Sensors and Actuators B 241 (2017) 255–261

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

# Bioluminescent detection of the total amount of viable Gram-positive bacteria isolated by vancomycin-functionalized magnetic particles

Xiaoxiao Su, Mengyao Wang, Hui Ouyang, Shijia Yang, Wenwen Wang, Yong He, Zhifeng Fu *

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

ARTICLE INFO

*Article history:*
Received 1 August 2016
Received in revised form
28 September 2016
Accepted 9 October 2016
Available online 17 October 2016

*Keywords:*
Bioluminescence
Adenosine triphosphate
Vancomycin
Magnetic particles
Gram-positive bacteria

ABSTRACT

A facile bioluminescent protocol was developed to detect the total amount of viable Gram-positive bacteria based on a novel antibiotic-affinity strategy. Vancomycin-functionalized magnetic particles were adopted to isolate Gram-positive bacteria from sample matrix, utilizing the five-point hydrogen bonds binding between vancomycin and D-Alanyl-D-Alanine moieties on the cell wall. Then the bioluminescence signal from the bacterial intracellular adenosine triphosphate was collected to quantify the captured bacteria after cells lysis. In this proof-of-principle work, the developed protocol was applied to detect four Gram-positive bacteria, including *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus* and *Streptococcus mutans*. It showed a linear range of $1.0 \times 10^2$–$1.0 \times 10^7$ CFU mL$^{-1}$ and a detection limit of 33 CFU mL$^{-1}$ for the four model bacteria. The whole assay process could be completed within 70 min. Gram-negative bacteria and dead bacteria all showed negligible interference to the detection of the viable Gram-positive bacteria. The method was successfully applied to quantify the amount of viable Gram-positive bacteria in environmental, food and pharmaceutical samples with acceptable recovery values ranging from 72.0% to 120.0%. The protocol possessed numerous advantages such as high sensitivity, low cost, ideal specificity and short detection time. It could also be extended to Gram-negative bacteria detection by utilizing other antibiotics acting on Gram-negative bacteria.



## 1. Introduction

Infectious diseases resulting from pathogenic bacteria are widespread and believed to be the leading causes of lethality in many developing countries. In addition, pathogenic bacteria have been used in biological warfare for a long term due to their low infectious dose and rapid spread/proliferation [1]. Therefore, there is an urgent need to develop fast, sensitive and reliable methods for the detection of pathogenic bacteria in such areas as food safety, clinical diagnosis, environmental sanitation and homeland security.

Although the traditional culture and colony counting-based method remains to be the gold standard for bacteria detection, it is believed to be labor-intensive and time-consuming [2]. Some molecular biology techniques such as polymerase chain reaction (PCR) [3] and fluorescence quantitative PCR [4] have attracted increasing concern since they improved the sensitivity and shortened the detection time. Nevertheless, sometimes they suffer from false-positive or false-negative results due to the interference from the exogenous contamination [5]. Most importantly, these PCR-based protocols cannot figure out dead bacteria from viable bacteria. In the recent years, the molecular recognition-based methods have been effective alternative approaches for specific detection of bacteria, in which some biological materials including antibody [6–8], aptamer [9,10] and antimicrobial peptide [11,12] are adopted to recognize the bacteria. Nevertheless, these molecular recognition regents show such limitations as high cost, poor stability and varying performances among batches and sources. Furthermore, they cannot distinguish between viable and dead bacteria, thus potentially lead to false-positive results.

In view of the above problems, some protocols have been established to figure out viable bacteria from dead ones, including electrical impedance technology [13], gas phase chromatography [14], and examination of membrane integrity [15], et al. The electrical impedance technology monitors the impedance change caused by the release of ionic metabolites from viable cells, to exclude the interference from the dead bacteria [13]. Unfortunately, sample matrix and exogenous contamination might influence the

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

http://dx.doi.org/10.1016/j.snb.2016.10.042
0925-4005/

results since no sample pretreatment is conducted [16]. Gas phase chromatography has been adopted for specific and rapid identification of viable bacteria by monitoring the characteristic metabolites. However it requires complicated instrumentation and highly trained personnel, and thus is not fit for field assay [14]. Through examination of membrane integrity with colorometric, fluorescent or electrical evaluations, bacteria viability can be determined based on the fact that dead cells lose the physical integrity of the plasma membrane. Practical challenges for membrane integrity detection include speed and portability [15].

Adenosine triphosphate (ATP) exists in all biologically active bacteria as the basic energy substance, and its level in viable cell remains fairly constant ($\sim 10^{-18}$ mol cell$^{-1}$) [17]. Luciferase, in the presence of luciferin and oxygen, catalyzes a reaction that consumes ATP and produces very intensive bioluminescence (BL) emission [18]. Since ATP decays by ATP converting enzyme shortly after the cells die, its level can be detected by the above mentioned sensitive BL method to quantify all metabolically active bacterial cells. Unfortunately, since ATP is a widely-existing substance in most environments, ATP-based method for bacteria detection inevitably suffers from noticeable interference from various sample matrix.

Vancomycin is a broad-spectrum glycopeptide antibiotic mainly acting on Gram-positive bacteria, which can inhibit the biosynthesis of microbial cell wall. It has been reported to bind specifically with D-Alanyl-D-Alanine (D-Ala-D-Ala) moieties of 5′-*N*-acetylmuramyl-pentapeptide on the cell wall through five-point hydrogen bonds [19,20]. Here, vancomycin-functionalized magnetic particles (vanc-MPs) were prepared to isolate rapidly Gram-positive bacteria from complex sample matrix with a novel antibiotic-affinity strategy. After cell lysis, the isolated bacteria were quantified by the intensive BL signal from the intracellular ATP. Thus a novel protocol that combining vanc-MPs isolation and BL detection was developed for the quantification of the total amount of viable Gram-positive bacteria. It could exclude the interference from Gram-negative bacteria, dead bacteria, and complex sample matrix.

## 2. Experimental

### 2.1. Reagents and materials

Carboxy groups-coated magnetic particles (MPs) with a diameter of 2.0 μm were purchased from Biospes Co., Ltd. (China). The bonding amount of carboxyl group on the MPs was about 400–500 μmol g$^{-1}$. MPs could be separated absolutely from its solution within 15 s. Tris (hydroxymethyl) aminomethane (Tris), ATP, d-luciferin, and vancomycin hydrochloride were provided by Sigma-Aldrich Chemical Co., Ltd. (USA). Bovine serum albumin (BSA) was obtained from Gibco (USA). 2-(*N*-morpholino) ethanesulfonic acid (MES) and *N*-hydroxysuccinimide (NHS) were both obtained from J&K Chemical Co., Ltd. (China). Trichloroacetic acid (TCA), benzalkonium chloride (BAC), hexadecyl trimethyl ammonium bromide (CTAB), beta-cyclodextrin (β-CD) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Aladdin Reagent Ltd. (China). Luria-Bertani medium was supplied by Oxoid, Ltd. (USA). Firefly luciferase was purchased from Promega (USA). Dithiothreitol (DTT), magnesium acetate and ethylenediaminetetraacetic acid (EDTA) were purchased from Chengdu Kelong Chemical Co., Ltd. (China). Strains of *Staphylococcus aureus* (*S. aureus*), *Micrococcus luteus* (*M. luteus*), *Bacillus cereus* (*B. cereus*) and *Streptococcus mutans* (*S. mutans*) were all obtained from China Center for Type Culture Collection (China). Strains of *Pseudomonas aeruginosa* (*P. aeruginosa*), *Salmonella typhimurium* (*S. typhimurium*), *Escherichia coli* (*E. coli*) and *Shigella dysenteriae* (*S. dysenteriae*) were provided by Guangdong Microbiology Culture Centers (China). Milk was purchased from the local supermarket. Lake water was collected from Chongde Lake in Southwest University campus. Glucose injection solution and physiological saline injection were purchased from the local pharmacy. The polystyrene high-affinity 96-well microplate was provided by Greiner Bio-One Biochemical Co., Ltd. (Germany).

The dilution buffer for bacteria was 25 mM Tris-acetate buffer at pH 7.8. The activation buffer for MPs was 10 mM MES buffer at pH 5.6. The coupling buffer for amidization reaction was 10 mM phosphate buffer at pH 7.4. The blocking buffer for vanc-MPs was 10 mM phosphate buffer (pH 7.4) containing 1.0% BSA. The ATP extraction solution was 25 mM Tris-acetate buffer (pH 7.8) containing 5.0 mM CTAB and 2.5 mM EDTA. BL reagent solution was 25 mM Tris-acetate (pH 7.8) containing 0.075 mg mL$^{-1}$ luciferase, 0.25 mg mL$^{-1}$ d-luciferin, 2.5 mM EDTA, 25 mM $Mg^{2+}$, 2.5 mg mL$^{-1}$ BSA and 2.5 mM DTT.

### 2.2. Instrumentations

The BL signals were collected using a MPI-A CL analyzer (Xi'an Remax Electronic Science & Technology Co., Ltd, China) equipped with a photomultiplier operated at −800 V. Ultrapure water was prepared by an ELGA PURELAB classic system (France). The scanning electron micrographs were obtained with an S-3000N scanning electron microscope (SEM) (Hitachi Instrument Co., Ltd., Japan). The captured bacteria were separated utilizing a magnetic separator with a magnetic field intensity of 0.35 T (Biocanal Scientific Co., Ltd, China).

### 2.3. Preparation of vanc-MPs

Vanc-MPs were prepared through an amide binding between the carboxy groups-coated MPs and vancomycin with an EDC/NHS amidization reaction. In brief, 1.0 mL of MPs suspension was washed thrice with the activation buffer, and then dispersed in 1.0 mL of activation buffer containing 20 mg of EDC and NHS. After 30-min activation, the MPs were washed thrice and dispersed in 1.0 mL of coupling buffer containing 2.0 mg of vancomycin hydrochloride. Then the suspension was allowed to react for 12 h at 4 °C. The resulted vanc-MPs were washed thrice and dispersed in 1.0 mL of blocking buffer.

### 2.4. Measurement of capture efficiency

Two milliliters of *S. mutans* solution at $1.0 \times 10^6$ CFU mL$^{-1}$ was incubated with 20 μL of vanc-MPs at 10 mg mL$^{-1}$ under constant shaking for 1 h at 37 °C. After a magnetic separation, 500 μL of the collected supernatant was plated on plain Luria-Bertani agar and grown under aerobic condition for 24 h. The un-captured bacteria were quantified by plate counting. Then the capture efficiency was calculated by the bacteria concentrations before and after *S. mutans* was captured by vanc-MPs.

### 2.5. Detection of gram-positive bacteria

Two milliliters of bacteria solution was mixed with 20 μL of vanc-MPs at 10 mg mL$^{-1}$, and incubated under constant shaking for 1 h at 37 °C. The bacteria-capturing vanc-MPs were washed thrice and dispersed in 50 μL of dilution buffer. Subsequently, 150 μL of ATP extraction solution was added into the MPs suspension, and allowed to stand for 2 min. After 150 μL of β-CD solution at 7.5 mM was added, 50 μL of the resulted suspension was moved into a microplate. At last, 50 μL of BL reagent solution was injected into the microplate to trigger BL signal for bacteria detection.