every year (González-Escalona, Brown, & Zhang, 2012; Liu et al., 2012). The incidence of *S. Enteritidis* infections and the number of related outbreaks have increased dramatically since 1970s. Besides, the accumulating evidences have indicated that eggs are the most important source of human *S. Enteritidis* infection (Braden, 2006; Reu et al., 2006; Zhang, Zheng & Xu, 2011). Intensive epidemiologic and laboratory investigations have identified that fresh eggs (obtained from the chicken farm directly without any process) can be contaminated easily with *S. Enteritidis* in any contaminated environment, such as the nest box, the hatchery environment or the hatchery truck. In addition, eggs can be contaminated by penetration through the eggshell from the colonized gut (Messens, Grijspeerdt, & Herman, 2005). *S. Enteritidis* can slower their own metabolism under the disadvantageous conditions on the dry eggshell surface. Therefore, *S. Enteritidis* can survive and grow on the eggshell in the absence of faecal contamination and will survive for a longer time even at a low temperature (Gantois et al., 2009; Messens, Grijspeerdt, & Herman, 2006; Radkowski, 2002). Above all, one of the effective measures to reduce the infection from *S. Enteritidis* on the eggshell is to develop cleaned eggs which have been processed by washing, disinfecting under ultraviolet irradiation, drying, coating preservative, and packing before selling (Xiao, Zhang, Chi, Feng, & Xiao, 2013). However, for most ordinary eggs, a rapid, simple, highly sensitive and selective detection method of *S. Enteritidis* on eggshell is in urgent need.

The conventional identification approaches include culture and colony counting (Allen, Edberg, & Reasoner, 2004), enzyme linked immunosorbent assay (Cheung & Kam, 2012), polymerase chain reaction (Martin, Garriga, & Aymerich, 2012; Salinas, Garrido, Ganga, Veliz, & Martinez, 2009), immunological techniques (Van, leven, Pattyn, Van, & Laga, 2001) and fluorescence-based assays (Fu, Huang, & Liu, 2009). Although these methods are error-proof and powerful, most of them are labour-intensive, complex and time-consuming for a complete analysis (Sanvicencs Pastells, Pascual, & Marco, 2009). Therefore, the highly-sensitive and rapid detection methods are expected.

Nowadays, fluorescence analysis, which is developed for rapid, technically-simple and efficient detection of the bacterial count from originals or pre-enriching samples without any expensive apparatus, is of great importance (Luo et al., 2009). Quantum dots (QDs), a family of nanosized particles, comprised of a few thousand atoms with typical sizes of 1--10 nm in radius. They have been...
widely used in the fields of chemistry and biomedical science as a fluorescence marker (Chan & Nie, 1998). Compared to conventional organic fluorophores, QDs have many advantages such as broad-band excitation, narrow and symmetric emission spectra, high signal-to-noise ratios, considerably greater resistance to quenching and photobleaching (Duong & Rhee, 2007; Xue, Pan, Xie, Wang, & Zhang, 2009). Thus, water-soluble QDs are the ideal fluorescence markers in the detection of total bacterial count (Agasti et al., 2010; Bae et al., 2010; Jackeray et al., 2011; Sanvicens et al., 2011; Tully, Hearty, Leonard, & O’Kennedy, 2006).

In this study, we prepared the monodisperse, homogeneous, highly-fluorescent and water-soluble CdTe QDs, and then conjugated prepared CdTe QDs with anti-S. Enteritidis antibodies. It was a novel method to use this bioconjugated CdTe QDs as fluorescent label for rapid, technically-simple and efficient detection of S. Enteritidis. The fluorescence intensity was linear with the bacterial count in the range of $3 \times 10^2$–$3 \times 10^7$ CFU/mL and the low detection limit was $1 \times 10^2$ CFU/mL in 1–2 h. The method was also applied to the direct detection of the S. Enteritidis count in real egg samples to prove its feasibility and practicability. The results demonstrated that the application of bioconjugated CdTe QDs as fluorescent probes in detecting S. Enteritidis count on eggshell was rapid, selective and feasible.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical grade and used without prior purification. Doubly-deionized water (DDW) was used throughout this work. NaBH₄, CdCl₂·2.5H₂O, NaOH, thioglycollic acid (TGA), N-hydroxysuccinimide (NHS), Nutrient broth medium (NB), nutrition agar medium and salts studied (Na⁺, K⁺), glucose, lactose were acquired from Guoyao Company (China). Glycine, cysteine, glutathione, L-serine, tyrosine, albumin, lysozyme, BSA were purchased from Biosharp Company (China). Te powder was acquired from Tianjin Delan Fine Chemical Reagent Company (China). 1-Thioglycerol (TG) was acquired from Aladdin Chemical Reagent Company Ltd. (China). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was acquired from Aladdin, Inc. (China). Rabbit anti-Salmonella antibody (3–4 mg/mL) was purchased from Gene Tex, Inc. (USA).

Phosphate buffered solution (PBS) with different pH values were prepared by mixing 1/15 mol/L Na₂HPO₄ and 1/15 mol/L NaH₂PO₄ according to certain proportions.

The egg samples used in the experiment were obtained from a local chicken farm and the supermarket in China. Three types of eggs were used in this experiment: fresh eggs from a local chicken farm, ordinary eggs from the supermarket and the cleaned eggs from the supermarket. The cleaned eggs had been processed by washing, disinfecting under ultraviolet irradiation, drying, coating preservative, and packing (Xiao et al., 2013). Each sample was prepared by using three eggs during the experiment. The egg samples were put into sterile plastic bags respectively. Their eggshells were rubbed with 200 mL sterile DDW for 1–2 min to detach the bacteria. The wash solutions from eggshell were collected for future detection. For the stability of the experiment, each sample was set in three parallel.

2.2. Synthesis and characterization of CdTe QDs

Highly-fluorescent and water-soluble CdTe QDs were synthesized in aqueous solution based on a previous public method with minor modification (Yang & Gao, 2005). Briefly, the oxygen-free NaHTe solutions were prepared by stirring a mixture of 36.4 mg NaBH₄, 26.7 mg Te powder and 1.5 mL DDW at room temperature until black Te powder disappeared. The freshly-prepared oxygen-free NaHTe solutions were reacted with a mixture of 160 mL 0.026 mol/L CdCl₂ and 140 mL TGA (pH 11.2 adjusted with 1 mol/L NaOH) at 97 °C for 1 h. The QDs concentration $c (A(\chi L))$ was measured according to Carion, Mahler, Pons, and Dubertret (2007). In this equation, $A$ was the absorption of the QDs sample measured at 350 nm, $L$ was the optical path (usually the dimension of the cuvette used for the measurement) and $e (M^{-1} cm^{-1}) = (1.438 \times 10^{26}) a^2$ was the extinction coefficient at 350 nm (a was the QD radius in centimetres).

The quantum yield (QY) of CdTe QDs was calculated according to literature (Brouwer, 2011).

Synthesized hydrophilic CdTe QDs were characterized by various techniques. Absorption spectra were obtained using a UV-1800 pharma spectrometer (Shanghai, China). Fluorescence intensity and emission spectra were recorded with the excitation wavelength fixed at 330 nm using an RF-5301 spectrophotometer (Hitachi, Japan). All the spectra were recorded with DDW as blank. The image of CdTe QDs were examined by an JEM-2100F high-resolution transmission electron microscopy (JEOL, Japan) with an acceleration voltage of 200 kV. The aqueous solutions of QDs were dropped on an 800-mesh carbon-coated copper grid and dried at room temperature for HRTEM sample preparation.

The X-ray powder diffraction spectra (XRD) patterns were obtained on a D/max-2500 X-ray diffractometer with Cu Kα radiation (Rigaku, Japan). XRD samples were prepared as follows. The QDs solution was concentrated to about one tenth using a rotary evaporator and then cold ethanol was added until turbidity occurred. The precipitate and supernatant were separated by centrifugation. To prepare the final powder sample, the ethanol precipitated and re-dissolved samples were dried overnight under vacuum at 60 °C.

2.3. Bioconjugation of CdTe QDs with anti-S. Enteritidis antibodies

Fig. 1 showed the coupling process between CdTe QDs and anti-S. Enteritidis antibodies. The water-soluble CdTe QDs were activated by using carbodiimide chemistry for conjugation with anti-S. Enteritidis antibodies. Particularly, 1 mL (QY 36%, about $1.02 \times 10^{-6}$ mol) of QDs stock solution was put into the mixed solutions containing 1 mL of PBS (0.01 mol/L, pH 7.2) and 100 μL (4 mg/mL) of EDC. The mixture
was under gentle stirring at room temperature for 5 min and it took about 15–20 min to fully activate free carboxylic acid groups on the QDs (Zhao et al., 2009). Then, 100 μL (0.15 mg/mL) of sulfo-NHS was added to the mixed solutions and the mixture was stirred for another 20 min. Thereafter, anti- S. Enteritidis antibodies were added into the system and reacted for at least 2 h in constant temperature incubator. In this step, CdTe QDs and anti- S. Enteritidis antibodies were conjugated through strong covalent bonds. The final bioconjugated CdTe QDs were collected and kept at 0–4 °C overnight. Conjugation process was optimized by varying reaction temperature and time as well as the mole ratios of CdTe QDs and anti-S. Enteritidis antibodies.

2.4. Bacterial inoculate preparation and surface plating methods

Stock cultures of S. Enteritidis were obtained from our laboratory culture preservation. Cultures were grown for 18–24 h at 37 °C in NB. Serial 10-fold dilutions were made in sterile PBS. The number of S. Enteritidis was determined by surface plating technology using 0.1 mL proper dilutions onto nutrition agar medium. After the incubation at 37 °C for 24 h, colonies on the plates were counted to determine the number of visible cells in the cultures in terms of colony forming units per millilitre (CFU/mL). For safety considerations, all of the bacterial samples were inactivated in an autoclave at 121 °C for 15 min, except for some special tests and characterizations.

2.5. Detection of S. Enteritidis by immunoassay procedure

The basic immunoassay procedures for detection of the S. Enteritidis were displayed in Fig. 2. After the S. Enteritidis cultures were centrifuged, poured out of the supernatant and suspended the precipitated S. Enteritidis in 1 mL of axenic PBS. The process was repeated twice to remove the NB. Then, the serial 10-fold of S. Enteritidis culture dilutions were made in sterile PBS. For quantitative determination, 200 μL of the bioconjugated CdTe QDs was put into certain amounts of S. Enteritidis culture (3 × 10^2 to 3 × 10^7 CFU/mL, respectively) and they were kept at 37 °C for 40 min. In this step, bioconjugated CdTe QDs were used as fluorescence labels to capture target bacterial cells through the immuno-recognition between antibodies and S. Enteritidis. 1 mL DDW was treated, using the same procedure, as a control. After the reactions, these mixtures were centrifuged at 8000 rpm for 10 min to precipitate the S. Enteritidis–bioconjugated CdTe QDs. The resulting supernates were used to monitor the change of emission fluorescence intensity by spectrofluorometer. The calibration graph set up in the study was used to quantify of the concentration of S. Enteritidis in the sample. The excitation wavelength was selected at 330 nm. The slit widths of both excitation and emission were 5.0 nm.

Fig. 3. UV–vis absorbance (a) and fluorescence spectra (b) of water-soluble CdTe QDs.

Fig. 4. HRTEM image of CdTe QDs.

Fig. 5. XRD images of CdTe QDs.

Fig. 6. Fluorescence spectra of CdTe QDs before (a) and after (b) bioconjugation.
2.6. Fluorescence microscopy

A fluorescence microscope (Olympus, BX71), equipped with a digital CCD camera (Olympus DP70) and a broadband mercury lamp (OSRAM HBO 100 W) with an ultraviolet excitation filter, was used to capture images of the samples on the glass slides.

3. Results and discussion

3.1. Synthesis and characterization of CdTe QDs

Fig. 3 shows UV–vis absorbance (a) and fluorescence spectra (b) of CdTe QDs. The absorption spectrum indicates that CdTe QDs has a wider range of absorption with the absorption peak at 460 nm. Their broad absorption spectra allow for the efficient excitation at any wavelength with a single light source. The emission spectrum shows characteristically narrow and symmetric emission peak at 512 nm. The CdTe QDs provide sufficient spectral resolution for quantitative detection of the fluorescence intensity with 40 nm of FWHM (full width at half maximum) and high QY (36%).

3.2. HRTEM image of CdTe QDs

Fig. 4 depicts the HRTEM image of the CdTe QDs. The shape of these QDs was spherical, crystalline, sufficiently-monodisperse and well-separated, with the average size about 2–3 nm. It also shows that lattice planes are extended across the entire particle, which confirms a well-crystallized structure of the CdTe QDs (He & Gu, 2006).

3.3. XRD spectra of the CdTe QDs

The XRD resulted in Fig. 5 confirms the formation of cubic structure of CdTe QDs. The peaks observed at 2θ are 24.4°, 41.6°, 46.9°, corresponding to the (111), (220) and (311) crystalline planes of cubic CdTe (Huang, Liu, Han, Mi, & Xu, 2012). The considerably broad width of the diffraction peaks indicates that the CdTe QDs have a nano-size distribution which is consistent with the image of HRTEM.

3.4. Optimum conditions for detection

Fig. 6 displays the fluorescence emission spectra of CdTe QDs before (a) and after (b) being bioconjugated with anti-S. Enteritidis antibodies. After being bioconjugated, the fluorescence intensity increases and shifts slightly towards higher wavelength because of the surrounding organic layer (anti-S. Enteritidis antibodies) of CdTe QDs (Goldman et al., 2004).
The coupling process conditions were optimized by varying the concentration of anti-Salmonella antibodies, reaction temperature and pH. Fig. S1 shows the effect of the concentration of anti-S. Enteritidis antibodies on bioconjugation system. Maximum fluorescence intensity occurs at the concentration of 7.5 μg/mL. When concentration is higher than 7.5 μg/mL, the limited QDs molecules cannot occupy all binding sites of anti-S. Enteritidis antibodies coexisting in the system. When the concentration is lower than 7.5 μg/mL, the fluorescence intensity may decrease because of the self-quenching effect of excessive QDs. Fig. S2 shows the influence of reaction temperatures on bioconjugation. As is shown, the reaction was completed within 120 min at 37 °C. Fig. S3 shows the effect of pH on the bioconjugated process. From Fig. S3, we can see that when pH is 7.2, the fluorescence intensity reaches a maximum and therefore the pH of 7.2 is consequently selected for further studies.

3.5. Detection of S. Enteritidis using bioconjugated CdTe QDs

3.5.1. The interaction of bioconjugated CdTe QDs with S. Enteritidis

Changes of fluorescence spectra of the bioconjugated CdTe QDs in the presence of different concentrations of S. Enteritidis are presented in Fig. 7. The fluorescence signals decrease rapidly with the rising S. Enteritidis concentrations (from 3 × 10^2 to 3 × 10^7 CFU/mL). Thus, the more S. Enteritidis bacterial cells are in the sample, the more fluorescence intensity decreases because of the electrostatic repulsion between electronegative S. Enteritidis and bioconjugated CdTe QDs. The maximum value of fluorescence intensity is reached when the system has been detected at 0 °C (changes of the fluorescence intensity in the presence and absence of S. Enteritidis). Therefore, 0 °C was adopted in this work. Fig. 9C shows the effect of pH on the system. It is found that the optimum pH ranges are from 7.2 to 7.5. When pH is lower than 7.2 or greater than 7.5, the fluorescence intensity is comparatively lower. The reason may be explained as follows: in low pH value, the fluorescence intensity decreases as a possible result of the deconstruction of the CdTe–TGA complexes’ annulus due to the protonation of the surface-binding thiolates. When pH value is too high, the fluorescence intensity decreases partly because of the electrostatic repulsion between electronegative S. Enteritidis and bioconjugated CdTe QDs.

### Table 1

Interference of coexisting substances.

<table>
<thead>
<tr>
<th>Coexisting substances</th>
<th>Concentration (10^-8 mol/L)</th>
<th>Change of fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>10000</td>
<td>+3.9</td>
</tr>
<tr>
<td>K⁺</td>
<td>10000</td>
<td>+4.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>400</td>
<td>+2.6</td>
</tr>
<tr>
<td>Lactose</td>
<td>4000</td>
<td>+2.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>300</td>
<td>+2.3</td>
</tr>
<tr>
<td>Glutathione</td>
<td>100</td>
<td>+5.0</td>
</tr>
<tr>
<td>L-serine</td>
<td>200</td>
<td>+4.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>200</td>
<td>+4.3</td>
</tr>
<tr>
<td>Albumin</td>
<td>3</td>
<td>+4.9</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.3</td>
<td>+4.8</td>
</tr>
<tr>
<td>BSA</td>
<td>0.2</td>
<td>+3.9</td>
</tr>
</tbody>
</table>

### Table 2

Comparison of the linear ranges and LODs of several selected fluorimetric methods for determination of S. Enteritidis.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Linear range (CFU/mL)</th>
<th>LOD (CFU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum dots coated with streptavidin Q dot 705 streptavidin conjugate</td>
<td>1.0 × 10^3–1.0 × 10^5</td>
<td>1.0 × 10^4</td>
<td>Yang and Li (2005)</td>
</tr>
<tr>
<td>CdTe/ZnS QDs</td>
<td>ND^a</td>
<td>10 CFU/g</td>
<td>Wang et al. (2012)</td>
</tr>
<tr>
<td>CdTe quantum dots</td>
<td>3.0 × 10^2–3.0 × 10^2</td>
<td>1.0 × 10^2</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Table 3

Results for the determination of S. Enteritidis in synthetic sample.

<table>
<thead>
<tr>
<th>Synthetic sample</th>
<th>Main interferences (10^-6 mol/L)</th>
<th>Change of fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Na⁺ 10000, K⁺ 10000, glutathione 50, BSA 0.1, glucose 1500, lactose 1500</td>
<td>-4</td>
</tr>
<tr>
<td>2</td>
<td>Na⁺ 10000, L-serine 150, glucose 1500, glutathione 50, BSA 0.1</td>
<td>-1.3</td>
</tr>
<tr>
<td>3</td>
<td>Na⁺ 10000, K⁺ 10000, glutathione 50, BSA 0.1</td>
<td>10.6</td>
</tr>
</tbody>
</table>

bioconjugated CdTe QDs could bind. The total detection time, from adding a sample solution to obtaining the final result, is less than 2 h.

3.5.2. Fluorescence microscopy

Fluorescence microscopy image of S. Enteritidis cells attached to bioconjugated CdTe QDs with emission wavelengths of 512 nm (green) are showed in Fig. 8. The fluorescence signals of the results were definitively confirm that the S. Enteritidis were captured by bioconjugated CdTe QDs.

3.5.3. Optimum conditions for detection

In order to perfect the fluorimunoassay method of S. Enteritidis, the test conditions were improved by studying the effect of various factors such as reaction time, temperature and pH. The results were shown in Fig. 9. Fig. 9A shows that the immunologic process reaction can be completed within 40 min. As the temperature is an important factor in the fluorescence measure, it has been investigated how the fluorescence intensity changes at varying temperatures during detection, and the results are shown in Fig. 9B. The maximum value of fluorescence intensity is reached when the system has been detected at 0 °C (changes of the fluorescence intensity in the presence and absence of S. Enteritidis). Therefore, 0 °C was adopted in this work. Fig. 9C shows the effect of pH on the system. It is found that the optimum pH ranges are from 7.2 to 7.5. When pH is lower than 7.2 or greater than 7.5, the fluorescence intensity is comparatively lower. The reason may be explained as follows: in low pH value, the fluorescence intensity decreases as a possible result of the deconstruction of the CdTe–TGA complexes’ annulus due to the protonation of the surface-binding thiolates. When pH value is too high, the fluorescence intensity decreases partly because of the electrostatic repulsion between electronegative S. Enteritidis and bioconjugated CdTe QDs.

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<td>-4</td>
</tr>
<tr>
<td>2</td>
<td>Na⁺ 10000, L-serine 150, glucose 1500, glutathione 50, BSA 0.1</td>
<td>-1.3</td>
</tr>
<tr>
<td>3</td>
<td>Na⁺ 10000, K⁺ 10000, glutathione 50, BSA 0.1</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Fig. 10. Linear relationships of the changes in fluorescence intensity versus the log-transformed cell population during the detection of S. Enteritidis, at populations ranging from 3 × 10^2 to 3 × 10^7 CFU/mL. Concentration of CdTe QDs: 1.02 × 10^-6 mol/L, sex = 330 nm; pH 7.2.
Enteritidis are shown in Fig. 10. The linear ranges for changes of S. Enteritidis population are identical with the conventional plate count method. The study demonstrates that the application of CdTe QDs as immunoassays fluorescent probes is feasible to detect the total bacteria count.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2013.06.025.

References


