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Dual-recognition detection of *Staphylococcus aureus* using vancomycin-functionalized magnetic beads as concentration carriers

Shijia Yang, Hui Ouyang, Xiaoxiao Su, Hongfei Gao, Weijun Kong, Mengyao Wang, Qi Shu, Zhifeng Fu *

Key Laboratory of Luminescence and Real-Time Analytical Chemistry (Ministry of Education), College of Pharmaceutical Sciences, Southwest University, Chongqing 400716, China

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ABSTRACT

Vancomycin, which has a strong antibacterial effect to Gram-positive bacteria, was adopted as one molecular recognition agent for bacterial detection. Magnetic beads (MBs) were functionalized with this antibiotic to effectively concentrate *Staphylococcus aureus* (*S. aureus*). In addition, alkaline phosphatase (ALP)-tagged rabbit immunoglobulin G (ALP-IgG) was used as the second recognition agent to improve the specificity based on the binding between the Fc region of rabbit IgG and protein A in the cell wall of *S. aureus*. MBs-concentrated sandwich complex of vancomycin/*S. aureus*/ALP-IgG was formed with a one-step incubation protocol. Then ALP chemiluminescent reaction was triggered by injecting substrate solution to quantitate *S. aureus*. Based on the sandwich molecular recognition mechanism and MBs concentration, an ultrasensitive, specific and rapid method was developed for *S. aureus* detection. The linear range for *S. aureus* detection was $12–1.2\times10^6$ CFU mL^{-1}, with a very low detection limit of 3.3 CFU mL^{-1}. The whole detection process could be completed in 75 min. Other Gram-positive bacteria and Gram-negative bacteria, including *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Bacillus cereus* and *Bacillus subtilis*, showed negligible interference to *S. aureus* detection. This method was successfully used to quantitate *S. aureus* in lake water, milk, human urine and human saliva with acceptable recoveries ranging from 70.0% to 116.7%.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a widely distributed Gram-positive bacterium (Wertheim et al., 2005). It has been one of the major foodborne and iatrogenic pathogens involved in a variety of dangerous diseases including septicemia, osteomyelitis, pneumonia, toxic shock syndrome, and endocarditis (Gorwitz et al., 2008; Lowy, 1998). Thus, it is of great importance to develop rapid, sensitive, specific and low-cost methods for *S. aureus* detection.

The conventional culture and colony counting method remains the most reliable and inexpensive for *S. aureus* detection (Banada et al., 2009). Nevertheless, it requires rather long time ranging from several hours to days, thus is not appropriate for field assay and rapid screening of pathogens (Velusamy et al., 2010). Nucleic acid-based approaches such as polymerase chain reaction (Cheng et al., 2006), surface plasmon resonance sensor (Naimushin et al., 2002) and fluorescence in situ hybridization (Oliveira et al., 2002), are also scaled up due to their multiplex detection capability and high specificity for pathogens detection. The main disadvantage of these nucleic acid-based approaches is that they demand cell disruption and nucleic acid extraction. Recently, some novel methods based on molecular recognition of bacterial whole cells are proposed to overcome the shortages of the conventional approaches for *S. aureus* detection. For example, immunological assays such as enzyme-linked immunoassay (Sandhu et al., 2012), amperometric immunosensor (Escamilla-Gómez et al., 2008) and piezoelectric quartz crystal microbalance immunosensor (Iijima et al., 2011) are frequently used in rapid assay of *S. aureus*. Some electrochemiluminescent (Lian et al., 2015), fluorescent (Duan et al., 2012) and potentiometric sensors (Zelada-Guillén et al., 2012) are also developed to quantitate *S. aureus* by using aptamer as molecular recognition agent. Undoubtedly, the above methods can be developed to portable devices and field assay protocols. However, they bear the limitations of high cost and poor stability of biomacromolecular recognition agents, restricting the widespread utilization (Alocilja and Radke, 2003; Jain et al., 2012; Subramanian et al., 2012). Hence, further efforts are still urgently needed in developing more approaches with low cost, easy manipulation, as well as high stability, sensitivity and specificity.

Recently, some improvements in bacterial detection have been

* Corresponding author.
E-mail address: fuzf@swu.edu.cn (Z. Fu).

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achieved by adopting magnetic beads (MBs) as the concentration carriers. MBs functionalized with some specific recognition agents can be used for magnetic separation and concentration of target pathogens, which leads to high sensitivity and simple manipulation (Chang, Lu 2013; Gu et al., 2003; Richardson et al., 2001). For these protocols, antibodies (Wen et al., 2014) and aptamers (Abbaspour et al., 2015) are the most frequently adopted agents for MBs functionalization due to their ideal specificity, whereas they still suffer from high cost and poor stability.

Antibiotics are a group of small molecular compounds used for the treatment and prevention of bacterial infection. Glycopeptide antibiotics, such as vancomycin, exhibit strong antibacterial effect to Gram-positive bacteria. Its mechanism is based on the fact that vancomycin can bind with the D-alanyl-D-alanine (D-Ala-D-Ala) dipeptide moieties in the cell wall of bacteria via five hydrogen bonds (Healy et al., 2000; Hubbard, Walsh 2003; Kell et al., 2008). Thus, this antibiotic can be adopted to functionalize MBs and used to concentrate and detect *S. aureus*. Compared with the previously reported recognition agents such as antibody and aptamer, it shows greatly improved stability and greatly lowed cost. However, as a broad-spectrum antibiotic for Gram-positive bacteria, vancomycin is lack of ideal specificity for recognizing *S. aureus*. Fortunately, there exists large amount of protein A in the cell wall of *S. aureus*, which can bind with Fc region of IgG specifically (Langone, 1982; Xiao et al., 2007).

Utilizing the two different recognition mechanisms as described above, a chemiluminescent (CL) method was developed for sensitive and specific detection of *S. aureus*. Briefly, vancomycin-functionalized MBs (MBs-vanc) were applied to recognize and concentrate *S. aureus*. Meawhile, alkaline phosphatase (ALP)-tagged rabbit IgG was adopted as the second recognition agent and CL tracer for *S. aureus*.

2. Experimental

2.1. Reagents and Materials

Carboxy groups-coated MBs with a diameter of 100 nm were purchased from Biospes Co., Ltd. (China). Vancomycin hydrochloride was provided by Sigma-Aldrich Chemical Co., Ltd. (USA). Bovine serum albumin (BSA) and glycine were obtained from Gibco (USA). 2-(*N*-morpholino) ethanesulfonic acid (MES) and *N*-hydroxysuccinimide (NHS) were both obtained from J&K Chemical Co., Ltd. (China). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Aladdin Reagent Ltd. (China). NaCl, NaOH, HCl and $Na_2HPO_4 \cdot 12H_2O$ were purchased from Kelong Chemical Co., Ltd. (China). ALP CL substrate solution composed of disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo(3.3.1.1^{3,7}) decan}-4-yl) phenyl phosphate and Sapphire-IITM enhancer was purchased from Boson Biotech Co., Ltd. (China). ALP-tagged rabbit IgG (ALP-IgG), fluorescein isothiocyanate (FITC)-tagged rabbit IgG and phycoerythrin (PE) were all provided by Boster Biotechnology Co., Ltd. (China). Polystyrene 96-well microplate was obtained from Greiner Bio-One Biochemical Co., Ltd. (Germany). Luria-Bertani medium was supplied by Oxoid, Ltd. (USA). Strains of *S. aureus* (CCTCC AB 91093), *Escherichia coli* (*E. coli*) (CCTCC AB 212355), *Salmonella* (CCTCC AB 91105), *Pseudomonas aeruginosa* (*P. aeruginosa*) (CCTCC AB 93078), *Micrococcus luteus* (*M. luteus*) (CCTCC AB 91100), *Bacillus cereus* (*B. cereus*) (CCTCC AB 2011085) and *Bacillus subtilis* (*B. subtilis*) (CCTCC AB 90008) were all obtained from China Center for Type Culture Collection. Strains of *Staphylococcus epidermidis* (*S. epidermidis*) (GIM1.444), *Staphylococcus saprophyticus* (*S. saprophyticus*) (GIM1.771) *and Staphylococcus aureus subspecies aureus* (*S. aureus subsp. aureus*) (GIM1.174) were provided by Guangdong Microbiology Culture Center. Milk was purchased from the local supermarket. Lake water was collected from Chongde Lake in Southwest University. Human urine and human saliva were obtained from healthy adult volunteers. Ultra-pure water (18.2 MΩ) used in the whole investigation was purified by ELGA PURELAB classic system (France).

The dilution buffer for all proteins and bacteria was 0.01 M Tris–HCl buffer at pH 7.4 (0.01 M Tris, 0.01 M NaCl, adjusted pH using HCl). The activation buffer for MBs was 0.01 M MES buffer at pH 5.5 (0.01 M MES, adjusted pH using NaOH). The coupling buffer for vancomycin was 0.01 M phosphate buffer saline at pH 7.4 (0.01 M Na_2HPO_4 and 0.05 M NaCl, adjusted pH using NaOH). The washing buffer was 0.01 M Tris–HCl buffer containing 0.05% Tween-20.

2.2. Apparatus

The CL signal detection was performed on an Infinite 200 PRO multifunctional microplate reader equipped with a solution injection module (Tecan, Austria). The scanning electron micrographs were obtained with an S-3000N scanning electron microscope (SEM) (Hitachi Instrument Co., Ltd., Japan). The purification of PE-tagged vancomycin was accomplished on an AKTAprime protein purification system (GE healthcare Co., Ltd., USA) equipped with a Sephadex G-25 column. The fluorescent images of *S. aureus* were obtained using a LSM710 confocal laser scanning microscopy (Carl Zeiss AG, Germany). The treatment of *S. aureus* was conducted in a biosafety cabinet (biosafety level II) (Suzhou Antai Airtech Co., Ltd., China).

2.3. S. aureus culture, pretreatment and counting

S. aureus was grown in Luria-Bertani broth with vigorous shaking under aerobic conditions at 37 °C for 10 h. Two milliliters of *S. aureus* culture was centrifuged at 4500 g for 15 min and washed thrice with 2.0 mL of washing buffer to remove the culture medium. The collected bacterial pellet was then resuspended and diluted serially into with Tris–HCl buffer. The result of agar plate count for colony-forming unit (CFU) showed that the concentration of the cultured *S. aureus* was approximately 1.2×10^9 CFU mL^{-1}.

2.4. Preparation of MBs-vanc

MBs-vanc were prepared using a classic EDC/NHS amidization reaction. Firstly, 1.0 mL of carboxy groups-coated MBs suspension was washed thrice with 1.0 mL of activation buffer, then resuspended in 1.0 mL of the same buffer containing 20 mg of EDC and NHS. Following 15-min activation, the MBs were washed thrice with 1.0 mL of coupling buffer and dispersed in 1.0 mL of coupling buffer containing 2.0 mg of vancomycin hydrochloride. After 12-h reaction under constant shaking, the resulted MBs-vanc were washed thrice with 1.0 mL of washing buffer and dispersed in 1.0 mL of Tris–HCl buffer containing 1% BSA.

2.5. Preparation of PE-tagged vancomycin

PE-tagged vancomycin were also prepared using EDC/NHS amidization reaction. Briefly, 500 μL of 20 mg mL^{-1} vancomycin hydrochloride were mixed with 300 μL of 10 mg mL^{-1} NHS and 300 μL of 20 mg mL^{-1} EDC. After 5-min activation at room temperature, 250 μL of 1.0 mg mL^{-1} PE was added into this activated vancomycin solution. The reaction was continued for 12 h at 4 °C, and then stopped by adding 40 μL of 2.0 M glycine solution. Lastly, the product was purified using an 11-cm Sephadex G-25 column