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Quantum Dots: Application for the Detection of *Salmonella typhimurium*

Munawwar Ali Khan[1*] • Sultan Mohammed Faheem[2]

[1] Department of Natural Science and Public Health, College of Arts and Sciences, Zayed University, P. O. Box 19282, Dubai, United Arab Emirates
[2] Department of Biotechnology, Manipal University Dubai campus, Block-7, Dubai International Academic City, P. O. Box 345050, Dubai, United Arab Emirates
Corresponding author: * **Munawwar.Khan@zu.ac.ae**

ABSTRACT

Quantum dots (QDs) or nanocrystals 5 to 50 nm in size have recently emerged as a novel and promising class of fluorophores for cellular imaging. Unlike conventional organic dyes, QDs can be excited by a wide spectrum of wavelengths to give enhanced photostability, and their emission spectra, which differ according to size and material composition, are narrow, symmetrical, and tunable. This novel detection strategy can significantly improve the efficiency of molecular techniques used in the identification of important microbes related to human diseases. The aim of this study was to apply QDs linked antibodies to detect pathogenic *Salmonella typhimurium* cells. The signal, photostabilty and efficiency of QDs linked antibodies was compared with the organic fluorophore linked antibody for the detection of *Salmonella* cells. The use of QDs as fluorophores has shown greater enhancement in photostability and brighter signal than conventional organic dyes in detecting *S. typhimurium* cells. This approach might also be extended for the simultaneous detection of more than one pathogenic microorganism in the clinical, food or environmental samples by using specific antibodies linked with the QDs of different sizes and colors.

Keywords: antibody, fluorescence, quantum dots, photostability, *Salmonella typhimurium*

INTRODUCTION

Quantum dots (QDs) are luminescent semiconductor nanocrystals with several significant optical properties and have recently emerged as a promising tool in various fluorescence based biomedical applications in cellular imaging (Bruchez *et al*. 1998; Chan and Nie 1998; Goldman *et al*. 2002; Jaiswal *et al*. 2003; Jurgen and Thomas 2004; Rawsthorne *et al.* 2009; Agasti *et al.* 2010; Byers and Hitchman 2011). The reasons QDs are being preferred over conventional organic fluorophores such as fluorescein isothiocyanate (FITC) or Tetra methylrhodoamine isothiocyanate (TRITC) is due to their photostability, size tunability with narrow, symmetric emission spectra and broad continuous excitation which allow excitation of multiple QDs with a single wavelength (Bruchez *et al*. 1998; Chan and Nie 1998; Goldman *et al*. 2002; Jaiswal *et al*. 2003; Jurgen and Thomas 2004). These features make QDs very robust luminescent labels for various biological applications (Kloepfer *et al*. 2003; Rawsthorne *et al.* 2009; Agasti *et al.* 2010; Byers and Hitchman 2011). The use of QDs in applications related to the detection of microorganisms have been conjugated to biomolecules such as biotin, streptavidin, avidin, transferrin, immunoglobulin G (IgG), nucleic acids, peptides and wheat germ agglutinin (Chalmers *et al*. 2007). These QDs and biomolecule conjugates have been shown to bind with enhanced sensitivity to a variety of target microorganisms (Kloepfer *et al*. 2003; Chalmers *et al*. 2007; Beckman *et al*. 2008; Decho *et al*. 2008).

There have been several reports on the application of QD conjugated antibodies for labeling, detection, and quantification of human oral microbial communities, *Mycobacterium bovis*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar *typhimurium*, *Cryptosporidium parvum*, *Giardia lamblia* and phage-based assays for the detection of slow growing bacteria (Kloepfer *et al*. 2003; Zhu *et al*. 2004; Lee *et al*. 2004; Yang and Li 2005; Yang and Li 2006; Chalmers *et al*. 2007). These QDs based detection approaches have reported higher detection sensitivity and specificity than that achievable with conventional fluorophores. QDs in a biocompatible form can be prepared by several methods reported in the literature (Bruchez *et al*. 1998; Chan and Nie 1998; Goldman *et al*. 2002). Furthermore, QDs are currently available commercially as streptavidin conjugates in a water-soluble form and could be applied for the detection of pathogenic microorganisms if a specific antibody to that particular microorganism is available (Zhu *et al*. 2004; Lee *et al*. 2004).

In this study, QDs were applied in labeling and detection of *Salmonella typhimurium* cells using immunofluorescent antibody based assay. To investigate a QD-based immunofluorescence assay to detect *Salmonella typhimurium* cells, biotinylated anti-*Salmonella* polyclonal antibodies (PAb) were conjugated to the streptavidin coated QDs. The photostability of the QDs conjugated antibody was compared with the FITC conjugated anti-*Salmonella* PAb.

MATERIALS AND METHODS

Microbial strains

All organisms used were purchased from the Microbial Type Culture Collection (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh, India. All media and general chemicals were purchased from either Hi-media or Sigma-Aldrich. The incubation temperatures were 37°C for *Salmonella typhimurium* (MTCC 98) and *Pseudomonas fluorescence* (MTCC 103). All strains were maintained as pure cultures on Luria-Bertani agar plates and grown with shaking in Luria-Bertani broth at 37°C.

Labeling of bacteria with QDs

Bacterial cells were grown in Luria-Bertani broth under recommended growth conditions until they reached the exponential

Received: 28 April, 2010. Accepted: 15 February, 2011.

Original Research Paper

growth phase. The biotinylated anti-*Salmonella* and FITC labeled anti-*Salmonella* antibodies were polyclonal antibodies raised against the mixture of *Salmonella enteriditis*, *Salmonella typhimurium*, and *Salmonella heidelburg* (Abcam, UK). The *Pseudomonas fluorescens* strain was used as a negative control in all immunofluorescence based assays.

The strategies used in earlier studies related to the detection of pathogenic protozoans such as *Cryptosporidium parvum* and *Giardia lamblia* and were used to label *Salmonella typhimurium* cells (Zhu *et al*. 2004; Lee *et al.* 2004). Briefly biotinylated anti-*Salmonella* PAb and FITC labeled anti-*Salmonella* PAb were first reacted with target cells and then conjugation of streptavidin coated QD605 (Invitrogen, USA) was performed to the cells. In detail, 10 µl of target *Salmonella* cells (~10^5 cells/ml) were spotted on a poly-L-Lysin coated glass slide (Vermicon Identification technology, Munich, Germany). After air dry, 20 µl of blocking buffer (Abcam, UK) was added onto the fixed cells and incubated for 20 min. After washing with phosphate-buffer saline (PBS, pH 7.4) three times each for 5 min, cells were incubated with 20 µl of 1X biotinylated Anti- *Salmonella* PAb for 30 min at 37°C in a humidified hybridization chamber (supplied with fluorescence *in situ* hybridization (FISH) based kit for the *Salmonella* detection, Vermicon Identification technology, Munich, Germany). The cells were again washed with PBS three times each for 5 min, and incubated with 20µl of various concentration of QDs solution (dilutions in the range of 5 to 40 nanomoles, nM) for 30 min at 37°C in a humidified chamber. After a final wash with PBS for 5 min three times, the slide mounted with mounting solutions (Abcam and VIT kit) was observed with epifluorescence microscope. Image analysis was performed using ProgRes digital camera system (JENOPTIK, Germany).

In another strategy (Zhu *et al.* 2004) Streptavidin- QD605 were linked with anti-*Salmonella* antibodies followed by the reaction with *S. typhimurium* cells. Diluted preparation of (1X) biotiny1ated antibodies was first incubated with QDs in the QDs incubation buffer (Invitrogen) for 30 min. After fixing and blocking the target cells, 20 µl of the pre-incubated solution was added onto the slide and incubated at 37°C in a humidified chamber for 30 min. After washing with PBS for 5 min at least three times, the slides were observed under the fluorescent microscope.

Epifluorescence microscopy

An epifluorescence microscope (NIKON eclipse 80i, JAPAN) with a 100W high pressure mercury lamp was used to examine the QD605-streptavidin and FITC anti-*Salmonella* PAb tagged *Salmonella* cells. Slides were examined with the filter sets G-2E/C for TRITC and QD605 (excitation wavelength, 540 to 565 nm; dichroic wavelength, 565 nm; emission, 605-655 band pass) and B-2E/C for FITC (excitation wavelength, 465 to 495 nm; dichroic wavelength, 505 nm; emission, 515-555 band pass). Images were captured and analyzed using ProgRes digital camera system (JENOPTIK, Germany).

RESULTS

Detection of *Salmonella* cells by QDs

The two strategies used for labelling target cells with QDs-antibody bioconjugates are illustrated in **Fig. 1**. The *S. typhimurium* cells from the 10-µl of exponentially grown culture (~10^5/ml) were detected with FITC-labeled anti-*Salmonella* PAb and QD605 conjugated anti-*Salmonella* PAb. The numbers of *S. typhimurium* cells were counted by the method described in the literature. The concentration of QD605-streptavidin was increased from 5 to 30 nM and this has resulted in an increased number of *Salmonella* cells being detected. The detection sensitivities with 10 nM QD605-streptavidin concentration were found to be enough for the detection of a maximum number of *Salmonella* cells. However, the detection sensitivities achieved at 20 and 30 nM QD605-streptavidin had not affected the number of detected cells. Furthermore, with FITC-labeled anti-*Salmonella* polyclonal antibodies a higher concentration up to 20-40 nM was required to detect a comparable number of *Salmonella* cells. Thus, 10 and 30 nM were the optimum concentrations required for the detection of *Salmonella* cells by QD605-streptavidin and FITC-labeled anti-*Salmonella* PAb, respectively. **Fig. 2** shows epifluorescence images of QD605-labeled *Salmonella* cells with 5 nM (**Fig. 2A**) and 10 nM QD605-streptavidin (**Fig. 2B**). As indicated in **Fig. 2**, the number of detected *Salmonella* cells increased by increasing the concentration of QD605-streptavidin from 5 to 10 nM. However, a further increase in the concentration of QD605-streptavidin up to 30 nM had no significant effect on the number of detected cells.

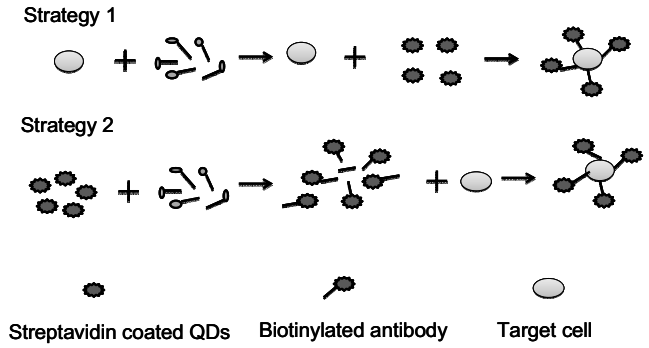

Fig. 1 Strategies for labeling target cells with QDs.

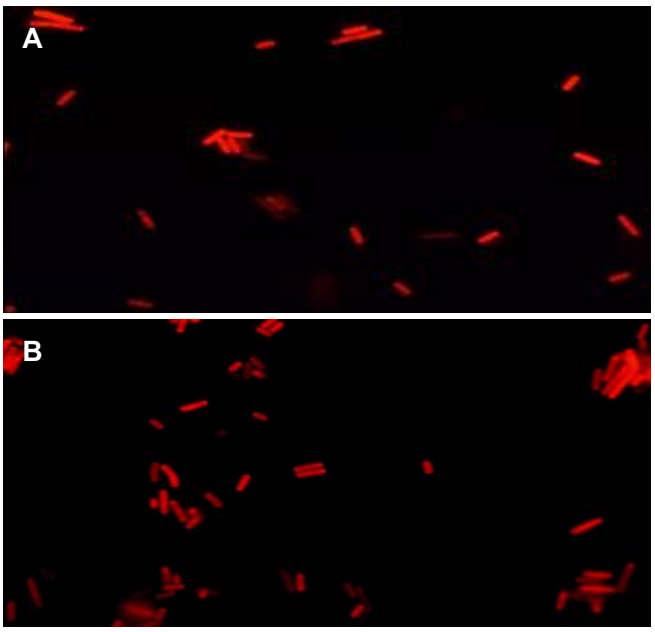

Fig. 2 Fluorescence images of *Salmonella typhimurium* cells labeled by QD605-streptavidin tagged-biotinylated anti-*Salmonella* PAb with (**A**) 5 nM QD605-streptavidin (**B**) 10 nM QD605-streptavidin. Each bar is equal to 10 µm.

Photostability of QD605-conjugated anti-*Salmonella* antibodies

The photostability and increased brightness are the main advantages of QDs over traditional organic fluorophores. In several studies in the past, it has been observed that the brightness of QDs could differ for QDs that are conjugated and used in staining experiments (Wu *et al.* 2003). Both streptavidin-QD605 and FITC-anti-*Salmonella* PAb labeled *Salmonella* were continuously illuminated for 3 min with a Nikon epifluorescence microscope fitted with a 100-W mercury lamp. The images were captured with a ProgRes digital camera system (JENOPTIK, Germany) at every 15-s interval, and the intensity was analyzed with the imaging system supplied by Nikon (Japan). In this study, it was noted that QD605-streptavidin labeled *Salmonella* cells exhibited brighter signals even at the lower concentration of QD605.

The FITC-PAb labeled *Salmonella* cells have shown good signals at the higher concentration. However, the FITC-labeled *Salmonella* cells signal bleached significantly after 2 min of illumination. In contrast, it was observed that there was insignificant reduction in the signal for QD605-labeled *Salmonella* cells. That is, the QD605-labeled *Salmonella* cells remained photostable even after 3 min of continuous illumination.

DISCUSSION

Salmonella typhimurium has been recognized worldwide as the major cause of most cases of human salmonellosis transmitted through food, water and other environmental sources (Carlson *et al.* 1999; Sharma and Carlson 2000; Baggesen *et al.* 2000; Baudart *et al.* 2000). Rapid and sensitive detection of pathogenic *Salmonella* strains in various environments is important for preventing the outbreaks of food/waterborne diseases associated with its infection. The detection of *Salmonella* is usually achieved through specific labels that selectively stain its cells according to its biochemical, structural or nucleic acid properties using antibody-based, oligonucleotide based Fluorescent in situ hybridization (FISH) and PCR-based methods (Carlson *et al.* 1999; Sharma and Carlson 2000; Baggesen *et al.* 2000; Baudart *et al.* 2000). Due to their high sensitivity and specificity, fluorescently labeled antibody based probes are most commonly used among all these methods. However, different environmental samples can contain inert objects or living or dead cells that have strong fluorescence and light scatter characteristics similar to, for example, fluorescently labeled pathogenic *Salmonella* cells, interfering with the detection of *Salmonella.* Therefore, careful selection of fluorescent dyes is the key to the detection and identification of pathogenic *Salmonella* strains.

Recent studies have revealed quantum dots (QDs) as a novel and promising class of fluorescent reporting systems for cellular imaging. QDs, which are nano scale inert particles (~5-50 nm), provide much higher photostability than conventional organic dyes and can be excited by a wide spectrum of wavelength from UV to red (Bruchez *et al.* 1998; Chan and Nie 1998; Goldman *et al.* 2002; Jaiswal *et al.* 2003; Kloepfer *et al.* 2003; Jurgen and Thomas 2004; Zhu *et al.* 2004; Lee *et al.* 2004; Yang and Li 2005; Yang and Li 2006; Chalmers *et al.* 2007; Rawsthorne *et al.* 2009; Agasti *et al.* 2010; Byers and Hitchman 2011). Since the emission spectra of QDs, which differ according to the size and material composition, are narrow, symmetrical and tunable, the use of QDs as a fluorescence reporting system can potentially and significantly minimize the interference from natural auto fluorescent particles, and provide multiplexing detections on different target cells with a clear discrimination from extraneous particles (Zhu *et al.* 2004). Recent progress on the conjugation techniques of QDs with biotin, avidin, protein transferring and antibodies have greatly increased the potential of QDs in detecting different molecular targets at different sub-cellular locations of various biological specimens like live cells, fixed cells and tissue sections.

The two strategies used in this investigation were applied for the detection of *Cryptosporidium parvum* and *Giardia lamblia* in a previous study (Zhu *et al.* 2004).There was no significant effect observed on the labeling of *Salmonella* by streptavidin coated QD605 by these two strategies. As suggested in the Zhu *et al*. (2004) study, in strategy 2, excessive amounts of QDs were needed to reduce the numbers of antibodies bound onto one QD before conjugating the antibodies would bind QDs to cells. As a result, the signal brightness should decrease rapidly with the increase in the dilution of QDs concentration. However, in this study both strategies achieved almost the same signal brightness at the 10 nM QDs working concentration. In addition, an increase in the QD working concentration in both strategies did not affect the brightness of QDs labeled *Salmonella* cells. This particular observation was inconsistent with the earlier study (Yang and Li 2006) where 10 nM working concentration of QD605 and QD525 failed to generate detectable signal for detection of *E. coli* O157:H7 and *S. typhimurium.* This might be due to the different working concentrations of biotinylated antibody used in this study. In addition, this observation could also be attributed to the use of polyclonal antibodies as compared to the monoclonal antibodies used in a study conducted by Yang and Li (2006). The polyclonal antibodies used in this study were targeted at multiple O and H antigens displayed by most of the members of the *Salmonella* family. The polyclonal antibodies used were able to bind *Salmonella* cells more effectively by QDs tagged PAb even at the lower concentration of 5nM or 10nM ODs. However, further study is needed to compare the efficiency of anti-*Salmonella* monoclonal and PAb to label *Salmonella* cells at different QDs working concentrations. In another closely related study, a 30 nM working concentration of QD655 was found to generate stable signals for the detection of *Streptococcus gordonii* in oral biofilms (Chalmers *et al.* 2007). In the current study, 5-10 nM working concentration of QD-605 was found to be sufficient to detect bacteria in pure culture.

The effect of various antifade agents on the photostability of QDs was also investigated in this study. These mounting solutions included immersion oil, antifade solutions used in the VIT (Vermicon Identification technology, Munich, Germany) kit, 10% BSA in 1X PBS used in reference 10 and 90% glycerol. The antifade agent supplied with FISH based VIT-*Salmonella* kit gave the best results. However, there was not much reduction in the signal in the case of QDs labeled *Salmonella* cells. The immersion oil which was found to be the best antifade mounting solution in the Zhu *et al*. (2004) study was found to be inadequate in the case of FITC labeled *Salmonella* cells. Other mounting solutions including 1X PBS and pure glycerol were further tested, and all gave poorer results than that of the VIT kit supplied antifade agent.

CONCLUSIONS

This study successfully demonstrated labeling of *Salmonella typhimurium* cells with streptavidin-QDs conjugated with polyclonal antibodies against *Salmonella*. QDs were shown to have increased photostability and brightness over conventional organic dyes in the detection of *S. typhimurium* cells. This approach might also be extended for the simultaneous detection of different members of the *Salmonella* family in the same sample by using QDs with different sizes and colors. Therefore, QDs labeling is a novel, promising and durable method for the identification and detection of pathogenic or environmentally relevant microorganisms in clinical, food and other environmental samples.

ACKNOWLEDGEMENTS

We are thankful to the Department of Biotechnology, Manipal University Dubai campus for providing imaging facilities. We thank Dr. Carol Campbell for critical reading of the manuscript.

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