A fluorescent biosensor based on quantum dot-labeled streptavidin and poly-L-lysine for the rapid detection of *Salmonella* in milk

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ABSTRACT

Salmonella, as a common foodborne pathogen in dairy products, poses a great threat to human health. We studied a new detection method based on quantum dots (QD). A fluorescent biosensor with multiple fluorescent signal amplification based on a streptavidin (SA) biotin system and the polyamino linear polymer poly-L-lysine (PLL) were established to detect *Salmonella* in milk. First, *Salmonella* was captured on a black 96-well plate with paired *Salmonella* mAb to form a double-antibody sandwich. Second, SA was immobilized on biotin-modified mAb by SA-biotin specific bond. Then, the biotin-modified polylysine (BT-PLL) was bound on SA and specifically bonded again through the SA-biotin system. Finally, water-soluble CdSe/ZnS QD-labeled SA was added to a black 96-well plate for covalent coupling with BT-PLL. The fluorescent signal was amplified in a dendritic manner by the layer-by-layer overlap of SA and biotin and the covalent coupling of biotinylated PLL. Under optimal conditions, the detection limit was 4.9×10^3 cfu/mL in PBS. The detection limit was 10 times better than that of the conventional sandwich ELISA. In addition, the proposed biosensor was well specific and could be used for detecting *Salmonella* in milk samples.

Key words: quantum dots, milk, *Salmonella*, fluorescence

INTRODUCTION

Salmonella is a pathogenic bacterium with over 2,500 serotypes identified that can cause zoonotic diseases (Foley and Lynne, 2008; Liébana et al., 2009); it is widely distributed in nature. *Salmonella* Anatum is one of the serotypes of *Salmonella*. Since *Salmonella* Ana-

tum was first identified as causing infection in human infants (1948), it has become an important zoonotic pathogen and has caused widespread outbreaks (Hartmann and West, 1995; Singh et al., 2010). Its effect on human health and safety is greater than other foodborne pathogens (Wu et al., 2021) because it is commonly present in human food and animal-derived food (e.g., milk, meat, and eggs). Milk is an important part of the human diet. However, because it contains different nutrients (Zhou et al., 2017; Murasova et al., 2020), milk is the best medium for the growth and proliferation of various bacteria (Angela et al., 2006; Bano, 2020). Drinking milk contaminated with *Salmonella* can easily cause abdominal pain, diarrhea, acute gastroenteritis, and even death (Ni et al., 2018; Wang et al., 2018). Therefore, an accurate, simple, and efficient method for detecting *Salmonella* in milk should be established.

Established techniques for determining *Salmonella* include bacterial culture, PCR (Kubo et al., 2020), loop-mediated isothermal amplification (Chen et al., 2015b; Wen et al., 2020), and immunofluorescence detection (Yang et al., 2018; Yu et al., 2019). Bacterial culture, which is a gold standard method for detecting bacteria, has high accuracy but is time consuming and labor intensive (Yu and Stopa, 1996; Majdinasab et al., 2018; Xiong et al., 2019). The PCR method is sensitive and specific (Chhalliyil et al., 2020) but requires skilled technicians and expensive instruments (Tsen et al., 2013; Yu et al., 2019). High requirements for pretreating food samples can easily result in false negatives (Mercanoglu Taban et al., 2009). Loop-mediated isothermal amplification has high sensitivity and short response time (Fan et al., 2015) but is susceptible to aerosol contamination. This process is also dependent on unobvious turbidity, and color changes may lead to false-negative results (Kaewphinit et al., 2013; Momin et al., 2020).

Recently, with the advancement of nanomaterial technology and biotechnology, various biosensors for detecting pathogenic bacteria have been developed, including optical (Wang et al., 2020), magnetic (Chen

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et al., 2015a; Qi et al., 2021), and electrochemical (Feng et al., 2021). As an important part of the optical sensor, the fluorescence sensor has many advantages including simple operation, high sensitivity, fast speed, and easy readout (Xue et al., 2018). Furthermore, the fluorescent label is very important for the detection performance of the fluorescence sensor. Among different fluorescent labels, quantum dots (QD) have been widely used in fluorescence sensors because of their high stability and high fluorescence performance (Chen et al., 2019). Fluorescence technology relies on the output of light signal when the target has been identified and labeled by the probe. The presence of more targets indicates more labeling by the probe and greater output light signal. The realization of the high sensitivity of the fluorescence immunoassay technology mainly depends on the amplification of the fluorescence signal (Wu et al., 2020b). Currently, many methods have been proposed based on fluorescence signal amplification strategies, including rolling circle amplification (Zhang et al., 2018b; Xiao et al., 2020), silver nanocluster amplification (Zhang et al., 2021), polymerization-induced nanobeads (Wu et al., 2020b), and nucleic acid fragment amplification (Liu et al., 2016; Yu et al., 2019). However, few studies have been done on the amplification of fluorescent signals based on polymer and streptavidin-biotin systems.

In this study, *Salmonella* Anatum was used as the research model, a fluorescent sensor using polyamino linear polymer poly-L-lysine (PLL) macromolecule as the probe skeleton, water-soluble QD as fluorescent probes, and the streptavidin-biotin system was applied for rapidly detecting *Salmonella* in milk. The water-soluble QD (PEGylated, CdSe/ZnS/–COOH) used in this study enabled the synthesized QD-SA fluorescent probes to maintain high stability and reduced nonspecific adsorption (Bentzen et al., 2005; Zhang et al., 2018a). The PLL is a linear polymer containing many amino groups and can be used as a multifunctional molecular carrier. Several studies have been reported that the covalent modification of PLL on its surface can increase the loading of probes (Zou et al., 2019; Wu et al., 2020a). Thus, to enhance the output fluorescent signal and identify more targets, PLL were introduced in the proposed biosensor to load more fluorescent probes. If the PLL macromolecules are directly modified on the surface of QD, cross-linking PLL and QD can be easily carried out. Thus, the QD become abnormally large and agglomerate because PLL is very large. Therefore, PLL was selected for covalent coupling with biotin. The PLL contains a large number of amino sites. After coupling with biotin, the PLL can amplify biotin. In addition, the SA-biotin system has also been introduced into fluorescent sensors to improve sensitivity. One molecule of biotin can bind to 4 molecules of biotin through high

affinity (Yue et al., 2021). Biotin based on polymer amplification can be combined with more fluorescent probes (QD-SA) through the SA-biotin system, facilitating the loading of more fluorescent markers on the surface of PLL to a certain extent. Meanwhile, PLL can also use SA as a bridge to bind more biotin-modified monoclonal antibody (BT-mAb) to amplify signals to recognize more target bacteria, thereby increasing the sensitivity of detection. In general, the amplification strategy of using polymer-amplified biotin to achieve fluorescence signal is based on the signal amplification of particles. Compared with a single fluorescent probe, the fluorescence signal is amplified as a whole in this method.

We amplified multiple fluorescent signals during the detection process by synthesizing QD-SA probes, biotin-modified polylysine (BT-PLL), and overlapping SA and biotin layers to form the dendrite structure (BT*mAb-SA-BT*PLL-QD*SA; * indicates amide bond). A schematic diagram of the detection process is shown in Figure 1(C). We first used the high specificity of the mAb to capture *Salmonella* in black 96-well plates. Then, we modified the SA on the BT-mAb and the BT-PLL on the SA. Finally, we attached the QD-SA fluorescent probes to BT-PLL to form dendritic structures. Thus, through the layer-by-layer overlapping of SA and biotin and multiple-loaded fluorescent label of PLL, we achieved multiple amplification of the signal to lower the detection limit (LOD) and made the detection more sensitive.

MATERIALS AND METHODS

Materials and Reagents

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl) and N-hydroxysuccinimide sodium salt (NHSS) were provided by Aladdin. Water-soluble CdSe/ZnS QD (item no. Q3605, PEG/–COOH) were obtained from Wuhan Jiayuan Quantum Dot Technology Company. The PLL hydrobromide (30–70 kDa) was supplied by Sigma-Aldrich. The SA was provided by the Shanghai Hualan Technology Company. Bovine serum albumin, agarose, horseradish peroxidase-labeled SA (HRP-SA), and chromogenic fluid (tetramethylbenzidine and H₂O₂) were purchased from Solarbio Science Company. In addition, (+)-biotin N-hydroxysuccinimide ester (BT) was purchased from Thermo Fisher Scientific. Paired *Salmonella* mAb were obtained from Alpha Lifetech Co. The mAb is specific to the following *Salmonella* species: *Salmonella* Typhi A, *Salmonella* Agona, *Salmonella* Typhimurium, *Salmonella* Paratyphi B, *Salmonella* Thompson, *Salmonella* Blockley, *Salmonella* Kentucky (C3), *Salmonella* Enter-

itidis, *Salmonella* Typhi, *Salmonella* Dublin, *Salmonella* Anatum, and *Salmonella* Arizonae. Pasteurized milk was bought at a convenience store (Meiyijia, Jiangxi, China). Carbon film copper mesh (300 mesh) was purchased from Zhongjing Science & Instrument Co. Ltd.

Equipment

A multifunctional reader Varioskan LUX microplate was obtained from Thermo Fisher Scientific Company. In addition, a 96-well black polystyrene plate and 96-well transparent microtiter plate were purchased from Corning Co. The refrigerated centrifuge was supplied by Eppendorf. The particle size of the QD-SA complex and the zeta potential of BT-PLL were measured by a Zetasizer Nano ZS90. Agarose gel electrophoresis of the QD-SA image was performed in a Chemi Doc

MP imaging system (Bio-Rad). Transmission electron microscopy (TEM) images of the QD and QD-SA were obtained by the JEM-2100 transmission electron microscope (JEOL Ltd.). Agarose gel electrophoresis of QD-SA was measured by DYY-6C Electrophoresis system (Bio-Rad).

Bacteria Culture Conditions and Counting

We used *Salmonella* Anatum (ATCC 9270) in this study as target bacteria, and *Shigella sonnei* (Jiangxi CDC isolates), *Escherichia coli* O157:H7 (CMCC 44102), *Listeria monocytogenes* (ATCC 19115), *Pseudomonas aeruginosa* (CMCC 11997), and *Staphylococcus aureus* (CMCC, 2603) were used as nontarget bacteria in the experiments. These strains were inoculated in Luria-Bertani medium and cultured at 37°C for 14 to

Figure 1. Detection of *Salmonella* by fluorescence sensor: (A) the preparation of quantum dot (QD)-streptavidin (SA), (B) the preparation of biotin-modified polylysine (BT-PLL), and (C) the fluorescence detection assay for detection of *Salmonella* in milk. EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHSS = N-hydroxysuccinimide sodium salt; Ex = excitation (wavelength); Em = emission (wavelength).

18 h in a shaker incubator and then cultivated for 18 to 20 h on agar solid medium for plate counting. The results of plate counting were expressed as colony-forming units. After the experiment, all experimental strains were sterilized in a sterilization pot at 121°C for 30 min for the safety of the laboratory.

Synthesis of QD-SA

A synthesis schematic diagram of QD-SA is shown in Figure 1(A). The QD-SA was prepared according to the methods of Shao et al. (2018) with slight modifications. The molar ratio of QD/EDC/NHSS/SA was 1:2,000:1,000:20. First, the QD were diluted to 1 μ M with 0.01 M sterile borate buffer solution (BBS, pH 7.4), and then 15 μ L of freshly prepared EDC (5 mg/mL in BBS) and 6.96 μ L of freshly prepared NHSS (5 mg/mL in BBS) were added in sequence. These mixtures were gently mixed at room temperature for 30 min to activate the carboxyl groups on the surface of the QD. Then, SA was added to the mixture and mixed for 2 h at room temperature. After mixing for 2 h, 1% (wt/vol) BSA was used to block unreacted carboxyl groups on QD. Then, the reacted mixture was centrifuged at $10,000 \times g$ for 3 min at room temperature to remove possible agglomeration. Then, a 100-kDa ultrafiltration membrane was used to centrifuge at $8,000 \times g$ at room temperature for 5 min to remove excess SA. After 5 consecutive centrifugations, purified and concentrated QD-SA was collected in a low adsorption centrifuge tube, and then the collected purified and concentrated QD-SA was stored at 4°C in the dark for future use.

Characterization of QD-SA by Agarose Gel Electrophoresis

The electrophoretic mobility of agarose gel electrophoresis can determine the successful synthesis of QD-SA. The first step was the preparation of 0.5% wt agarose gel (0.3 g of agarose dissolved in 60 mL of $1 \times$ Tris-acetate-EDTA buffer). Then the side of the gel with the sample well was placed on the negative electrode of the electrophoresis tank. The sample well in the gel was added with 10 μ L of 1 μ M QD, physical mixture of QD and SA, and QD-SA, respectively. The parameters of the electrophoresis instrument were set at 90 V for 50 min. When the electrophoresis was completed, the gel was analyzed using a Chemi Doc MP imaging system, and then bands appeared.

Synthesis of BT-mAb and BT-PLL

The synthesis of BT-mAb had been reported in a previous study (Jin et al., 2020; Li et al., 2020) and

was slightly modified. The active esterified biotin was reacted with the antibody at a molar ratio of 20:1. The coupling reaction, which was carried out in a sterilized centrifuge tube for coupling reaction wrapped in foil, was continuously stirred at 32 rpm for 2 h at room temperature. After the complete reaction, the mixture was centrifuged ($8,000 \times g$ for 5 min at 4°C) 5 times to remove excess biotin. The purified BT-mAb was collected after centrifugation, mixed with an equal volume of sterile glycerin, and stored at -20°C .

The BT-PLL was prepared according to the methods of Chirra et al. (2011) with slight modifications [Figure 1(B)]. First, 50 μ L of PLL (2 mg/mL in sodium bicarbonate buffer) in 0.01 M PBS was mixed with 3.96 μ L of freshly prepared active esterified biotin (1 mg of esterified biotin in 180 μ L of ultrapure water). The mixture was incubated for 45 min at room temperature. After the reaction, unreacted and excess biotin of the mixture was removed through centrifugation 5 times ($8,000 \times g$, 8°C, 5 min). After 5 centrifugation steps, the purified BT-PLL was collected and stored at 4°C.

Fluorescence Immunosorbent Assay

A schematic diagram of the fluorescence immunosensor is shown in Figure 1(C). First, the *Salmonella* capture antibody was resuspended to 2.5 μ g/mL with 0.01 M PBS. Then, 100 μ L of this antibody (2.5 μ g/mL) was coated on black 96-well plates and incubated at 4°C overnight. The antibody that was not fixed on the black 96-well plate was washed thrice with 0.01 M PBST (pH 7.4, containing 0.05% Tween 20) and spin-dried. The nonspecific binding sites were blocked with 1% (wt/vol) BSA (200 μ L/well) at 37°C for 2 h. Then the black 96-well plate was washed thrice with 0.01 M PBST and spin-dried. A 10-fold gradient of the *Salmonella* solution (10^1 – 10^7 cfu/mL, 100 μ L/well) was used as the positive group. The negative group (100 μ L/well) was added with sterile PBS and incubated at 37°C for 1 h. After the black 96-well plate was washed thrice with 0.01 M PBST, 100 μ L of 0.625, 1.25, 2.5, 5, 7.5, 10, 12.5 μ g/mL BT-mAb was added to each well and incubated at 37°C for 1 h. The black 96-well plate was washed 4 times. Then, 100 μ L of SA was added to each well and incubated for 30 min at 37°C. The black 96-well plate was washed 4 times. Then, 100 μ L of BT-PLL (molar ratio of SA:BT-PLL of 2:1, 2:3, 2:5, 2:7, 2:9, 2:11, and 2:13) was added to the black 96-well plate and incubated for 30 min at 37°C. The black 96-well plate was washed 5 times. Finally, 100 μ L of QD-SA (molar ratio of QD:SA of 1:5, 1:10, 1:15, 1:20, and 1:25; 1:100 dilution) was added to the black 96-well plate and incubated for 30 min at 37°C. The black 96-well plate was washed thrice and spin-dried. The fluorescent

signal from the immune complexes (excitation wavelength, 320 nm; emission wavelength, 605 nm) from the dried plates was directly read using the multifunctional microplate reader. The relative increase in fluorescence signal (F/F_0) was determined as follows:

$$F/F_0 = F_1 - F_{\text{blank}}/F_2 - F_{\text{blank}},$$

where F_1 and F_2 are the fluorescence intensities of the immune complex on the plate with and without *Salmonella*, respectively, and F_{blank} is the fluorescence intensity of the blank reagent.

The LOD of the method was calculated as $\text{LOD} = M + 3 \times \text{SD}$, where M is the fluorescence intensity of the negative control groups, and SD is the standard deviation of these groups (Duan et al., 2021).

Traditional ELISA Calibration Curve

The traditional ELISA method was performed according to the method of Guo et al. (2016) with slight modifications. *Salmonella* capture antibody (2.5 µg/mL, 100 µL/well) was coated onto 96-well polystyrene plate at 4°C overnight. After washing the 96-well plate twice with 300 µL of 0.01 M PBST, 1% (wt/vol) BSA blocking buffer (200 µL/well) was added to 96-well plate and incubated at 37°C for 2 h to block unspecific binding sites. After washing thrice, 100 µL of the diluted bacterial solution (10^1 – 10^7 cfu/mL) was added into the positive well and incubated in a 96-well plate for 1 h at 37°C. Sterile PBS (100 µL/well) was added to the negative well as a control. The 96-well plate was washed thrice with 0.01 M PBST, and biotinylated anti-*Salmonella* mAb (2.5 µg/mL, 100 µL/well) was added into 96-well plate and incubated for 1 h at 37°C. The 96-well plate was washed 5 times with 0.01 M PBST. The HRP-SA [diluted 5,000 times with 0.01 M PBS (100 µL/well)] was added to the 96-well plate. After the 96-well plate were incubated at 37°C for 45 min and washed 7 times with 0.01 M PBST, tetramethylbenzidine/ H_2O_2 (100 µL/well) was added and incubated at 37°C for 15 min. Then, 50 µL of 2 M H_2SO_4 was added to stop the reaction, and the 96-well plate was placed in the multifunctional microplate reader. The wavelength was adjusted to 450 nm to read the absorbance. All of the experimental data came from 3 parallel tests.

Specific Detection

We selected *Salmonella* and 5 other nontargeted strains (*Shigella sonnei*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) to assess the specificity of the

proposed method under optimized conditions, and the concentration of *Salmonella* and 5 other nontargeted strains (10^8 cfu/mL) was calculated by plate counting. The concentration of all experiment strains was diluted to 10^7 cfu/mL in sterile PBS. Sterile PBS was used as a negative control. All of the experimental data came from 3 parallel tests.

Sensitivity Detection of *Salmonella* in Whole Milk

Salmonella at a concentration of 4.9×10^8 cfu/mL was added to sterile whole milk; then, the spiked milk was serially diluted to make concentrations of 4.9×10^1 , 4.9×10^2 , 4.9×10^3 , 4.9×10^4 , 4.9×10^5 , 4.9×10^6 , and 4.9×10^7 cfu/mL. The concentration of *Salmonella* was calculated by plate counting. Milk without *Salmonella* was used as the negative control. All of the experimental data came from 3 parallel tests.

Data Analysis

All of the experimental data were obtained from 3 parallel tests. Version 19 SPSS (SPSS Inc.) was used to perform final statistics and analysis.

RESULTS AND DISCUSSION

Characterization of QD-SA

Optical Properties Analysis. The QD and QD-SA were scanned with a multifunctional microplate reader to determine their emission wavelengths. The fluorescence spectra of QD and QD-SA were measured at an excitation wavelength of 320 nm and an emission wavelength range of 505 to 705 nm. Figure 2(A) shows the original QD had maximum fluorescence intensity at 605 nm. After being covalently coupled with SA, it retained its maximum fluorescence intensity at 605 nm. In addition, the fluorescence intensity of the QD-SA is slightly lower than that of the QD, which may be caused by the dilution and consumption of QD in the reaction process (Zhou et al., 2021). Therefore, no obvious shift was found in the maximum emission peak compared with that of the uncoupled QD. This result showed that the coupling of SA did not change the fluorescence properties of the QD.

Particle Size Analysis. The successful synthesis of QD-SA was verified according to the hydrated particle sizes of QD and QD-SA, agarose gel electrophoresis, and TEM. The results of the particle size are shown in the Figure 2(B). Dynamic light scattering analysis showed that the free QD had a particle size of 15 nm. After the covalent coupling with SA, QD-SA was enlarged to 25 nm. In addition, the particle size of the synthesized

QD-SA was uniformly dispersed, and the small increase in particle size also indicated that no agglomeration occurred in the synthesized QD-SA, confirming the successful coupling of SA on the surface of the QD.

Agarose Gel Electrophoresis Analysis. Figure 2(C) shows 2 sets of parallel results of agarose gel electrophoresis before and after CdSe/ZnS QD was coupled with SA. Compared with the QD: lane 1, the physical mixture of QD and SA: lane 2 had no obvious change in migration distance, migration speed, and shape. By contrast, the QD-SA: lane 3 changed dramatically. First, its electrophoresis speed was slower than those of the lane 1 and the lane 2. Second, the migration distance became shorter. In addition, the lane 3 had a

tailing phenomenon. These changes are related to the increase in the overall size and reduction of the surface negative charge caused by the covalent coupling of the QD and SA (Sahoo et al., 2019; Shandilya et al., 2021). Because SA was conjugated to the surface of the QD, the overall size of the coupling is larger than those of the QD without conjugation [Figure 2(B)]. In addition, SA and QD are coupled by an amide bond. Due to excessive SA, a large number of carboxyl groups were consumed on the surface of QD, resulting in a decrease in the negative charge of the coupling. In the alkaline buffer system, negatively charged surface nanoparticles migrated from the negative electrode to the positive electrode (Yan et al., 2020a). Therefore, the migration

Figure 2. Characterization of quantum dot (QD)-streptavidin (SA): (A) fluorescence spectrum (a.u. = arbitrary units) of QD and QD-SA; (B) particle size of QD and QD-SA; (C) agarose gel electrophoresis of QD, physical coupling of SA and QD and QD-SA (the serial number is 1, 2, 3). (D) Transmission electron microscopy (TEM) image: (I), (II), and (III) are the TEM images of QD at 200, 50, and 10 nm, and (IV), (V), and (VI) are TEM images at 200, 50, and 10 nm. The white and gray arrows are the selected representatives. Vertical bars represent SD (n = 3).

Figure 3. Characterization of biotin-modified poly-L-lysine (BT-PLL): (A) zeta potential of PLL and BT-PLL; (B) Fourier-transform infrared spectra of PLL and BT-PLL. The arrow indicates the position of the absorption peak. Vertical bars represent SD ($n = 3$).

distance of the QD-SA in the gel is smaller than that of the QD and physical mixture of QD and SA. These results were consistent with a previous study (Zhang et al., 2017) on the functionalized QD probe, proving the successful coupling of the QD-SA probe.

TEM Analysis. The dispersion state, particle size, and lattice structure of the free QD and the QD marked with SA were evaluated by TEM to verify whether SA was successfully marked on the QD. As shown in Figure 2(D, IV–VI), the QD-SA was not agglomerated and was in a uniformly dispersed state. Similar phenomena have been reported in previous studies (Yan et al., 2020b). In Figure 2(D, III), the lattice structure of the QD was clear. In Figure 2(D, VI), the outermost lattice of QD-SA structure was slightly fuzzy, which was related to the modification of SA on the QD. In addition, QD-SA was larger than the QD. These results demonstrated the successful coupling of the QD-SA probe.

Characterization of BT-PLL

Zeta Potential Analysis. To verify the successful synthesis of BT-PLL, we measured the zeta potential and Fourier-transform infrared spectra (FTIR) spectra of PLL and BT-PLL. The zeta potential of PLL was $+7.98 \pm 0.0971$ mV ($n = 3$) because the surface of PLL had numerous amino groups (Figure 3[A]). After coupling with biotin, the zeta potential of BT-PLL decreased to -1.61 ± 0.6129 mV ($n = 3$) because the formation of the amide bonds between biotin and PLL consumed the amino groups on the surface of PLL,

thereby reducing the zeta potential of BT-PLL. Thus, the changes in zeta potential verified the successful connection of BT-PLL.

FTIR Analysis. The FTIR spectroscopy of PLL and BT-PLL is illustrated in Figure 3(B). The features of the BT-PLL spectrum at 1,641 and 1,462 cm^{-1} indicated the C=O and N–H vibrations, which were related to the formation of an amide bond (Shan et al., 2009). Given that biotin and PLL are coupled through an amide bond, C–O stretching vibrations were observed at 1,104 and 1,252 cm^{-1} , C–N stretching vibration at 1,420 cm^{-1} , and N–H stretching vibration at 3,450 cm^{-1} (Ling et al., 2014). In addition, the absorption peak at 3,270 cm^{-1} on the FTIR spectrum of PLL was the amino absorption peak (characteristic peak of PLL; Dutta et al., 2020), but there was no absorption peak at 3,270 cm^{-1} on BT-PLL, which showed that the formation of amide bond consumes the amino group on PLL. These changes proved the successful coupling of BT-PLL.

Optimization of Experimental Parameters

To receive optimal effect of the method, we optimized the SA:QD synthesis molar ratio, concentration of biotinylated antibody, amount of BT-PLL, and QD-SA incubation time.

Optimization of QD-SA Synthesis Molar Ratio. The concentration of *Salmonella* detected was 10^7 cfu/mL. Figure 4(A) illustrates the influence of the synthesis ratio of QD-SA fluorescent probes on F/F₀. When the SA:QD molar ratio was from 5:1 to 20:1,

Figure 4. Optimization of the experimental parameters: (A) the concentration of quantum dot (QD)-streptavidin (SA) synthesis molar ratio, (B) the concentration of biotin-modified monoclonal antibody (BT-mAb) concentration, (C) biotin-modified polylysine (BT-PLL) concentration, and (D) the incubation time of the QD-SA fluorescent probe. F/F_0 represents the relative increase in fluorescence signal. Vertical bars represent SD ($P < 0.05$, $n = 3$).

F/F_0 increased substantially. When the SA:QD molar ratio was 20:1, F/F_0 reached its maximum value. When the molar ratio continued to increase, F/F_0 showed obvious change. To obtain the maximum F/F_0 and save the amount of SA, we choose the SA:QD synthetic molar ratio of 20:1.

Optimization of BT-mAb Concentration. Figure 4(B) shows the effect of QD-SA incubation time on F/F_0 . When the concentration of BT-mAb increased from 0.625 to 2.5 $\mu\text{g/mL}$, F/F_0 increased significantly. When the concentration of BT-mAb continued to increase, its F/F_0 decreased significantly. This phenomenon indicated that the orifice plate could only bind a certain amount of antibody. When the amount of antibody was excessive, the excessive BT-mAb competes with the antigen recognition site on the surface

of *Salmonella*, leading to the failure of the reaction, which adversely affects the subsequent SA binding, thus reducing the probe marker amount and reducing the F/F_0 . In addition, it can also lead to the hook effect (Li et al., 2018). Here, we selected the concentration of BT-mAb at 2.5 $\mu\text{g/mL}$.

Optimization of BT-PLL Concentration. Figure 4(C) shows the effect of SA-QD incubation time on F/F_0 . When the concentration ratio of SA and BT-PLL was increased from 2:1 to 2:7, its F/F_0 increased. The maximum F/F_0 (best value) was reached when the concentration ratio was 2:7. The amount of biotin on PLL was proportional to the fluorescence intensity in a certain concentration range. When the amount of biotin on PLL was beyond this range, the amount was not directly proportional to the fluorescence intensity.

Optimization of QD-SA Fluorescent Probe Incubation Time. Figure 4(D) shows the effect of QD-SA incubation time on F/F_0 . Incubation times of 10, 30, 45, and 60 min were chosen. When the QD-SA incubation time in 96-well plate was increased from 10 to 30 min, its F/F_0 significantly increased. However, continued increase in incubation time did not change F/F_0 . To obtain the best effect and shorten the total experiment time, we chose 30 min as the best incubation time for the fluorescent probe.

Results of Specificity

To evaluate the specificity, *Salmonella* and 5 non-*Salmonella* strains were tested under optimal experimental conditions. The concentration of the 6 strains was 10^7 cfu/mL. Figure 5(B) shows that the F/F_0 group that captured *Salmonella* was clearly higher than the other 5 non-*Salmonella* strains, which indicated that the fluorescence intensity of *Salmonella* group was significantly higher than that of the control group ($P < 0.05$), whereas the fluorescence intensity of other 5 non-*Salmonella* species was similar to the control group ($P > 0.05$). These results indicated the method had good specificity.

Calibration Curve of Traditional ELISA for the Detection of Salmonella

Under optimized conditions, calibration curves (Figure 5[A]) were established with known *Salmonella* concentrations and absorbance values. The linear range was 4.9×10^4 to 4.9×10^7 cfu/mL. The linear equation was $y = 0.01834x - 0.02194$ ($R^2 = 0.99148$). When the concentration of *Salmonella* was 4.9×10^4 , the absorbance value of the experimental group was slightly equal to the absorbance value corresponding to the LOD [$0.0978 (N + 3SD)$], where N is the mean of the negative group. Therefore, the LOD concentration of *Salmonella* was 4.9×10^4 cfu/mL.

Results of Sensitivity Detection in PBS Buffer and Whole Milk

Under optimized conditions, the sensitivity of *Salmonella* in PBS was determined using this proposed method. Then the curve (Figure 6) of the *Salmonella* concentration and F/F_0 was established, and the linear range was 4.9×10^3 to 4.9×10^7 cfu/mL using the equation $F/F_0 = 0.4324\text{Log}_{10}(S) + 0.0284$ with $R^2 = 0.994$. When the *Salmonella* concentration was

Figure 5. (A) Calibration curve of absorbance versus concentration of *Salmonella* from 4.9×10^1 to 4.9×10^7 cfu/mL and (B) specificity of fluorescence detection method for 10^7 cfu/mL of *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Shigella sonnei*, and *Pseudomonas aeruginosa*. F/F_0 represents the relative increase in fluorescence signal. Vertical bars represent SD ($P < 0.05$, $n = 3$). a.u. = absorbance units.

Table 1. Detection results of *Salmonella* in the spiked milk samples (n = 3)

Target bacteria	Sample	Plated count method (cfu/mL)	Proposed method (cfu/mL)	Recovery rate (%)	RSD ¹ (%)
<i>Salmonella</i>	1	4.9×10^4	4.12×10^4	84	9.28
	2	4.9×10^5	4.85×10^5	98	6.55
	3	4.9×10^7	5.63×10^7	114	9.14

¹RSD was defined as relative standard deviation divided by the mean and multiplied by 100.

4.9×10^3 cfu/mL, the fluorescence intensity of the positive group was 0.1814, which was slightly greater than that corresponding to the critical fluorescence intensity value of negative group of 0.1793174 ($N + 3SD$), resulting in F/F_0 of 1.32855. Therefore, the LOD concentration of *Salmonella* in the PBS buffer was 4.9×10^3 cfu/mL. The LOD of the proposed method was lower by 10 times compared with that of the traditional ELISA. In addition, the detection of *Salmonella* by using the PLL macromolecules coupled to QD via the SA-biotin system to amplify the fluorescence signal was more sensitive to a certain extent than using antibodies directly coupled to the surface of QD as fluorescent probes (Zhang et al., 2017). To further evaluate the applicability of the fluorescence sensor in actual samples, whole milk samples containing *Salmonella* concentration in the range of 4.9×10^1 to 4.9×10^7 cfu/mL were tested. As shown in Figure 6, the linear range of the standard curve is 4.9×10^4 to 4.9×10^7 cfu/mL using the equation of $F/F_0 = 0.39563 \log_{10}(S) - 0.00214$ with $R^2 = 0.987$. Furthermore, LOD was determined by the mean value of background signal plus 3 times the mean standard deviation (Yu et al., 2019). Thus, the LOD concentration of *Salmonella* in the whole milk was 4.9×10^4 cfu/mL. The diverse results from the PBS buffer may be related to the failure to pretreat the whole milk sample. In addition, the concentrations of *Salmonella* in milk of 4.9×10^4 , 4.9×10^5 , and 4.9×10^7 cfu/mL were measured by the proposed method. Experimental data came from 3 parallel groups. As shown in Table 1, the detection results of the proposed method were compared with the plate counting results. In addition, the recovery rate and RSD results of different concentration of *Salmonella* in milk indicate that this proposed

method has great potential for detecting *Salmonella* in actual samples. Some methods for detecting *Salmonella* in milk have been reported in previous studies, but they have limitations, such as long detection time, high detection limits, complex operations, and the need for professional knowledge. Thus, the proposed method was compared with other methods for the detection of *Salmonella* (Mazumdar et al., 2007; Xu et al., 2015; Xia et al., 2016; Zou et al., 2019; Table 2). The LOD and detection time of the proposed fluorescence detection method were lower than those of the other detection methods.

CONCLUSIONS

We developed an accurate, simple, rapid, and efficient fluorescence immunoassay method. In our fluorescence detection sensor, we used paired *Salmonella* mAb and *Salmonella* target to form a double-antibody sandwich structure, resulting in a very specific sensor. The water-soluble carboxylated QD could maintain high stability and effectively reduce nonspecific adsorption after being covalently coupled with SA. In addition, SA had high affinity for biotin. One molecule of SA had 4 molecules of biotin binding sites, and the signal could be amplified by the SA-biotin system. The PLL as a macromolecule carrier could carry many fluorescent probes. Thus, we overlapped SA and biotin layer by layer and used PLL loaded with multiple fluorescent labels to form a dendritic structure to amplify the signal. The linear range detected by the proposed method was 4.9×10^3 to 4.9×10^7 cfu/mL, and the LOD was 4.9×10^3 cfu/mL. Therefore, the proposed method could accurately and quickly detect *Salmonella*

Table 2. Comparison of this method with other reported methods¹

Detection method	Sample	Detection time (h)	LOD (cfu/mL)	Reference
NMR biosensor	Milk	2	10^5	Zou et al., 2019
IMS-microfluidic chip	Lettuce	3	10^4	Xu et al., 2015
GMBN-ICTS	Milk	20	5×10^5	Xia et al., 2016
SPR	Milk	1	1.25×10^5	Mazumdar et al., 2007
Fluorescent sensor	Whole milk	1	10^3	This study

¹LOD = limit of detection; NMR = nuclear magnetic resonance; IMS = immunomagnetic separation; GMBN-ICTS = gold magnetic bifunctional nanobeads; SPR = surface plasmon resonance.

Figure 6. Sensitivity detection of *Salmonella* in PBS (red circles) and in whole milk (black squares). F/F_0 represents the relative increase in fluorescence signal ($P < 0.05$, $n = 3$).

in milk. In future work, we will directly and covalently couple the macromolecular ligand to the surface of QD as fluorescent probes and change the detection system. Such a process may enhance the sensitivity of detecting *Salmonella* and decrease the LOD.

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