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Full Length Research Paper

Detection of live/dead *Staphylococcus aureus* cells based on CdSe quantum dots and propidium iodide fluorescent labeling

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A method of detecting live/dead *Staphylococcus aureus* cells was developed based on CdSe quantum dots-immunoglobulin G and propidium iodide fluorescent labeling. CdSe quantum dots were synthesized and surface-modified with immunoglobulin G and subsequently subjected to label *S. aureus.* The live *S. aureus* cells were stained by CdSe QDs-IgG using *S. aureus* protein A as target. The results showed that CdSe quantum dots with carboxyl were highly luminescent, stable, and successfully conjugated with the immunoglobulin G after staining (40 min). The optimal concentration of the immunoglobulin G coupled with CdSe quantum dots (1.0 mg/ml) was 80 ng/ml. Quantum dots-immunoglobulin G had high affinity to the surface protein A of *S. aureus* and exhibited high recognition property for three pathogenic *S. aureus* compared to *Escherichia coli*, *Streptococcus thermophilus*, and one non-pathogenic *S. aureus*. The fluorescence intensity of CdSe quantum dots decreased with an increasing ratio of dead *S. aureus*, while the fluorescence intensity of propidium iodide increased.

Kay words: *Staphylococcus aureus*, CdSe quantum dots, *Staphylococcus aureus* protein A, immunoglobulin G, propidium iodide, fuorescence resonance energy transfer.

INTRODUCTION

Staphylococcus aureus is one of the most common and broad spectrum pathogenic bacteria, which can cause of food contamination and responsible for many food-borne diseases. *S. aureus* food-borne diseases are estimated to cause suppurative types of infections and lead to deaths (Arcuri et al., 2010; Hennekinne et al., 2011).

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Abbreviations: QDs, Quantum dots; **IgG,** immunoglobulin G; **PI,** propidium iodide; **SpA,** *Staphylococcus aureus* protein A; **FRET,** Fuorescence resonance energy transfer; **TGA,** thioglycolic acid; **EDC,** 1-Ethyl-3-(3-dimethyllaminopropyl)-carbodiimide; **PBS,** phosphate buffered saline tablets; **FDA,** fluoresceinediacetate.

Because of the low infectious dose and high health risk, *S. aureus* have a differential ability to spread and cause outbreaks throughout various food items (Weiler et al., 2011). In general, *S. aureus* can be killed or damaged and partial to loss of activity by heat-treated, high pressure, radiation, or a chemical etc. Dead *S. aureus* cells can not cause clinical infections and food-borne infections, but there are closely related bacterial pathogens with the potential to cause infections by secreting a variety of extracellular toxins (Murray et al., 2005). Staphylococcal toxins residues can also cause severe gastrointestinal illness at relatively low concentrations and retain toxigenicity even after heat treatment (Ostyn, 2010). Therefore, monitoring and detection of live, sublethal and dead *S. aureus* cells in the environment, and food are currently being pursued to direct clean contamination, produce food safety and

reduce disease transmission (Zhao et al., 2009; Kahlisch et al., 2010).

S. aureus protein A (SpA), a cell wall product of certain pathogenic *S. aureus* strains, is a specific receptor for the Fc portion of many mammalian immunoglobulin G (IgG) subclasses (Yamada., 1996; Nguyen et al., 2010), and can be used as a specific anti-IgG reagent. Specific detection of *S. aureus* using IgG as bioprobes based surface protein A target has been demonstrated (Liu et al., 2007; Wang et al., 2011).

Currently, QDs are widely used in biological and even in medical studies because they are attractive fluorophores for multicolor imaging due to their broad absorption and narrow emission spectra, and the fact that they are brighter and far more photostable than those of conventional organic dyes (Jaiswal et al., 2003; Gao and Nie, 2005; Weng et al., 2006). The most popular and successful use of QDs is their application as a tool for cell-labeling, especially for monitoring living cell trafficking. Fluorescence immunoassays methods were successfully used to detect live bacteria by QDs labeling antibodies (Yang et al., 2006; Zhao et al., 2009). Propidium iodide (PI) is a traditional organic dyes, and cannot penetrate the intact cell membranes, whereas can penetrate damaged cell membranes and produce fluorescence when combined with DNA (Prakash, 2006; Kahlisch et al., 2010). The emission wavelength of green CdSe QDs can excited PI to cause excitation spectrum. Therefore, fluorescence resonance energy transfer (FRET) system can be constructed between CdSe QDs (donor) and PI (acceptor), making it possible for monitoring and detection of live and damaged *S. aureus* simultaneously (Ma et al., 2005).

For this reason, we prepared highly luminescent CdSe QDs with carboxyl and coupled with IgG to label *S. aureus* and used CdSe QDs-IgG and PI as labeling to identify live/dead *S. aureus*. This method provides a new way of thinking to detect both live and dead bacterial cells and extend the FRET applications in detection of bacterial.

MATERIALS AND METHODS

General supplies

S. aureus protein A (SpA; 42.7 KD), propidium iodide, were purchased from Sigma (St. Louis, MO, USA), while goat anti-rabbit immunoglobulin G was purchased from Sino-American Biotechnology Company (Shanghai, China). Thioglycolic acid (TGA, Tianjin Chemical Reagents Co., Tianjin, China) was used for surface modification of CdSe-QDs. Other inorganic reagents were provided by Shanghai Chemical Reagents Company. Ultra pure water was used throughout. Microbial strains of *S. aureus* 1 (ATCC25923) were obtained from the Institute of Microbiology Chinese Academy of Sciences. Microbial strains of pathogenic *S. aureus* 2 to 4 were separated from the mastitis milk and kept in Key Laboratory of Analysis and Detection Technology for Food Safety, Anhui, China. *Escherichia coli*, *Streptococcus thermophilus* were also obtained from key laboratory of analysis and detection technology for food safety.

Synthesis of water-soluble CdSe quantum dots and coupled with IgG

Colloidal CdSe QDs with carboxyl was prepared as reported in reference (Xue et al., 2009) by using TGA as stabilizer. The TGA-modified CdSe QDs was used to immobilize the IgG. For measuring the likely concentrations of the IgG coupled with CdSe QDs and integration times, 60 to 120 ng/ml IgG, 1.0 mg/ml CdSe QDs and 100 µl 1.0 mg/ml EDC were reacted together for 10 to 70 min at 37℃, and then the mixtures were purified using Dialysis bags to wash away the excess IgG. In the solution state, the fluorescence intensity was measured and time data was directly obtained from the F-970 spectrofluorometer (Shanghai, China).

Bacterial culture and aerobic plate count

Six bacteria strains were cultured in 100 ml of Luria Bertani (LB) broth, respectively. After incubation overnight at 37℃, the bacterial cells were centrifuged for 10 min at 1 600×*g*. The bacteria were washed twice with the sterilized PBS (pH 7.4) and then resuspended in 10 ml PBS by vortex for approximately 10 s. For live/dead analysis, *S. aureus* were killed and damaged by the heated treatment, and the counts of live *S. aureus* cells were examined with Aerobic plate count. Aerobic plate count was determined by inoculating 1 ml of samples onto nutrient agar plates and incubated aerobically at 37℃ for 24 h and colonies counted. After logarithmic transformation, live/dead ratio and standard deviation were calculated for the different incubates and plotted.

CdSe QDs-IgG and PI coupled with live/dead *S. aureus* cells

10 ml bacteria suspension (10^5 CFU/ml) was placed in a 100 ml beaker and mixed with 600 µl QDs-IgG (1.0 mg/ml). The mixture was incubated on the beaker for 10 to 60 min, and then rapidly washed with PBS. The fluorescence intensity of the bacteria suspension coupled with QDs-IgG was measured and compared to obtain the high specific bind on bacteria strains. The best conditions of QDs-IgG coupled with live *S. aureus* cells were further measured.

A stock solution of the propidium iodide stains was prepared by diluting both stains in 5 ml of filter sterilized sheath fluid. From this, a staining solution was made by diluting the stock solution 1/10. After incubation, 500 µl *S. aureus* cell suspensions, 500 µl CdSe QDs-IgG and 500 µl PI staining solution were mixed and incubated for 30 min at 37℃ in the dark, and then the mixtures were purified using ultra-filtration membrane (0.22 µm) to wash away the excess CdSe QDs. Further, the fluorescence intensity and fluorescence images of live/dead *S. aureus* were measured by the spectro-fluorometer and Olympus microscope system (Tokyo, Japan).

RESULTS

The fluorescence emission spectra of CdSe QDs and PI

Figure 1 shows the fluorescence emission spectra of CdSe QDs with carboxyl and PI in PBS buffer solution (pH 7.0). The maximal emission spectrum peaks of CdSe QDs and PI are at 519 and 590 nm after excited with 400

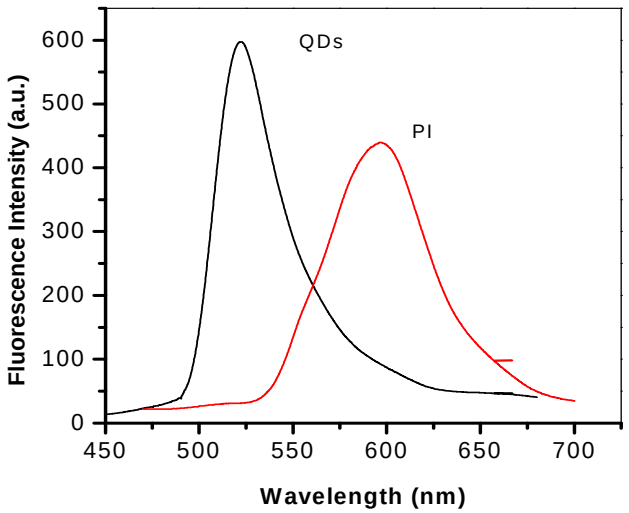

Figure 1. Fluorescence emission spectra of CdSe quantum dots and PI solution.

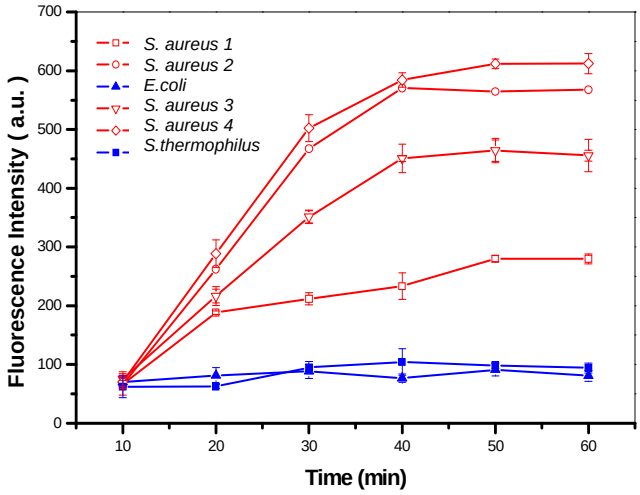

Figure 3. The binding isotherms for QDs-IgG onto different bacterial strains based on the variation of fluorescence intensity of stained bacterial.

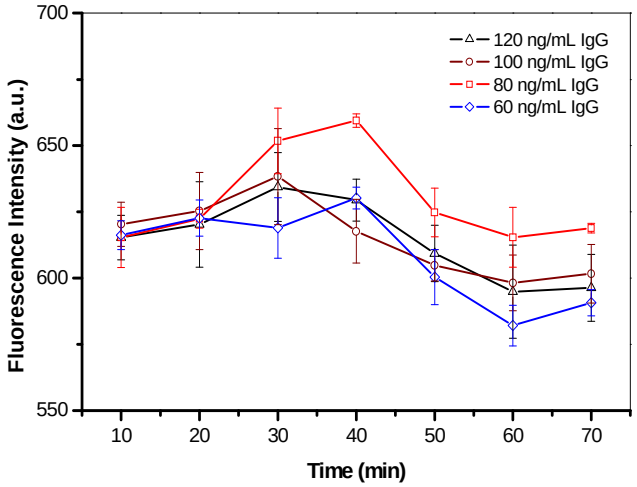

Figure 2. Effect of IgG concentrations and reaction time on the fluorescence intensity of CdSe QDs coupled with IgG.

and 520 nm excitation wavelength, and the distinguish-able emission colors are green and red, respectively. The emission wavelength of CdSe QDs may excited PI to cause excitation spectrum.

The IgG coupled with CdSe QDs and for live *S. aureus* labeling

As shown in Figure 2, within 30 to 40 min, the fluorescence intensity was increase with the integration time, but the fluorescence intensity was not increase approximately with the reaction time lengthened. The and 1.0 mg/ml CdSe QDs were respectively reacted together for 40 min at 37℃ to determine the likely coupled with antibody concentrations. As shown in Figure 2, fluorescence intensity of IgG coupled with CdSe QDs solution increased with antibody concentrations from 60 to 80 ng/ml, but CdSe QDs coupled with antibody was restricted with antibody concentrations and decrease when CdSe QDs concentrations achieved 100 and 120 ng/ ml. Thus, to prepare effective QDs-IgG conjugates, the minimum level of the concentration of the antiserum was 80 ng/ml, and integration times of CdSe CdSe QDs coupled with IgG was approximately 40 min in this level.

In order to identify whether the QDs-IgG specific bind on target pathogenic *S. aureus* cells, *E. coli*, *S. thermophilus* and nonpathogenic *S. aureus* were chosen as control bacteria, and the binding capabilities and recognition specificity were examined. Figure 3 shows the experimental binding isotherms for QDs-IgG onto 10^5 CFU/ml *S. aureus*, *E. coli*, and *S. thermophilus*, respectively. It can be seen that the binding capacity and recognition specificity of QDs-IgG on three pathogenic *S. aureus*, in this study, is high. On the other hand, QDs-IgG similarly showed low binding quantity and recognition specificity for *E. coli*, *S. thermophilus* and nonpathogenic *S. aureus*.

Labeling and detection of live/dead on *S. aureus* cells

The fluorescence microscopy images of live, 58% dead and all dead *S. aureus* cells treated with QDs-IgG, as seen shown Figure 4. Figure 4A demonstrates that a lot of green luminescent grape-shaped *S. aureus* cells were antibody in this study was 40 min. 60 to 120 ng/ml IgG best integration times of CdSe QDs coupled with

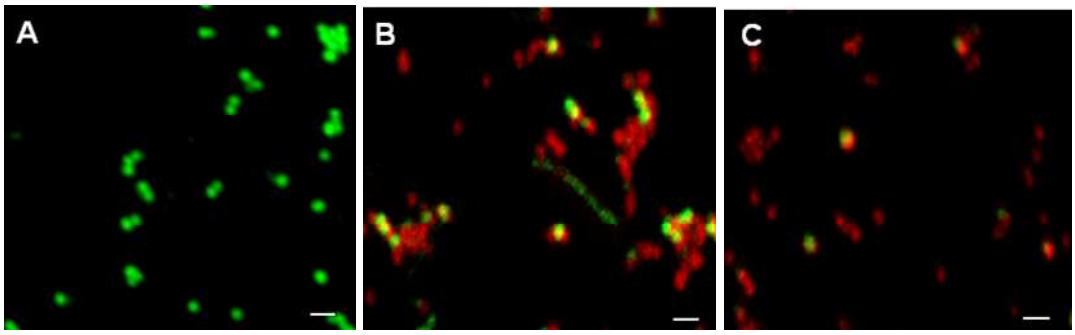

Figure 4. Fluorescence images (×400) of live and dead *S. aureus* cells treated with QDs-IgG and PI solution. (A), (B), and (C) indicate the images of live, 58% dead and all dead *S. aureus* cells, respectively. The scale bars are 1 µm.

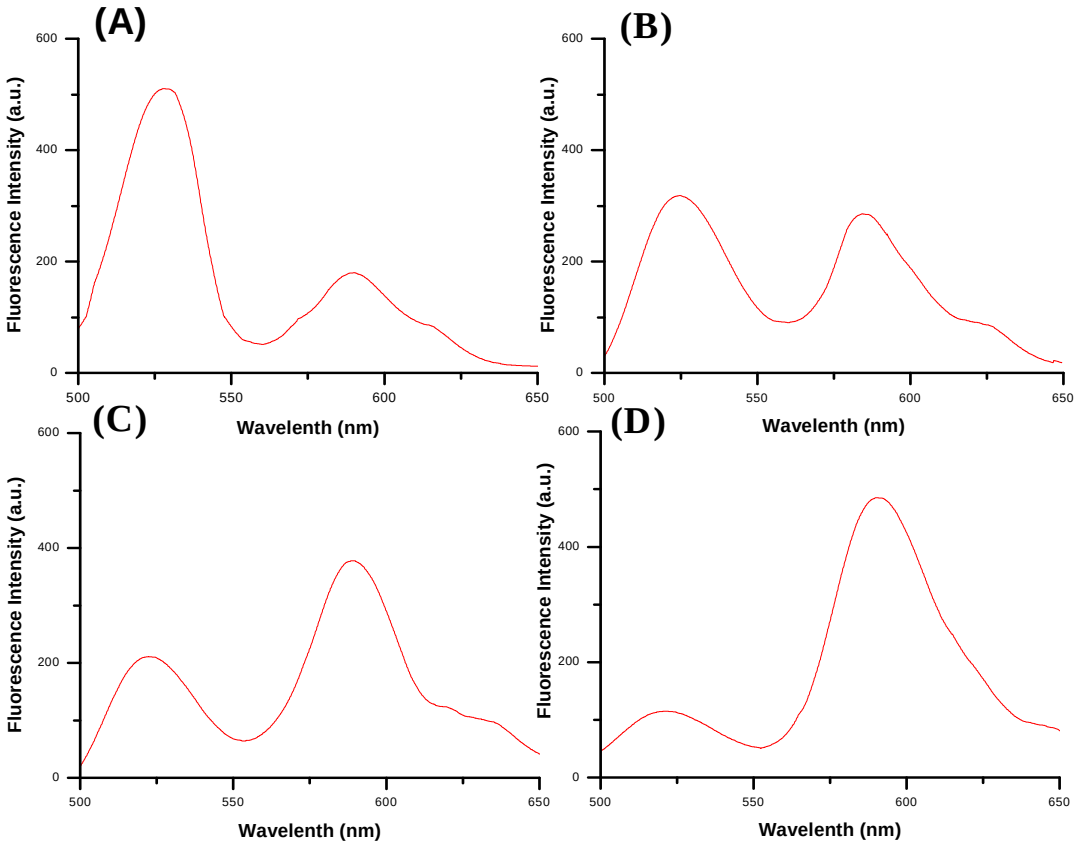

Figure 5. Variation of fluorescence intensity of live and Dead *S. aureus* coupled with QDs and PI. The fluorescence emission peaks of CdSe QDs and PI are at 519 nm and 590 nm, respectively. (A), (B), (C) and (D) indicate the fluorescence emission spectra of 10, 25, 58 and 82% dead *S. aureus*, respectively.

clearly seen approximately 30 min after the addition of the CdSe QDs-IgG solution to live *S. aureus*. In contrast, a lot of red luminescent dead *S. aureus* cells were observed under a fluorescent microscope (Figure 4C), which might be PI excited by CdSe QDs to cause red excitation fluorescence. Figure 4B demonstrates that a lot of red luminescent bacterial cells were dead *S. aureus*, few green luminescent dots were live *S. aureus*.

Figure 5 shows the fluorescence spectrum of CdSe QDs and PI coupled with 10, 25, 58 and 82% dead

S. aureus cells, respectively. The results showed that there are two fluorescent emission spectrum peaks at 519 and 590 nm after excited by blue Sapphire laser, which are the emission peaks of CdSe QDs and PI, respectively. Figure 5A indicated that CdSe QDs-IgG coupled with *S. aureus* have high fluorescence intensity when the ratio of dead *S. aureus* was 10%. However, the fluorescent emission spectrum of CdSe QDs become invisible approximately the after addition of the QDs-IgG and PI solution to the 82% dead *S. aureus* cells (Figure 5D), but fluorescent emission spectrum peak of PI was high, which might be due to the more PI penetrating damaged cell membranes and accepted energy from CdSe QDs. The results indicate that the fluorescence intensity of CdSe QDs was decrease with increasing of the ratio of dead *S. aureus*, but the fluorescence intensity of PI was increase.

DISCUSSION

S. aureus food poisoning is one of the most common food-borne diseases representing a potential hazard for consumers. In order to decrease false negative rate in detection, some surface proteins of *S. aureus* are expected to become novel target for developing new labeling probes. In this study, we have prepared highly luminescent CdSe QDs coupled with IgG to label *S. aureus* and used CdSe QDs-IgG and PI as labeling to identify live/dead *S. aureus*. This is the first demonstration that live/dead *S. aureus* were detected by labeling of CdSe QDs-IgG and PI.

SpA, which in the cell wall of 99% pathogenic *S. aureus* (Nguyen et al., 2010), is a cell wall-associated adherence protein that are important for colonization, tissue invasion, evasion of host defenses, and nutrient acquisition. As a cell wall product of certain pathogenic strains of *S. aureus*, SpA is a specific receptor for the Fc portion of many mammalian immunoglobulin G (IgG) subclasses (Yamada, 1996). Surface protein A of *S. aureus* was used in this research as labeling target; the IgG was selected to stain live *S. aureus*. The goat anti-rabbit immunoglobulin were successfully coupled with the surface of CdSe QDs after stained (30 min at 37℃), the protei n A of live *S. aureus* appears to be mediated by interaction with the IgG. QDs-IgG, having high affinity to the surface protein A of *S. aureus*, exhibited high recognition property for three pathogenic *S. aureus*. On the other hand, QDs-IgG similarly showed low binding quantity and recognition specificity for *E. coli*, *S. thermophilus* and nonpathogenic *S. aureus*. One possible reason is that the lack of proteins anchored on the surface of *E. coli*, *S. thermophilus,* and the interaction would not happen between IgG and bacteria. Lack of SpA or IgG-binding domain region in surface of nonpathogenic *S. aureus* partly accounted for the loss of the fluorescence (Moodley et al., 2008). SpA, a cell wall component of pathogenic *S. aureus* strains has found many uses as a research tool, as a diagnostic reagent and even as a possible IgG labeling receptor to monitor pathogenic *S. aureus* (Liu et al., 2007; Wang et al., 2011).

Experimentally, the concentrations of QDs and antibody are very important in order to enhance the sensitivity for immunoassay. In this study, fluorescence intensity of IgG coupled with CdSe QDs solution increased with antibody concentrations from 60 to 80 ng/ml, but was restricted with antibody concentrations and decrease when CdSe QDs concentrations achieved 100 and 120 ng/ml. Indeed, literature reports demonstrated that the coating of CdSe QDs with histidine-tagged biomaterial, such as proteins, DNA, antibodies etc. leads to a luminescence enhancement, as a result of the significant change in the local environment around the emissive inorganic particles (Kloepfer et al., 2005; Gazouli et al., 2010; Majithia, 2011). QDs fluorescence intensity enhancement is related to the high quantum yield of QDs surface area. Due to the conjugation of the antibody, the high surface area of CdSe QDs, a large number of atoms are exposed at the QDs surface (Biju, et al., 2008), which helps to improve quantum yield, but the effect may be partially counteracted by the additional antibody concentrations. The SpA contained a number of functional regions and conserved domains previously described in *S. aureus*, including four IgG-binding domains and apolymorphic X-region (Moodley et al., 2008). On the one hand, if there are too little QDs-IgG, a limited amount of antibody is coupled with binding sites, and lower concentrations of QDs-IgG can lead to a lower fluorescence single with bacteria (Wang et al., 2011). On the other hand, if there is too much QDs-IgG, a binding reaction for all binding sites is carried out, and higher concentrations of QDs-IgG can lead to a higher inhibition of fluorescence intensity with bacteria.

Monitoring and detection of live/dead food-borne pathogens are very important to control contamination of *S. aureus* and reduce transmission of diseases. Propidium iodide (PI) is a traditional organic dyes, and cannot penetrate the intact cell membranes, but can penetrate damaged cell membranes and produce fluorescence when combined with DNA. For the present study, there are some literature reports about the application of organic fluorescent dyes in live and dead bacteria detection. Kahlisch et al. (2010) used SYTO9 and PI as a staining procedure indicating membrane-injured cells to distinguish live and dead bacteria at the taxonomic level. Two fluorescent dyes, fluoresceinediacetate (FDA) and PI were used to selectively stain living and dead cells (Hannig, 2007). But PI and other conventional dyes are less suitable for extended periods of bio-imaging using fluorescent and confocal microscopy because the organic fluorophores tend to quench rapidly and the presence of strong background autofluorescence (De Grand et al., 2006). Our research result shows that PI have low fluorescence

intensity, and the emission spectrum is relatively broad spectral width, which provided sufficient spectral resolution for quantitative detection of the fluorescence intensity. Quantum dots (QDs) are a new class of fluorescence label and allow the use of a single excitation wavelength, which greatly simplified the optical system used to make simultaneous detection easier. QDs have been used for the sensitive detection of target bacterial analytes (Kloepfer et al., 2003; Huang et al., 2008). The outstanding photophysical properties of semiconductor QDs have encouraged the development of binding assays, based on fluorescence resonance energy transfer (FRET), for the detection of a diversity of biorelevant analytes (Shi et al., 2007; Wang and Xia, 2011). Our experiments have shown that the maximal emission spectrum peak of prepared CdSe QDs with carboxyl was at 519 nm after excited with 400 nm. The emission wavelength of green CdSe QDs can excited PI to cause excitation spectrum. Therefore, the combined use of green CdSe QDs and PI enables the identification of both viable and dead bacterial cells simultaneously.

In this research, QDs-IgG and PI were used to selectively stain living (green) and dead (red) *S. aureus*. This study results indicate that the fluorescence intensity of CdSe QDs was decrease with increasing of the ratio of dead *S. aureus*, but the fluorescence intensity of PI was increase. According to the variation of fluorescence intensity of CdSe QDs-IgG and propidium iodide, the live/dead *S. aureus* were detected.

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