Talanta 146 (2016) 299–302

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Lysozyme as a recognition element for monitoring of bacterial population

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form
18 August 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Lysozyme
Recognition element
Bacterial population
Bacterial detection

ABSTRACT

Bacterial infections remain a significant challenge in biomedicine and environment safety. Increasing worldwide demand for point-of-care techniques and increasing concern on their safe development and use, require a simple and sensitive bioanalysis for pathogen detection. However, this goal is not yet achieved. A design for fluorescein isothiocyanate-labeled lysozyme (FITC-LYZ), which provides quantitative binding information for gram-positive bacteria, *Micrococcus luteus*, and detects pathogen concentration, is presented. The functional lysozyme is used not only as the pathogenic detection platform, but also as a tracking reagent for microbial population in antibacterial tests. A nonlinear relationship between the system response and the logarithm of the bacterial concentration was observed in the range of 1.2×10^2–1.2×10^5 cfu mL^{-1}. The system has a potential for further applications and provides a facile and simple method for detection of pathogenic bacteria. Meanwhile, the fluorescein isothiocyanate -labeled lysozyme is also employed as the tracking agent for antibacterial dynamic assay, which show a similar dynamic curve compared with UV–vis test.

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1. Introduction

Pathogens may be suspected of playing a key role in that disease and some believe a substantial portion of chronic diseases may in part be caused by infectious agents [1,2]. The determination, identification, and quantification of pathogen for food safety, water environmental analysis, or clinical diagnosis are crucial for public health protection [3]. Traditional techniques for monitoring bacterial populations, such as plating and culturing [4] and polymerase chain reaction [5], involve a pre-enrichment step followed by a biochemical test, and the sophisticated series of assays required to complete. Though promising, these methods are still in the development phase and several problems may occur when they are used in situ and in real time. Rapid technologies for bacterial analysis are essential to food safety, prevention of environmental contamination, and clinical diagnosis of food poisoning and food-borne and water-borne diseases [3]. Biosensors have emerged as extremely useful tools for pathogenic detection. Various sensitive, reliable and rapid methods, such as electrochemical impedance spectroscopy [3,6–8], surface plasma resonance [9,10], quartz crystal microbalance [3,11], have been reported for the determination and monitoring of microorganisms. The specific and direct detection of bacteria using monoclonal antibody and aptamer as recognition receptors immobilized covalently onto functionalized interface of biosensor or biochip are widespread developed. However, the application of aptamer [12]-based elements suffers from the limitation of discrimination between viable and dead pathogen, while for monoclonal antibody [3,6,13,14]-based recognition elements, the limitation are expensive cost, susceptibility to natural/human environmental stresses and cross-binding to other pathogen, which may cause several positive results [15–17]. Compared to monoclonal antibody and aptamer, bacteriophages are not uncommon in natural environment, relative highly specific to pathogen and thus harmless to human health, much less complicated technique to prepare than antibodies or aptamer, and show a far longer shelf life at room temperature [18].

The continuous development of specific recognition element-based ultrasensitive signal labels for use in bioanalysis is critically important for disease prevention, medical diagnosis, food safety, and environmental remediation. Recent decade witnessed the development of a number of biological/chemical functional molecules used as specific recognition elements for the detection and determination of bacteria. Examples of such biomaterials include lectin [19], carbohydrate [20], antibiotic [21,22], bacteriophages [23], crystal violet [24], antenna [25], and antimicrobial peptides

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http://dx.doi.org/10.1016/j.talanta.2015.08.056
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[26,27]. Unlike monoclonal antibodies, aptamer and bacteriophages, these recognition elements are significantly more stable and exhibit broadband detection for a range of pathogenic bacteria. The crystal violet with trans-cyclooctene can be used to render gram-positive bacteria magnetic by their visual staining properties using bioorthogonal chemistry [24]. The antibiotic, such as vancomycin conjugate [28–30] and daptomycin [21], can also specifically detect gram postive bacterial infections, discriminate bacterial infections from sterile inflammation in vivo in the lower leg of a human cadaver to culture- and label-free detection and drug-resistant testing of pathogen in early clinical samples. We have recently shown that antibiotic can be utilized to fabricate vancomycin-functionalised antibacterial materials that had a higher selective phototoxicity and bioanalysis for Van-sensitive bacteria [31]. This has enabled the generation of bifunctional probe containing a relative specific recognition moiety combined with an antibiotic binder to hydrogen bonds-based self-assemble [32]. Based on self-assembly of antimicrobial peptides on direct interfacing of graphene nanosensors onto biomaterials, the researches further show bioselective detection of bacteria at single-cell levels [33].

Lysozyme (LYZ), which is known as muramidase or *N*-acetylmuramide glycanhydrolase, does this by binding to the peptidoglycan molecule, a hexasaccharide, in the binding site within the prominent cleft according to Phillips-Mechanism [34–36]. The myriad application of lysozyme as an antimicrobial agent in pharmaceuticals, home appliances, food preservatives, potential aseptics and therapeutic use is an interesting area of research [37–40]. There is no report for LYZ used as a recognition element for bioanalysis or bacterial detection. In this paper, a LYZ-based sensor used as recognition element for bacteria detection was developed for the first time. Our results suggested that the LYZ-based technique could be readily applied to bacteria detection. This low cost, portable sensor is simple, long-term stability, and could also meet the growing demand for bacteria detection.

2. Experimental

2.1. Reagents

Lysozyme (LYZ) and Fluorescein isothiocyanate (FITC) were ordered from Sigma-Aldrich (St. Louis, MO). Unless otherwise noted, all reagents for bacterial cultivation were purchased from Sinopharm Group Company Limited. Fluorescence spectra of bacterial detection and antibacterial tracking were obtained with a Hitachi 3500 fluorospectrometer with xenon lamp excitation source. UV–vis absorption spectra of FITC-LYZ were obtained in water with a Hitachi U-2900 spectrophotometer (shown in Fig. S1). Ultrapure water was produced by Storage & Distribution system (Millipore Corporation, USA). All chemicals and solutions used throughout this study were dissolved or diluted with ultrapure water.

2.2. Preparation of FITC-labeled lysozyme

250 ug FITC was added into 5 ml of 10 mg/ml lysozyme solution in 0.1 M sodium carbonate buffer at pH 9.5. After incubation for 4 h in the dark at room temperature, the reaction was stopped by being removed to 4 °C. And the excess FITC was removed from the reaction mixture by dialysis using dialysis membrane (300 Da).

2.3. Bacterial cultures

All bacteria were obtained from the Key Laboratory of Experimental Marine Biology. Bacteria were seeded and cultured in suspension using the following media: *Escherichia coli* (*E. coli*) in Luria–Bertani media; and *Micrococcus luteus* (*M. lutues*) in yeast extract peptone dextrose medium. For these bacteria, a single colony was inoculated in bacterial medium at 30 °C overnight with shaking at 200 rpm. After centrifugation (3000 rpm) for 10 min and phosphate-buffered saline (PBS) washing, the bacterial cells were diluted to the desired concentration or optical density in phosphate buffer. Bacterial cell numbers were estimated by plating onto standard agar plates.

2.4. Bacterial detection

Bacterial cells were first washed with PBS containing 2 mg mL^{-1} bovine serum albumin. For labeling using directly FITC-LYZ, bacterial cells were incubated with 10 μg mL^{-1} FITC-LYZ in PBS-B for 5 min at room temperature. Excess unbound FITC-LYZ was removed by washing the bacterial cells with centrifugation (3000 rpm) for 10 min in three times. Relative fluorescence ratio (RFR) are estimated as $RFR=(F_{sample}-F_{control})/F_{control}$, where F_{sample} and $F_{control}$ are intensity values of bacterial sample and control sample, respectively. All labeling experiments were carried out at three times to obtain the reproducibility of our approach.

2.5. Antibacterial tracking

The antimicrobial activity tracking of LYZ in solution was characterized using *M. lutues*, a well-studied substrate organism for LYZ, and *E. coli*. The assay was performed according to the recommended procedure (Sigma L6876, Enzymatic assay for LYZ). Mixing 0.1 mL of LYZ with 0.9 mL of freshly prepared cell suspension ($OD_{450\ nm}=1.0$) resulted in a decrease in turbidity of the suspension, allowing us to continuously monitor the LYZ activity in real time. For FITC-LYZ's antibacterial tracking, the Bacterial cells were first washed with PBS. After centrifugation (3000 rpm) for 10 min and PBS washing, the bacterial cells were diluted to the desired concentration in phosphate buffer. Then, the bacterial cells were incubated with 10 μg mL^{-1} FITC-LYZ in PBS-B range from 1 min to 60 min at room temperature. Excess unbound FITC-LYZ was removed by washing the bacterial cells with centrifugation (3000 rpm) for 10 min in three times. All labeling experiments were carried out at three times to obtain the reproducibility of our approach. Relative antibacterial ratio (RAR) are estimated as $RAR=(F_{sample}-F_{control})/F_{control}$, where F_{sample} and $F_{control}$ are intensity values of bacterial sample and control sample, respectively. All labeling experiments were carried out at three times to obtain the reproducibility of our approach.

3. Results and discussion

3.1. Measurement principle

One of the most important approaches for enzyme labeling is to incorporate functionalities into proteins at specific sites for enzymatic reactions. Many companies offer horseradish peroxidase, alkaline phosphatase, and other substrate enzymes in purified, activated, and kit formats for easy conjugation to proteins and for the preparation of bioconjugates for immunoassays and related applications [41]. Our group recently develops that synthesize lysozyme-DNA conjugates, and tested its application in bacteria detection (unpublished data). However, it is known to me that there is no report for lysozyme used as a recognition element for bioanalysis or bacterial detection. In this study, Fig. 1 shows the schematic representation of this LYZ-based detection method for bacteria detection. Functional FITC-LYZ was used to link fluorescence analysis to detect our targets, such as pathogen, and the platform was used to quantify pathogen present in the samples. This method avoids the

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