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Simple and rapid preparation of red fluorescence and red color *S. aureus* derived nanobioparticles for pathogen detection

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ABSTRACT

In this study, a simple and rapid method was developed to transform protein A producing *Staphylococcus aureus* cells into red color and red fluorescent nanobioparticles, which were homogeneous, dispersive and relatively stable with a uniform size of 800 nm. The method consists of reaction with a monotetrazolium redox dye at 25 °C for 15 min and heat inactivation at 65 °C for 30 min. This method provided the first *S. aureus* nanobioparticles with the dual property of red color and red fluorescence. Attributed to the IgG binding site known as protein A on their surface, the nanobioparticles could be used as vectors for immunoassays of many bacteria and viruses. Coagglutination test of *Escherichia coli* O157:H7 observed by naked eyes showed that the detection limitation of the nanobioprobes was $1 * 10^6$ CFU/ml, which was about 100 times more sensitive than the natural uncolored *S. aureus* bioprobes. Red fluorescence detection and analysis of the coagglutination product by a microplate reader lowered the detection limit to $2.5 * 10^4$ CFU/ml.

1. Introduction

Agglutination tests have been used widely in clinical and biochemical labs for detecting antigens because they are rapid, inexpensive, and simple to interpret (Daurat, 2008; Khazenson, 1983; Picardeau et al., 2014). Among the different forms of agglutination tests, coagglutination tests based on protein A producing *Staphylococcus aureus* particles are unique for their convenience of conjugating with IgG antibodies since protein A can react with the Fc (Fragment crystallizable region) fragment of the antibodies from human and most of mammal animals. *S. aureus* particles could be easily conjugated with different antibodies (Akatov, 1977) to detect specific bacteria or viruses (Sumithra et al., 2014; Varshney et al., 2007) without the need of any additional chemical modifications or physical treatments. Normally, natural *S. aureus* cells are used in current coagglutination tests (Mendes Ribeiro and Araujo, 2009). Since the natural *S. aureus* cells are pale-white and hard for naked eyes to judge agglutination results, there is a need to develop colored or fluorescent *S. aureus* nanoparticles for enhancing the detection sensitivity.

Giemsa staining (Varshney et al., 2007) and triphenyl tetrazolium chloride (TTC) (Sumithra et al., 2014) have been employed to stain the *S. aureus* cells into blue and purple, respectively, for coagglutination tests of bacteria, such as *Bacillus anthracis*. In recent years, as an emerging field in bionanotechnology, the synthesis of nanomaterials by living organisms, such as yeast, fungi and bacteria, has demonstrated great potential for different applications (Dhillon et al., 2012; Jan et al., 2014; Wang et al., 2011; Zhang, 2011). Biosynthesis of quantum dots (QDs) inside *S. aureus* cells has successfully transformed *S. aureus* into fluorescent nanobioprobes for sensitive detection of pathogens, such as H9N2 virus (Xiong et al., 2014).

In this study, we present a much simple and rapid way to transform *S. aureus* into red color and red fluorescent nanobioprobes for pathogen detection. We found that live *S. aureus* cells could react rapidly with a monotetrazolium redox dye, 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) at room temperature, and form insoluble formazan (CTF) inside the cells. As a result, the *S. aureus* cells became bright red and fluorescent. After heat deactivation, the bioparticles were ready for conjugating with antibodies for pathogen detection. This method provided the first *S. aureus* nanoparticles with the dual property of red color and red fluorescence. The coagglutination test of *Escherichia coli* O157:H7 by naked eyes showed that the nanobioprobes prepared by sensitizing the *S. aureus* nanobioparticles with a monoclonal antibody were about

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100 times more sensitive than the natural uncolored *S. aureus* bioprobes. Utilizing the fluorescence of the nanobioprobes to analyze the coagglutination result, the detection limitation was $2.5 * 10^4$ CFU/ml, which was 40 times lower than that observed by naked eyes.

2. Materials and methods

2.1. Preparation of red color and red fluorescent S. aureus nanobioparticles

Because protein A could be produced on its surface, *S. aureus* strain N315 (lab collection) was used in the study. 5-Cyano-2, 3-ditolyl tetrazolium chloride (CTC) was purchased from Sigma Aldrich Inc. (US). To prepare the red and fluorescent *S. aureus* nanobioparticles, N315 was first inoculated into 100 ml LB broth and incubated at 37 °C overnight until the $OD_{600\ nm}$ of the culture reached about 1.0–1.5. Before CTC staining, the concentration (CFU/ml) of the bacteria suspension was determined by plate counting. Then, CTC was added into the rest of the bacteria culture to make the final concentration of CTC of 2.0 mM. After further incubation at 25 °C for 15 min, the mixture turned bright red. After the reaction, the red bacterial cells were collected by centrifugation at 10000 ×*g* for 10 min, and resuspended in a certain volume of Tris–Cl buffer (0.1 M, pH 8.0) to make the final concentration of the bacteria suspension of $1 * 10^{10}$ CFU/ml based on the result of the plate counting. Finally, the suspension was heat-inactivated at 65 °C for 30 min and the novel red and fluorescent nanobioparticles with a concentration of $1 * 10^{10}$ particles/ml were ready for use. Natural *S. aureus* bioparticles were also prepared following the same procedure except without reaction with CTC. All the bioparticles were kept at 4 °C until use.

2.2. Characterization of the novel nanobioparticles

To test the dispersion and uniformity of the prepared *S. aureus* nanobioparticles, transmission electron microscopy (TEM) was used to observe the suspension of the nanobioparticles. First, 20 μl of the suspension containing the *S. aureus* nanobioparticles was added to a clean plastic membrane. Then, the droplet was covered by a copper grid with the formvar-carbon coated side. After 5 min of absorption, the grid was placed on a droplet of 2% sodium phosphotungstate (PTA) solution and stained for 3 min. The prepared grid was air dried for more than 12 h before observation by a 100 KV TEM instrument (Hitachi H-7000FA, Japan).

The fluorescence of the *S. aureus* nanobioparticles was measured using a microplate reader (Synergy H1 Hybrid Reader, BioTek, US) with excitation wavelength at 550 nm and emission wavelength at 630 nm. Briefly, aliquots of *S. aureus* culture (about 10^8 CFU/ml) were mixed with 2 mM CTC and allowed to react at room temperature for different times. Then, the mixtures were heat-deactivated at 65 °C for 30 min. Finally, 100 μl solutions from each of the mixtures were removed into different wells in a 96 well microplate for measuring the fluorescence.

The fluorescence of the individual *S. aureus* nanobioparticles was also measured using a two-photon confocal microscope (Nikon Eclipse Ti, Japan) after placing a drop (2 μl) of the reaction mixture on a clean glass slide.

2.3. E. coli O157:H7 coagglutination test

A murine monoclonal antibody (mAb) of O157:H7 (purchased from Prajna Biology Technique Ltd., Shanghai, China) was used to conjugate with the red *S. aureus* nanobioparticles prepared above. Briefly, the mAb (1 mg/ml) was added into the novel nanobioparticle suspension ($1 * 10^{10}$ particles/ml) at a volume ratio of 1:50 (Xiong et al., 2014). Then the mixture was placed in an oven at 37 °C for 30 min. Following that, the mixture was centrifuged at 4000 rpm for 5 min and the pellet was collected and washed three times using a Tris–Cl buffer (0.1 M, pH 8.0). Finally, the bioprobes (the bioparticles coated with the antibody) were blocked at 37 °C for 30 min using 1% BSA and washed 3 times using the Tris–Cl buffer by centrifugation.

To detect *E. coli* O157:H7, 10 μl of the bacteria sample was mixed with 20 μl of the mAb-sensitized bioprobes (10^{10} particles/ml) on a glass slide to test coagglutination. At the same time, a blank solution (0.1 M Tris–Cl without *E. coli* O157:H7) was also mixed with the mAb-sensitized nanobioprobes as a blank control on the same glass slide. A sample was considered positive only when it exhibited obvious coagglutination and the blank control did not show coagglutination within 5 min. The sensitivity was determined by testing a series of diluted *E. coli* O157:H7 suspensions in the Tris–Cl buffer. In order to compare with the red nanobioprobes, the sensitivity of unstained N315 derived bioprobes was also evaluated with the same protocol.

The specificity of the nanobioprobes was evaluated using different types of *E. coli* (O91, O97, O100, O149 and BL21) and several other types of bacteria (*Salmonella enteritidis*, *Streptococcus suis* and *Pseudomonas aeruginosa*). 10 μl of individual bacterium sample at the concentration 10^7 CFU/ml (10^5 CFU/reaction) was mixed with 20 μl of the mAb-sensitized bioprobes (10^{10} particles/ml) on a glass slide to test coagglutination. Bacteria mixtures consisting of *E. coli* O157:H7 and one other bacterium (10^7 CFU/ml each) were also tested. The coagglutination products between *E. coli* O157:H7 and the mAb-sensitized nanobioprobes after the reaction were imaged by TEM to confirm the result and observe the coagglutination pattern. The sample preparation for TEM was similar to the procedure for characterization of the prepared nanobioparticles described above.

The stability of the mAb-sensitized nanobioprobes was tested after storage at 4 °C and room temperature for one month. After culturing on a LB plate overnight at 37 °C, one colony of *E. coli* O157:H7 grown on the plate was picked into 0.1 ml PBS buffer. The bacteria suspensions obtained were tested using the mAb-sensitized nanobioprobes before and after the storage.

The results of the coagglutination tests described above are all observed by naked eyes since the nanobioprobe is bright red in color. Considering the red fluorescence property of the nanobioprobe, we also designed an experiment to determine the result of the coagglutination test by fluorescence signal for a series concentration of *E. coli* O157:H7. First, we conducted a glass tube coagglutination test for a series of diluted *E. coli* O157:H7 suspensions in the Tris–Cl buffer. The glass tube coagglutination test was performed by mixing a 10 μl sample with 30 μl nanobioprobes for 5 min. Second, after coagglutination, 160 μl Tris–Cl buffer (0.1 M, pH 8.0) was added into the reaction mixture and then the mixture was transfered to centrifuge tubes gently. After centrifugation at 500 rpm for 5 min to remove the agglutination product, 100 μl of the supernatant of each tube was transferred to a 96 well plate. The fluorescence was read at λ_{ex} 550 nm and λ_{em} 630 nm using a microplate reader (Synergy H1 Hybrid Reader, BioTek). Control tests were also done simultaneously by mixing 10 μl of Tris–HCl buffer and 30 μl of the nanobioprobes. The decrease in the relative fluorescence unit (RFU) of the samples compared with that of the controls was used as the signal.

2.4. In vitro culture and in vivo biosafety evaluation of the novel nanobioparticles

To check the viability of the nanobioparticles after the heat inactivation, 100 μl of the nanobioparticles ($1 * 10^8$ particles/ml) was inoculated on LB plates and into 5 ml LB broth, respectively, and cultured at 37 °C for 72 h. Further checking the biosafety on the mouse model was also conducted. Briefly, 100 μl of the nanobioparticles ($1 * 10^{10}$ particles/ml in PBS buffer) was injected into 5 female BALB/c mice intraperitoneally. Negative controls (3 mice) and positive controls (6 mice) were also injected with 100 μl of PBS and the live N315 cells ($1 * 10^{10}$ CFU/ml in PBS buffer), respectively.