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Communication

Selective fluorescence labeling and sensitive determination of *Staphylococcus aureus* by microchip electrophoresis with a multiple-concentration approach

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ABSTRACT

The detection of *Staphylococcus aureus (S. aureus)* is very important as it is responsible for bacterial infectious diseases and food poisoning. In this paper we explored the application of fluorescently labelled vancomycin to specifically bind and detect *S. aureus*. In view of the specificity of vancomycin towards bacterial cell surfaces, we utilized Cy5 to label vancomycin (Cy5-Van) for the identification of *S. aureus*. Our experiments were designed to examine in greater detail the specificity of the reaction between Cy5-Van and *S. aureus*. Detection parameters such as the derivatization conditions, concentrations of buffer, pH value, response performance of Cy5-Van to bacterial surface, injection time and reversed-polarity time have been investigated and optimized. To develop a simple and quick assay for the detection of *S. aureus* at low concentrations, we propose to use the Cy5-Van for labeling the *S. aureus* coupled with an on-line multiple-concentration in microchip electrophoresis. Under the optimized conditions, the detection of *S. aureus* was achieved within 150 s with limit of detection (S/N = 3) of 981 CFU/mL, and 350-fold enhancement was obtained for *S. aureus* as compared to using the no concentration step. It is self-evident that this approach has great potential in the future for the analysis of *S. aureus*.

The detection and identification of pathogenic microorganisms, including bacteria [1], viruses [2], and fungi [3], are crucial to ensure the safety of the consumer and of the patient, maintain the product quality, and meet the regulatory requirements. Therefore, developing rapid, sensitive, and broadly applicable methods to identify and monitor such microorganisms is of paramount importance. Currently, several analytical methodologies have been made to test potentially dangerous microbial agents [4–11] while these methods present drawbacks such as high instrument or reagent cost, complexity, time consumption, or low detection limits. Microchip electrophoresis (MCE) has been explored for the possibility of analyzing bacteria and is currently of great interest because of its rapid response, high information exportation and the use of minute amount of samples and reagents. Though MCE has been proved to be used for bacteria analysis, in some cases, the sensitivity of MCE is not sufficient because of the low sample volume and short separation distance characteristic of the method [12,13]. Therefore, a method for rapider and more effective identification of low-abundance bacteria is still highly desired.

We have used the red fluorescent nucleic acid stain SYTO-62 for labeling bacteria, used a combination of chitosan sweeping, field-amplified sample stacking (FASS) and reversed-field stacking (FRS) for the ultrasensitive detection of bacteria using MCE with laser-induced fluorescence (LIF) [14]. Currently, SYTO-62 was mainly used to stain bacteria. However, SYTO-62 undergoes non-specific labeling of nucleic acids abundant in blood, plasma or urine. As a result, SYTO-62 is not suitable for the determination of microbial contamination in biological sample. Therefore, we try to find a new compound able to label bacteria for the detection at low-abundance bacteria by MCE.

Vancomycin is a glycopeptide recognized as an antibiotic drug because of its capacity to rupture the bacterial cell-wall by specific binding to the mucopeptide [15–22]. Williams and co-worker described the specific molecular recognition of vancomycin to the D-alanyl-D-alanine terminus of the stem pentapeptides present in the bacterial peptidoglycan *via* hydrogen bonding interactions between the basis of the skeleton of vancomycin and the terminal D-alanyl-D-alanine moieties in Gram-positive bacteria (Fig. 1)

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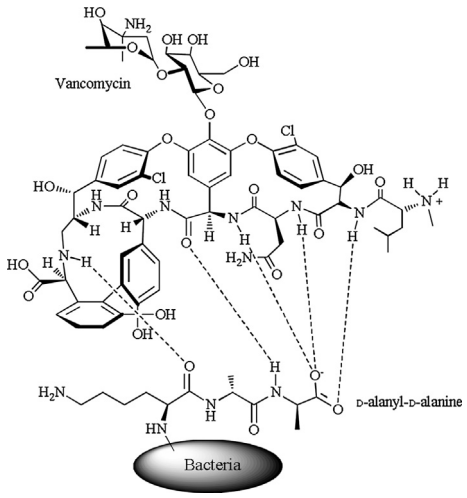

Fig. 1. Cartoon representation of the vancomycin-D-alanyl-D-alanine interactions. The critical components for the strong H-bonds both on the vancomycin molecule (the heptapeptide backbone) and the D-alanyl-D-alanine dipeptide exposed from the bacterial surface are highlighted.

[23,24]. Xing and co-worker reported that the vancomycin group antibiotics have been propelled to the front of fighting against the serious pathogen like *Staphylococcus aureus* (*S. aureus*) and methicillin resistant *S. aureus* (MRSA) because of its clinical significance in treating Gram-positive bacterial infections [25]. Lin *et al.* have employed vancomycin-modified magnetic nanoparticles as affinity probes to selectively trap Gram-positive pathogens from urine sample on the basis of specificity for the D-Ala-D-Ala units of the cell walls for vancomycin [26].

In the view of using the specific binding of vancomycin to bacterial cell surfaces, we utilized Cy5 to label vancomycin (Cy5-Van) for the identification of *S. aureus*. Our experiments were designed to detect in greater detail the specificity of the reaction between Cy5-Van and *S. aureus*. We investigated and optimized the detection parameters such as the derivatization conditions for Cy5 labeling of vancomycin and derivatization conditions for Cy5-Van labeling of *S. aureus*. In comparison with the method of nucleic acid labeling, this strategy avoids the background nucleic interference from *S. aureus*. This paper discusses the response performance of vancomycin to the bacterial cell surface. After a series of experiments, we combined field-amplified stacking (FASS) and reversed-field stacking (RFS) in a multiple-concentration approach for MCE detection of *S. aureus*. Because the proposed method ensures satisfactory sensitivity and excellent enrichment simultaneously, it provides a fast and feasible for the detection of *S. aureus*.

In order to achieve the best results, various experimental conditions were investigated and optimized. The experimental section was placed in the Supporting information.

The derivatization of vancomycin with Cy5 was tested in the first step of the experiment. The scheme of the labeling reaction is depicted in Scheme S1 (Supporting information). In the process of derivatization, influential factors including pH value and concentration of borate buffer solution, and time of the reaction were examined to obtain the maximum stable fluorescence yield. The reaction was performed by varying the pH (7.5–10.0) and the concentration of borate solution (10–90 mmol/L), while keeping the other parameters constant. The maximum LIF signal was obtained when increasing the pH value up to 8.5 above which the signal decreases due to the hydrolytic degradation of the Cy5 and the high ionic strength that may reduce the reaction rate of both cations and anions. The pH value of the buffer system and the variation of the buffer concentration may lead to the formation of boric acid ions and hydroxyl groups of vancomycin forming a strong negatively charged complex. Therefore the pH value of 8.5 was kept constant while optimizing the borate buffer concentration. The results demonstrated that a concentration of 20 mmol/L derivatization borate buffer was found to achieve maximum reaction efficiency in the labeling reaction (Fig. S2 in Supporting information). At the same time, the reaction temperature and the time phad an important influence on the derivatization yield. The experimental results also demonstrated that the reaction required darkness and 6 h of incubation at room temperature. Under optimal conditions, we can observe two well resolved peaks of Cy5 and Cy5-Van on the electropherogram. Fig. 2 illustrates the highest efficiency of the derivatization obtained.

In view of the fact that *S. aureus* has no native fluorescence, specific markers of *S. aureus* were detected by derivatizing with Cy5-Van. The derivatization procedure is optimized with regards to the peak area obtained under different conditions. The influence of pH on the derivatization is the most important factor affecting the focusing efficiency. We therefore studied the derivatization at pH values between 4.0 and 9.0. The experimental results demonstrated that the derivatization was mostly successful with the largest peak area at pH 6.0. Therefore, this pH value 6.0 was selected in this derivatization procedure (Fig. S3A in Supporting information). Furthermore, the influence of the duration of derivatization was also investigated. The mixture was vortexed for 30 s and then placed in the dark at room temperature for different durations (2–7 h). It was found that the maximum yield was obtained by applying 4 h of reaction time (Fig. S3B in Supporting information).

Secondly, the conditions of specific marking *S. aureus* by Cy5-Van were investigated to obtain the maximum fluorescence intensity. The amount of Cy5-Van had a significant influence on the derivatization efficiency. A bacterial suspension (1.5 mL of approximately 2.5×10^8 CFU/mL) was mixed with a solution of Cy5-Van (0.5 mmol/L) at different volumes ranging from 30 μL to 70 μL to obtain a final concentration. The derivatization reaction was concentration dependent, and increasing the reagent concentration would improve the derivatization efficiency. The experimental results illustrated that the fluorescence response of the selected analytes increases when increasing the Cy5-Van volume and levels off when it was higher than 50 μL of 0.5 mmol/L Cy5-Van. The maximum fluorescence intensity of the analytes was hard to obtain when using Cy5-Van volumes less than 50 μL. Therefore, 50 μL of 0.5 mmol/L Cy5-Van was used for all subsequent derivatizations.

After above marking procedure, MCE was used to separate *S. aureus* from the residues of Cy5 and Cy5-Van. In order to achieve higher detection sensitivity while maintaining high resolution, an on-line multiple-preconcentration strategy combining FASS and RFS has been developed and used in MCE [27,28]. The schematic

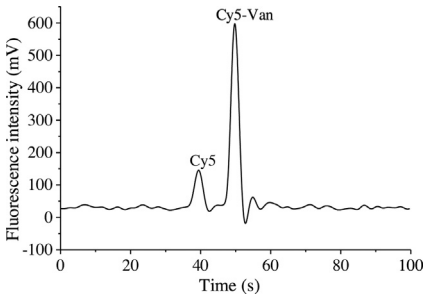

Fig. 2. Electropherogram of Cy5 labeling vancomycin. The running buffer was a 50 mmol/L borate solution.

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