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Cell-based fluorescent microsphere incorporated with carbon dots as a sensitive immunosensor for the rapid detection of *Escherichia coli* O157 in milk

Yong Zhao [a,b,1], Yanzhao Li [b,1], Pingping Zhang [a,b], Ziheng Yan [a,b], Yugui Zhou [a,b], Yipu Du [a,b], Chunyu Qu [b], Yajun Song [a,b], Ding Zhou [c,*], Songnan Qu [d,**], Ruifu Yang [a,b,***]

[a] State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, 100071, China
[b] Beijing Key Laboratory of POCT for Bioemergency and Clinic, Beijing, 100071, China
[c] State Key Laboratory of Luminescence and Applications, Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, Changchun, Jilin, 130033, China
[d] Joint Key Laboratory of the Ministry of Education, Institute of Applied Physics and Materials Engineering, University of Macau, Taipa, Macau, China

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ABSTRACT

The rapid and early detection of foodborne pathogens in contaminated food is important for ensuring food safety and quality. In this study, a highly sensitive fluorescent immunosensor was developed to detect *Escherichia coli* O157:H7 in milk, by using microspheres labeled with carbon dots (CDs). The CDs-microspheres were prepared with *Staphylococcus aureus* cells as the carrier to incorporate CDs particles. Characterization of the microsphere revealed strong intensity, good stability and high uniformity in fluorescence. With Staphylococcal Protein A (SPA) on the surface of *S. aureus* cells, the microsphere could be easily coupled with various antibodies (e.g., immunoglobulin G). In combination with the immunomagnetic beads technique, a CDs-microsphere immunosensor was established for the specific detection of *E. coli* O157:H7 in milk. The limit of detection for *E. coli* O157:H7 is 2.4×10^2 colony-forming unit (CFU)/mL, comparable to that of real-time PCR methods. Milk samples spiked with *E. coli* O157:H7 at concentrations from 2.4×10^2 to 2.4×10^7 CFU/mL could be detected within 30 min. The coefficients of variation of the intra-assay tests were less than 10%, indicating a good repeatability. Moreover, the method was able to detect trace amounts of *E. coli* O157:H7 (<10 CFU) in real milk samples, with a 100% (10/10) accuracy after bacterial enrichment. This CDs-microsphere immunosensor shows considerable potential as a rapid and sensitive tool to detect pathogens in milk and other foods.

1. Introduction

Escherichia coli O157:H7 is an important foodborne pathogen that can contaminate foods and threaten people's health. The early and rapid detection of this pathogen in foods such as ground beef, vegetables, and unsterilized milk is crucial for ensuring food safety and quality (Lim et al., 2010; Page and Liles, 2013). However, traditional detection methods such as culture methods, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) usually require a long bacterial culture time (2–3 days), and/or complicated procedures and instruments. The lateral-flow assay is a well-known rapid and simple method for point-of-care detection, but it often lacks sufficient sensitivity (Luo et al., 2017; Wang et al., 2016). Therefore, it is vital to develop more sensitive and simple methods to detect *E. coli* O157:H7 and other foodborne pathogens.

The on-going development of bio-functional nanomaterials has provided new opportunities for the sensitive detection of pathogens. For instance, quantum dots (QDs) have been widely used as sensitive fluorescent probes in various immunosensors (Bruno, 2014; Chen et al., 2016). The QDs can be incorporated into polymer microspheres to

* Corresponding author. Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, Changchun, Jilin, 130033, China.
** Corresponding author. Institute of Applied Physics and Materials Engineering, University of Macau, Taipa, Macau, China.
*** Corresponding author. Beijing Institute of Microbiology and Epidemiology, No. 20, Dongdajie, Fengtai District, Beijing 100071, China.
E-mail addresses: zhouding@ciomp.ac.cn (D. Zhou), songnanqu@um.edu.mo (S. Qu), ruifuyang@gmail.com (R. Yang).
[1] These authors contributed equally.

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further increase their fluorescence intensity and improve the detection sensitivity (Ren et al., 2014). However, these microspheres are typically prepared by physical and chemical methods, which are inefficient, expensive, and require hazardous chemicals.

Cell-based microspheres are an alternative strategy for preparing luminescent microspheres. Many studies have shown that QDs and other nanomaterials can enter or attach to living cells and make the cells luminescent (Liu et al., 2015; Shang et al., 2014). The use of such luminescent cells (e.g., bacteria, spores, and yeast) as probes in bioanalyses has been demonstrated in several studies (Xiang et al., 2018; Zeng et al., 2016; Zhang et al., 2015, 2016a). Compared with traditional microspheres, those with biological cells as carriers are simpler to prepare and produce on a large scale. They do not require complex control of particle parameters, because bacteria or spore cells are naturally uniform in shape and size. However, most cell-based microspheres developed to date require long period of spore collection (around 7 days), tedious surface treatments, and limited approaches to conjugate them with bioactive molecules (e.g., antibodies) (Zhang et al., 2015).

Here, we managed to address these issues by using *Staphylococcus aureus* cells as the carrier of microspheres. This bacterium is a Gram-positive coccus (about 0.8 μm in diameter) with a solid cell structure because of large amounts of peptidoglycan in the cell wall. Moreover, the cell surface is rich in Staphylococcal Protein A (SPA), which has high affinity with antibodies. Previous studies have shown that SPA can directly combine with most immunoglobulin G (IgG) antibodies without any chemical activation; and it can directionally combine with the fragment crytallizable region (Fc) of antibodies, leaving the fragment antigen-binding region (Fab) available for binding to antigens (Rigi et al., 2019). Given its easy preparation, solid structure, and high biological affinity, *S. aureus* could serve as a superior carrier for cell-based microspheres.

Carbon dots (CDs) are a new class of fluorescent nanomaterial that have attracted considerable interest as alternatives to conventional QDs. The CDs typically have a small particle size (<10 nm) and superior biological properties such as low toxicity and good biocompatibility (Devi et al., 2019; Du et al., 2019). It has been reported that CDs can enter cells of various bacteria, including *S. aureus*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, *Bacillus subtilis*, and *Clostridium sporogenes,* as well as bacterial spores (Zhang et al., 2016b). The incorporated CDs mainly localize in the bacterial cytoplasm, and they remain in the cells even after several washes. Therefore, CDs are well suited for cell-based fluorescent microspheres. The mechanism of CDs enter bacteria cells remains unclear, although many studies speculate that the uptake of CDs in eukaryotic cells is through endocytosis (Cao et al., 2007). Unlike eukaryotic cells, bacterial cells have a thick cell wall structure, especially for Gram-positive bacteria, leading it is difficult for CDs to enter the bacteria through endocytosis. Permeation is another possible way based on one previous study (Zhang et al., 2016b); however, the mechanism needs more researches.

In this study, we introduce a simple method to prepare cell-based fluorescent microspheres using inactive *S. aureus* cells as the carrier and CDs as the reporter. Characterization of the microspheres revealed strong fluorescence intensity, good photostability, and high affinity with antibodies, making them suitable for biological detections. In combination with the immunomagnetic beads (IMBs) technique, a CDs-microsphere immunosensor was established for the detection of *E. coli* O157:H7 in milk. The performance of the method was evaluated by testing bacterial cultures and real milk samples. As a result, the method showed good specificity and sensitivity for the detection of *E. coli* O157:H7 in 30 min. Moreover, trace amounts of *E. coli* O157:H7 in contaminated milk samples could be reliably detected after a short period of enrichment (6 h). Our results demonstrate that these cell-based microspheres can be used as a sensitive and specific fluorescent probe for pathogen detections. The proposed CDs-microsphere immunosensor shows considerable potential as a rapid tool for the detection of *E. coli* O157:H7 in milk and other foods.

2. Materials and methods

2.1. Chemicals and materials

Bovine serum albumin (BSA), Tween 20, casein, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 2-(N-morpholino)ethanesulfonic acid (MES) buffer were supplied by Sigma-Aldrich (St. Louis, MO, USA). Urea, citric acid, NaCl, $CaCl_2$, Na_2HPO_4, and NaH_2PO_4 were supplied by the Sinopharm Chemical Reagent Co., Ltd, (Shanghai, China). The modified *E. coli* broth containing novobiocin (mEC + n) was provided by the Beijing Land Bridge Technology Co., Ltd. (Beijing, China). Cy-5-labeled goat-anti-rabbit IgG antibodies were from Sangon Biotech (Shanghai) Co., Ltd, (Shanghai, China). The mouse monoclonal antibodies (mAb 7D9 and 9F4) against *E. coli* O157:H7 were prepared in our laboratory.

2.2. Synthesis of CDs

The CDs were synthesized using a space-confined vacuum heating approach (Zhou et al., 2019). Briefly, 0.5 g citric acid, 1 g urea, and 1 g $CaCl_2$ were first dissolved in 3 mL deionized water, and then dried at 120 °C under vacuum. The heating temperature was then increased to 250 °C. Then, the CDs were dissolved in a dilute aqueous hydrochloric acid solution, and purified by dialysis against deionized water. Finally, the solution was freeze-dried, and the CDs powder was collected and stored dry.

2.3. Bacterial cultures

The bacteria used in this study were *S. aureus* (ATCC 12598), *E. coli* O157:H7, *Salmonella paratyphi* A, *Salmonella enteritidis*, *Listeria monocytogenes*, *Vibrio cholera* O1, and *Vibrio parahemolyticus*. All bacteria were cultured in Luria–Bertani (LB) broth at 37 °C with shaking at 200 revolutions per minute (rpm). Pure cultures were collected in the logarithmic phase by centrifugation. The cells were resuspended in sterilized saline (0.9% NaCl, pH 7.2), and then the bacteria population size was determined by LB-plate counts.

2.4. Preparation of the cell-based CDs-microsphere with S. aureus

The strategy for developing cell-based CDs-microspheres is

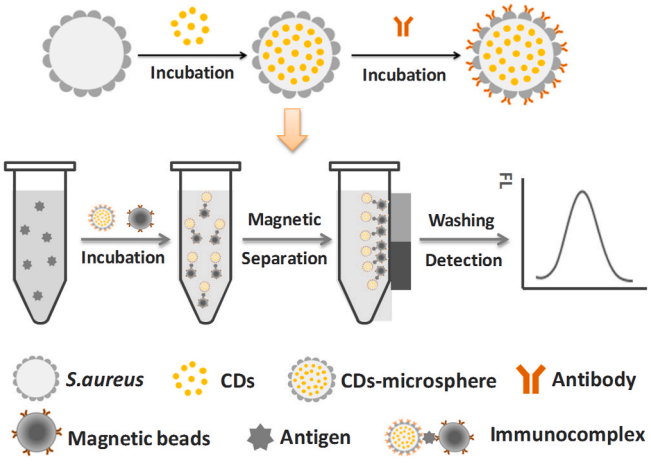

Fig. 1. Strategy for the development of cell-based CDs-microspheres with *S. aureus* cells and its application as a fluorescent immunosensor for pathogen detection. CDs-microspheres were prepared using *S. aureus* cells as the carrier to incorporate CDs particles. The inactivated cells could directly combine with antibody molecules via SPA proteins on the cell surface. The CDs-microsphere immunoassay was developed by combining immunomagnetic separation and CDs-microsphere fluorescence detection.

illustrated in Fig. 1. First, *S. aureus* cells were collected by centrifuging the culture solution at 4000×*g* for 5 min. Then, the bacterial pellet was resuspended in CDs solution (80 mg/mL, in sterilized saline). The mixture was incubated at 37 °C for at least 12 h with shaking at 200 rpm, then washed three times with sterile saline and centrifuged at 7000×*g* for 5 min. The pellet was collected and resuspended in phosphate buffered saline (PBS) to a final concentration of 100 mg/mL. Finally, the acquired CDs-microsphere solution was inactivated by heating at 65 °C for 30 min and then stored at 4 °C in dark. The inactivation was verified by plate culture.

2.5. Characterizations of CDs and microspheres

The chemical composition of CDs was investigated by energy dispersive X-ray spectrometry (EDS) with a ESCALAB 250 instrument (Thermo Scientific). A transmission electron microscope (TEM) and a scanning electron microscope (SEM) were used to observe the morphology of the CDs-microspheres. The fluorescence spectra and relative fluorescence units (RFUs) were determined using an F-7000 Fluorescence Spectrometer (Hitachi, Tokyo, Japan). Flow cytometry analyses were conducted using a BD FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a violet laser (405-nm, 30-mW solid state).

Comparison of the fluorescence intensity of individual CDs-microspheres and CDs particles was conducted by dropping highly diluted CDs-microspheres or CDs particles solution on a glass slide. The fluorescence imaging of single particles was obtained with a Nikon C2 confocal microscope coupled with an electron-multiplying charge-coupled device (EMCCD) camera. Then, the fluorescence intensity was analyzed with Image J software, thorough measuring the integrated density of individual CDs-microspheres or CDs particles ($n = 10$) after background subtraction.

Stability of the CDs-microsphere was evaluated over a 90-day storage at 4 °C in dark. 5 μL of the CDs-microsphere solution (100 mg/mL) was added to 300 μL of saline, and then fluorescence of the diluted CDs-microsphere was measured using an F-7000 Fluorescence Spectrometer. Each sample was tested in triplicate.

2.6. Conjugation of antibodies to the CDs-microsphere

First, 50 μL CDs-microsphere solution (100 mg/mL) was added to 700 μL PBS solution, followed by the addition of 0.5 mg mAb-9F4 against *E. coli* O157:H7. After incubation for 30 min at 37 °C with gentle shaking, the mixture was centrifuged at 7000×*g* for 5 min. Then, the bacterial pellet was resuspended in 1 mL PBS containing 1% (w/v) BSA and incubated for 30 min at 37 °C. The mixture was centrifuged at 7000×*g* for 5 min and washed three times with 1 mL PBST [PBS containing 0.05% (v/v) Tween 20]. Finally, the obtained bacterial pellet was resuspended in PBS to a concentration of 10 mg/mL and stored at 4 °C.

2.7. Preparation of the immunomagnetic beads

Carboxylated (COOH-) magnetic beads (1 μm in diameter) were obtained from Ocean NanoTech (Springdale, AR, USA). The conjugation of antibodies was performed according to the provided instruction with minor modifications. Briefly, the magnetic beads (5 mg) were washed twice and suspended with 0.5 mL MES buffer, followed by the addition of EDC and NHS with a final concentration of 2 mM and 5 mM, respectively. After incubation at 37 °C for 15 min, the mixture was washed three times and resuspended with 0.5 mL MES buffer. Then, 0.5 mg mAb-7D9 against *E. coli* O157:H7 was added and the mixture was incubated at 37 °C for 3 h. Subsequently, the beads were blocked with 1% (w/v) BSA. Finally, the IMBs were collected and washed five times with PBST. The IMBs were resuspended and preserved in 0.5 mL PBS solution containing 0.02% (w/v) NaN_3 and 0.5% (w/v) BSA.

2.8. Establishment and evaluation of CDs-microsphere immunosensor for detection of E. coli O157:H7

As illustrated in Fig. 1, the CDs-microsphere immunosensor for detection of *E. coli* O157:H7 was developed in combination of IMBs separation and CDs-microsphere fluorescence detection. Briefly, functionalized CDs-microspheres and IMBs were simultaneously incubated in the detection solution containing *E. coli* O157:H7 for 20 min; then fluorescence of the formed sandwich-structured immunocomplexes was measured after magnetic separation.

Solutions of *E. coli* O157:H7 at a range of concentrations from 2.4×10^2 to 2.4×10^7 colony-forming unit (CFU)/mL were prepared to evaluate performances of the immunosensor. For the test, 100 μL sample was mixed with 900 μL sample treatment buffer [PBS containing 0.1% (w/v) casein] before adding 0.1 mg IMBs and 0.1 mg CDs-microspheres. After incubation at 37 °C for 20 min with gentle shaking, the formed immunocomplexes (IMBs-bacteria-microspheres) were separated by a magnetic scaffold, and then washed twice with PBST and once with PBS. Finally, the immunocomplexes were resuspended in 300 μL PBS before detecting fluorescence. Under 410-nm excitation, the fluorescence intensity of the sample at the emission peak (515 nm) was taken as the detection result. The cut-off value was determined as the mean plus three times the standard deviation (SD) of the detection results from the blank. To confirm selectivity of the CDs-microsphere immunosensor for *E. coli* O157:H7, it was also tested against five other related foodborne pathogens at a high concentration (10^7 CFU/mL): *S. paratyphi* A, *S. enteritidis*, *L. monocytogenes*, *V. cholera* O1, and *V. parahemolyticus*.

2.9. Detection of E. coli O157:H7 in milk samples

To evaluate whether the method is suitable for the detection of pathogens in real samples, milk samples contaminated with *E. coli* O157:H7 at different concentrations (2.4×10^2–2.4×10^7 CFU/mL) were prepared by diluting an *E. coli* O157:H7 culture with sterile milk. The milk was purchased from a local supermarket. Then, the milk samples were tested using the method as described above, with standard samples as the control. Each sample was tested in triplicate.

Next, 15 milk samples, including 10 samples artificially spiked with trace amounts of *E. coli* O157:H7 (<10 CFU, as determined by the traditional plate counting method) and 5 samples without bacteria were prepared and pretreated according to the National Food Safety Standards in China (GB4789.36–2016). Briefly, 25 mL of each milk sample was added to 225 mL modified *E. coli* broth containing novobiocin (mEC + n) and homogenized for 4 min. Then, the broth was cultured at 37 °C with shaking at 200 rpm. After 4, 6, and 8 h of culturing, 100 μL of the culture was separately collected and detected with the CDs-microsphere immunosensor. For comparison, a commercial real-time PCR kit (Beijing Landbridge Technology Co., Ltd., Beijing, China) was also used to detect *E. coli* O157:H7. For this test, DNA was extracted from 1 mL of the culture using the water-boiling method. The reaction was performed with a Roche Light-Cycler 480 II fluorescent thermocycler according to the manufacturer's instructions.

3. Results and discussion

3.1. Characterization of cell-based CDs-microspheres

The prepared CDs particles had a narrow size distribution with an average diameter of 4.1 nm (Fig. 2a). The chemical composition of the CDs was determined by EDS analysis, which revealed the proportions of C (49.36%), N (12.41%), O (35.34%), and Ca (2.89%) (Fig. S1). As shown in the SEM and TEM images, the prepared CDs-microspheres had a typical coccus morphology and an average diameter of 0.8 μm (Fig. 2b and c). And, each microsphere had an intact cell wall structure about 40 nm thick. The prepared CDs-microspheres emitted green fluorescence under ultraviolet excitation (Fig. 2d). Fluorescence spectroscopy

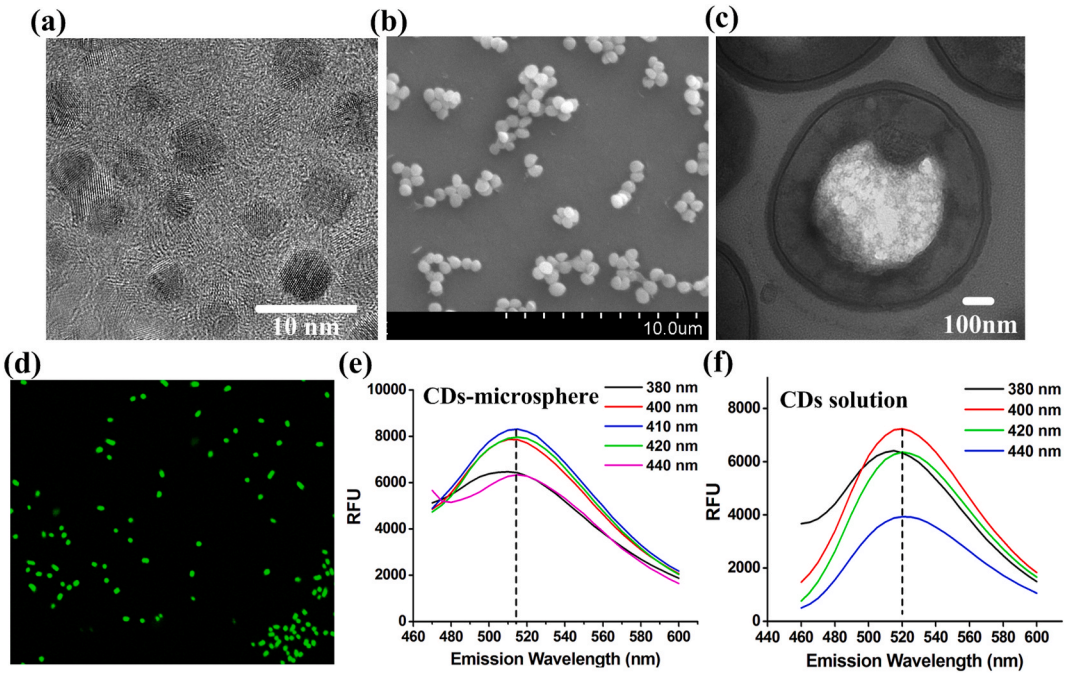

Fig. 2. Characterization of CDs particles and CDs-microspheres. (a) TEM image of CDs particles. (b) SEM image of CDs-microspheres. (c) TEM image of a single CDs-microsphere. (d) Fluorescence microscope image of CDs-microspheres under excitation by ultraviolet light. Fluorescence emission spectra of CDs-microspheres (e) and CDs particles (f) in saline solution excited at various wavelengths (380–440 nm).

analyses of the CDs-microspheres showed that their emission peak remained around 515 nm when the excitation wavelength varied from 380 to 440 nm, and the optimal excitation was at 410 nm (Fig. 2e). These results demonstrated that the spectrum of the CDs-microspheres has not changed significantly in comparison with that of CDs (optimal excitation and emission was at 400 nm and 520 nm, respectively) (Fig. 2f).

To obtain CDs-microspheres with high fluorescence intensity, the optimal density of CDs in solution (80 mg/mL) and incubation time (12 h) were determined as shown in Fig. 3a and b. The fluorescence intensity

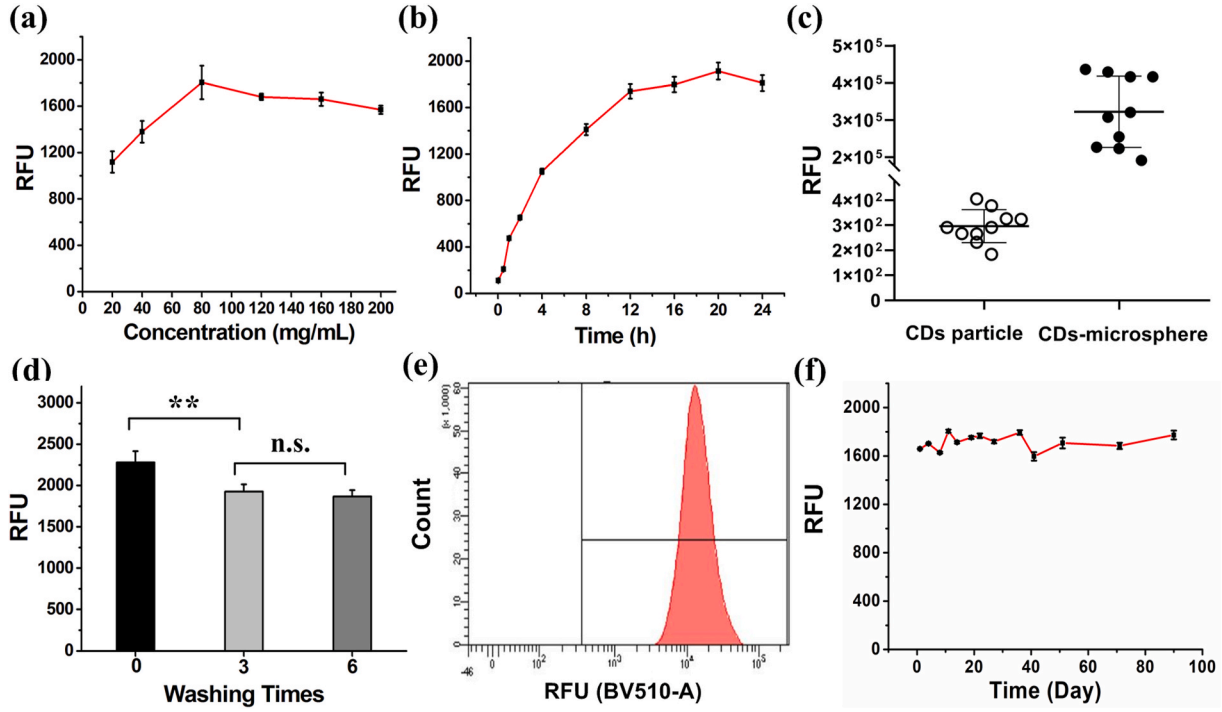

Fig. 3. Characterization of CDs-microsphere fluorescence. Optimization of CDs concentration (a) and incubation time (b) for CDs-microsphere preparation. (c) Comparisons of the fluorescence intensity between individual CDs-microspheres and CDs particles. Data were analyzed using Image J software (n = 10). (d) Fluorescence stability of CDs-microspheres after washing. (e) Flow cytometry analysis of CDs-microsphere solution (excitation at 405 nm, emission at 525 nm). (f) Long-term stability of CDs-microspheres (in PBS solution) over a 90-day storage. n.s., not significant. ** Significant difference at $p < 0.01$ (one-way ANOVA and LSD test).

of CDs-microspheres gradually increased to reach a plateau with increasing CDs concentrations and incubation time, suggesting that the microspheres became saturated with CDs particles. After optimization, the fluorescence intensity of individual CDs-microspheres was around 1000 times that of individual CDs particles (Fig. 3c), as determined by the confocal microscopy coupled with an EMCCD camera. After washing three times, the fluorescence intensity of CD-microspheres decreased slightly; but it no longer decreased after more washes (Fig. 3d), indicating that the CDs were stably adsorbed inside the microspheres. Moreover, there is no significant change in fluorescence intensity after 90 days of storage at 4 °C in dark, demonstrating excellent long-term storage stability of the CD-microspheres (Fig. 3f).

The results of flow cytometry analyses displayed that more than 99% of the prepared CDs-microspheres (1.68×10^7 particles in total) could emit fluorescent signals (Fig. 3e). The overall fluorescence distribution of the microspheres presented a significant normal distribution, showing that the CDs-microspheres were highly uniform in their fluorescence intensity.

3.2. Bioaffinity between CDs-microspheres and antibodies

One primary reason for choosing *S. aureus* cells as the carrier of CDs-microspheres is that it can directly combine with antibodies without any chemical modification. This property is attributed to the abundant SPA proteins on the *S. aureus* cell surface, which have high affinity with the Fc fragment of antibodies (IgG). To verify this ability, we analyzed the interaction between Cy-5-labeled antibodies (goat-anti-rabbit IgG) and CDs-microspheres. After a simple 30-min incubation, the CDs-microspheres emitted green fluorescence from the CDs at 405-nm excitation and red fluorescence from Cy-5 at 520-nm excitation (Fig. 4). The red fluorescence was visible as a hollow ring, revealing that the labeled antibodies were located at the cell surface. The green fluorescence was localized inside the ring-like structure, suggesting that the CDs were incorporated inside the microsphere.

We further incubated a certain amount of CDs-microspheres (2 mg) with different amounts of Cy-5 labeled antibodies (0.05, 0.1, 0.2, 0.3 mg) to determine the optimal antibody dose (Fig. S2). The results showed that with the increase of the antibody dose, the fluorescence intensity (Cy-5 emissions) of the microspheres gradually increased. The strongest fluorescence intensity was obtaind at an antibody dose of 0.2 mg, and did not increase with higher antibody concentrations.

In addition, the interaction between the antibody (mAb-7D9) against *E. coli* O157:H7 and the CDs-microspheres was evaluated in a slide-agglutination test (Fig. S3). In this analysis, the CDs-microspheres specifically captured *E. coli* O157:H7 and rapidly formed a visible agglomerated precipitate on the slide. In contrast, no agglomerated precipitate formed when the CDs-microspheres were reacted with *S. enteritidis* solutions. These results demonstrated that the CDs-microspheres were successfully functionalized with the anti- *E. coli* O157:H7 antibody.

3.3. Optimization of the CDs-microsphere fluorescent immunosensor

As illustrated in Fig. 1, the CDs-microsphere immunosensor for the detection of *E. coli* O157:H7 was established through combining IMBs separation and fluorescence detection. To achieve the best performance, three major factors of the reaction (1 mL) were optimized and confirmed as follows: 0.1 mg of IMBs, 0.1 mg of CDs-microspheres, and 20 min of incubation time. Our results showed that adding more than 0.1 mg IMBs to the reaction caused a lower signal-to-noise ratio (S/N; the ratio of the fluorescence intensity of a positive sample to that of a blank one) (Fig. S4a). We speculated that more IMBs interfered with the optical detection of fluorescent signals in the solution. We also found that 0.1 mg CDs-microspheres and 20 min incubation time were sufficient to capture and occupy the binding sites of *E. coli* O157:H7 at a high concentration of 10^7 CFU/mL (Fig. S4b). Larger amounts of CDs-microspheres lowered the S/N for low concentrations of *E. coli* O157:H7 mostly because of the increased opportunity for non-specific adsorption between IMBs and CDs-microspheres.

3.4. Detection of E. coli O157:H7 with CDs-microsphere immunosensor

After optimization, the CDs-microsphere immunosensor was evaluated with serial dilutions of *E. coli* O157:H7 ranging from 2.4×10^2 to 2.4×10^7 CFU/mL. Fig. 5a shows the fluorescence emission spectra of the formed immunocomplexes under 410-nm excitation. The fluorescence intensity at the emission peak gradually increased with the increase of bacterial concentrations. The limit of detection (LOD) of *E. coli* O157:H7 was 2.4×10^2 CFU/mL (24 CFU/test for a 100-μL sample), which generated a detectable fluorescence intensity above the cut-off line (Fig. 5b). The response curve between the bacterial concentration (2.4×10^4 to 2.4×10^7 CFU/mL) and fluorescence intensity displayed a good quantitative linear fit ($R^2 = 0.9218$, $p < 0.05$) (Fig. 5c). The coefficients of variation (CV) of the intra-assay tests were less than 10% (Table S1). These results confirmed that the immunosensor has a wide dynamic range and good repeatability.

Five other related foodborne pathogens were prepared to test specificity of the CDs-microsphere immunosensor (Fig. 5d). The test panel includes *S. paratyphi* A, *S. enteritidis*, *L. monocytogenes*, *V. cholera* O1, and *V. parahemolyticus*. No significant cross-reaction occurred when these bacterial strains were tested at a high concentration (10^7 CFU/mL), confirming that the immunosensor has a high selectivity for *E. coli* O157:

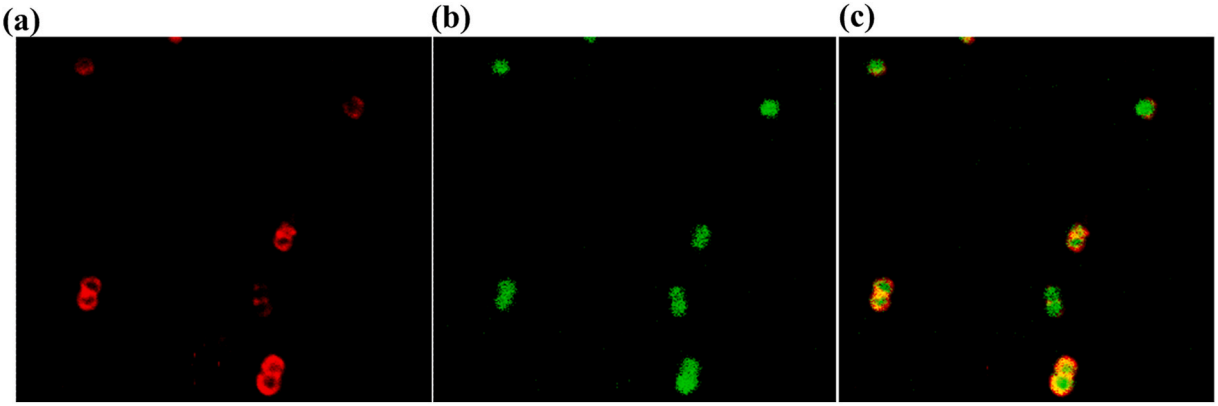

Fig. 4. Microscope images of CDs-microspheres coupled with antibodies (goat-anti-rabbit IgG) labeled with Cy-5. (a) Red fluorescence of Cy-5 at 520-nm excitation. (b) Green fluorescence of CDs at 405-nm excitation. (c) Merged green fluorescence and red fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

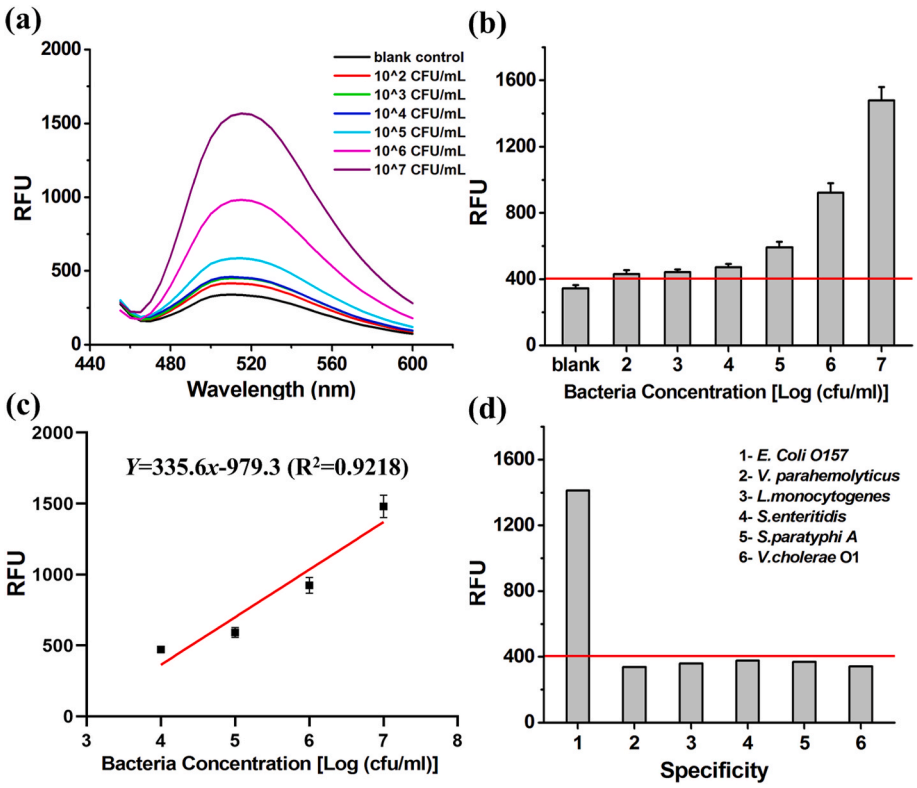

Fig. 5. Performance of the CDs-microsphere immunosensor for detection of *E. coli* O157:H7. Fluorescence spectra (a) and fluorescence intensity at 515 nm (b) of captured *E. coli* O157:H7 at different concentrations. (c) Linear range of the sensor for detecting *E. coli* O157:H7 from 2.4×10^4 to 2.4×10^7 CFU/mL. (d) Specificity of the CDs-microsphere immunosensor. The cut-off line is shown in red. Error bars show standard deviation of at least three independent tests. All data are mean ± SD ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

H7.

3.5. Detection of E. coli O157:H7 in milk samples

Milk samples spiked with *E. coli* O157:H7 at different concentrations (2.4×10^2 to 2.4×10^7 CFU/mL) were prepared and analyzed using the CDs-microsphere immunosensor. As shown in Fig. S5, the LOD remains 2.4×10^2 CFU/mL. And, there was no significant difference in the detection results between the contaminated milk samples and the standard bacterial samples, demonstrating that the method is suitable for analyses of real milk samples.

In many cases, milk samples or other foods are contaminated with pathogens at very low levels (<10 CFU), below the LOD of most detection methods. Therefore, pre-enrichment is usually necessary to improve the detection sensitivity and accuracy (Cho and Ku, 2017). Here, we prepared 10 milk samples spiked with trace amounts of *E. coli* O157:H7 (<10 CFU), and five milk samples as negative controls. After enrichment in mEC + n broth for 4–8 h, 1 mL of the culture was collected and tested using the CDs-microsphere immunosensor and the real-time PCR method (Fig. S6 and Table S2). As shown in Table S3, culturing for at least 6 h was required to ensure a reliable detection rate (10/10, 100%) for both methods. All the positive samples were confirmed by the traditional plate-counting method, which showed that the bacteria concentration reached appropriately 10^2–10^3 CFU/mL and 10^3–10^4 CFU/mL after culturing for 6 h and 8 h, respectively. These results indicate that the CDs-microsphere immunosensor has comparable detection sensitivity to that of the real-time PCR method.

Table 1 exhibits the comparisons of the proposed sensor with recently reported detection methods for *E. coli* O157:H7. The CDs-microsphere immunosensor has a lower LOD (2.4×10^2 CFU/mL, for a 100-μL sample) than those of the traditional ELISA method and lateral-flow assay for *E. coli* O157:H7. Compared to other highly sensitive methods, such as real-time PCR, electrochemical assay, and QDs-fluorescent sensor, this proposed sensor shows the merit of simplicity and speed, as it requires only 20 min for incubation and 10 min for washing and fluorescence detection.

4. Conclusions

We report a novel and simple method to prepare cell-based CDs-microspheres using inactive *S. aureus* cells as the carrier. Compared with current commercial fluorescent microspheres (e.g. QDs microspheres), the proposed CDs-microspheres have natural high affinity with antibodies, long-term storage stability, and are easier to produce on a large scale with low cost, which suggests a promising possibility for commercial viability. The developed CDs-microsphere immunosensor was

Table 1
Comparisons of the proposed sensor with recently reported methods for detection of *E. coli* O157:H7.

Methods	Label	Sample quantity (μL)	LOD (CFU/mL)	Linear range (CFU/mL)	Detection time	References
Real-time PCR	Taqman probe	2 (DNA)	10^2	10^2–10^7	2 h	Liang et al. (2019)
ELISA	[a]HRP	5	10^4	Not mention	2.5 h	Zhao et al. (2020)
Lateral-flow assay	[b]AIE FM	100	4×10^3	5×10^3–1×10^6	25 min	Zhang et al. (2019)
Electrochemistry	–	1000	10^1	10^1–10^8	1 h	Zhong et al. (2019)
Fluorescence	QDs@MnO_2	1000	1.5×10^1	1.5×10^1–1.5×10^6	2 h	Xue et al. (2020)
Fluorescence	CDs-microsphere	100	2.4×10^2	2.4×10^4–2.4×10^7	30 min	This study

[a] HRP represents horseradish peroxidase.
[b] AIE FM represents aggregation-induced emission fluorescent microsphere.

able to detect *E. coli* O157:H7 as low as 2.4×10^2 CFU/mL in milk; it has the advantages of speed (30 min), sensitivity, and simplicity for the detection of foodborne pathogens over conventional ELISA and real-time PCR assays. The main limitation of the method is the dependence on *S. aureus* cells; therefore, it must be ensured that *S. aureus* cells have been completely inactivated before real applications. Furthermore, the detection efficiency and throughput of the sensor will need to be improved through process integration on microfluidic chips in the future research.

CRediT authorship contribution statement

Yong Zhao: Methodology, and, Writing - original draft. **Yanzhao Li:** Methodology, and, Investigation. **Pingping Zhang:** Methodology. **Ziheng Yan:** Methodology. **Yugui Zhou:** Methodology. **Yipu Du:** Methodology. **Chunyu Qu:** Methodology. **Yajun Song:** Writing - review & editing. **Ding Zhou:** Methodology, and, Supervision. **Songnan Qu:** Methodology, and, Supervision. **Ruifu Yang:** Conceptualization, Writing - review & editing, and, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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