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Short communication

Label-free electrochemiluminescent biosensor for rapid and sensitive detection of pseudomonas aeruginosa using phage as highly specific recognition agent

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ABSTRACT

A virulent phage named as PaP1 was isolated from hospital sewage based on a lambda phage isolation protocol. This phage showed a strong and highly specific binding ability to *Pseudomonas aeruginosa* (*P. aeruginosa*). Using this isolated phage as a recognition agent, a novel electrochemiluminescent (ECL) biosensor was developed for label-free detection of *P. aeruginosa*. The biosensor was fabricated through depositing phage-conjugated carboxyl graphene onto the surface of a glass carbon electrode. After specific binding of the host bacteria through the adsorption of *P. aeruginosa* cell wall by phage tail fibers and baseplate, the ECL signal of luminol suffered a decrease since the formed non-conductive biocomplex obstructed the interfacial electron transfer and blocked the diffusion of the ECL active molecules. The ECL emission declined linearly with *P. aeruginosa* concentration in the range of 1.4×10^{2} –1.4×10^{6} CFU mL^{-1}, with a very low detection limit of 56 CFU mL^{-1}. The whole detection process could be completed within 30 min as a ready-for-use biosensor was adopted. This biosensor was successfully applied to quantitate *P. aeruginosa* in milk, glucose injection and human urine with acceptable recovery values ranging from 78.6% to 114.3%.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative bacterium with ubiquitous occurrence in most kinds of environment, which can rapidly spread through water, foodstuffs and animal feces (Frei et al., 2012; Gorityala et al., 2016). As one of the most common pathogens involved in many diseases, such as bloodstreams and urinary infections, severe burn wounds, metabolic disease and malignant tumor, it has long been afflicting people worldwide (Krithiga et al., 2016; Pastells et al., 2016). Therefore, a rapid, facile, reliable and low-cost platform for *P. aeruginosa* detection has been strongly needed for long time.

Presently, microbiological technique based on bacterial culture and plate counting is the gold standard for *P. aeruginosa* detection (Webster et al., 2014). However, this technique is time-consuming and well-trained personnel demanding, which limits its application in some field environment (Pahlow et al., 2016). Polymerase chain reaction-based approach has been widely used as more rapid alternate for clinical diagnosis in recent years (Lazcka et al., 2007; Zhao et al., 2005). Nevertheless it is performed with cell destruction and nucleic acid extraction, leaving bacterial sample easily been contaminated and thus leading to false results (Cai et al., 2011; Chen et al., 2016). Molecular recognition mode that utilizes recognition agents to anchor the whole cells of bacteria is attracting increasing interest because it is free of bacterial cell destruction. Aptamer (Kumar et al., 2016; Park et al., 2014), antibody (Shigematsu et al., 2007; Su et al., 2007) and antimicrobial peptide (Abdel-Sayed et al., 2016; Brunetti et al., 2016; Liu et al., 2016; Uzarski and Mello, 2012) are the most common used molecular recognition agents for rapid screening of *P. aeruginosa*. These agents always suffer from poor stability, high cost and unavailability to some source-limited institutes. More importantly, the reported methods based on molecular recognition mode usually depend upon time-consuming and labor-intensive labeling process that utilizes nanomaterial (Park et al., 2014), enzyme (Shigematsu et al., 2007; Su et al., 2007) or fluorescence dye (Kumar et al., 2016; Liu et al., 2016) as the signal probes. Thus further efforts are still urgently needed in searching more facile strategy to expand the application possibility of this mode.

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Phages are bacterial viruses which can infect the host bacteria and utilize the replicative machinery of host bacteria to produce the progeny phages (Chen et al., 2015; Le et al., 2014). They can specifically recognize and bind with the host bacteria via their tail fibers and baseplate. Due to the specificity of phage recognition, some phage-based methods have been developed to detect pathogens. For example, a phage amplification technique combined with immunomagnetic separation was developed for quantitation of *Escherichia coli* (*E. coli*) in food by detecting phage marker protein using liquid chromatography coupled with mass spectrometry (Martelet et al., 2015). However, this technique required expensive antibody for immunomagnetic separation and sophisticated instrument for protein detection. A phage display protocol had been designed to quantitate the same pathogen by displaying tetracysteine peptide tag on the capsid protein of phage M13KE, in which the displayed tag could be detected through its reaction with fluorescence-labeled biarsenical dye (Wu et al., 2011). Unfortunately, this strategy demanded a complicated gene manipulation to construct a recombinant phage by cloning a DNA sequence into the phage capsid protein gene.

Herein, a phage highly specific to *P. aeruginosa* was isolated from hospital sewage based on a lambda phage isolation protocol. This phage named as PaP1 was used as a recognition agent to fabricate a novel label-free electrochemiluminescent (ECL) biosensor for *P. aeruginosa* detection. Carboxyl graphene was adopted as the substrate for loading PaP1 due to its large surface area and signal enhancement to luminol ECL system (Li et al., 2014). The strong ECL emission was suppressed as the target pathogens were specifically captured by the fabricated biosensor due to the formed non-conductive biocomplex. Compared with other molecular recognition agents such as antibody, aptamer and antimicrobial peptide, phage shows lower cost as well as higher specificity, and can be easily cultured in most source-limited microbiological laboratories. Furthermore, the developed phage-based label-free biosensor is much more facile and user-friendly in comparison with the previously reported phage amplification technique (Martelet et al., 2015) and phage display protocol (Wu et al., 2011) which require sophisticated instrument and complicated gene manipulation.

2. Experimental

2.1. Preparation of ECL biosensor

The detailed information for the isolation and treatment of PaP1 is described in the Supporting Information. For the preparation of ECL biosensor, a glassy carbon electrode was sequentially polished with 1.0-μm, 0.3-μm, and 0.05-μm aluminum oxide powder and ultrasonically washed in ethanol and ultra-pure water for 5 min. After getting a clean and smooth surface, 5.0 μL of carboxyl graphene-PaP1 composite was dropped onto the electrode and dried at 4 °C to form film. Then the modified electrode was blocked by 30 μL of 3.0% bovine serum albumin (BSA) for 1.5 h at 37 °C. After thoroughly rinsing with phosphate buffered saline (PBS) at pH 7.4, the electrode was stored at 4 °C till use.

2.2. ECL detection of P. aeruginosa

The detailed information for the treatment of *P. aeruginosa* is described in the supporting information. To achieve label-free ECL detection of *P. aeruginosa*, 30 μL of sample solution was dropped onto the reaction region of the biosensor. After 20-min incubation at 37 °C and thorough rinsing with PBS at pH 7.4, the biosensor was inserted into an electrochemical cell filled with 6.0 mL of PBS at pH 8.5 containing 0.10 mM luminol. By a scan potential from −0.6 to 0.6 V and a scan rate of 100 mV s^{-1} applied on the biosensor, the ECL signal was triggered and collected for *P. aeruginosa* quantitation.

3. Results and discussion

3.1. Biological characterization of PaP1 and its binding behavior with P. aeruginosa

Phages can be categorized into temperate and virulent phages on the basis of the lifecycle. For temperate phages, their genome transfer and integrate into the genome of host bacteria to form a stationary state, and this state does not change until the rigid environmental conditions force the phages to enter a lytic cycle. While for virulent phages, they replicate only through one lytic cycle (Latino et al., 2014). In this life cycle of virulent phages, after the phages infect the host bacteria, they utilize the host cells as their own “machineries” to produce large quantities of progeny phages. To release the progeny phages, the host bacteria were disrupted by the phage holin and endolysin (Kutter and Sulakvelidze, 2005). As shown in Fig. S1A, numerous transparent plaques were observed on the lawn of *P. aeruginosa* after the isolated phages were mixed with *P. aeruginosa* and cultured overnight. The result demonstrated the successful isolation of phages specific to *P. aeruginosa*. Meanwhile, it also proved that the isolated PaP1 belongs to virulent phage group since the phages lysed the host bacteria to release progeny phages under suitable conditions for bacteria growth. The isolated PaP1 was also observed using transmission electron microscopy (TEM). As seen in Fig. S1B, it was composed of a 70-nm icosahedral symmetrical head and a 130-nm tail. Using one-step growth curve protocol, the capture time and complete lysis time of PaP1 to *P. aeruginosa* were measured to be 15 min and 60 min, respectively.

To investigate the binding behavior between PaP1 and the host bacteria, fluorescein isothiocyanate (FITC)-tagged PaP1 was incubated with *P. aeruginosa* and observed under the laser confocal fluorescence microscope (detailed process described in the supporting information). As seen in Fig. S1C and S1D, green fluorescence from FITC was observed on the bacterial cells, demonstrating that the isolated phages could bind with the cell wall of their host bacteria. The phenomenon also displayed the feasibility of label-free detection of pathogens based on capture of *P. aeruginosa* to the ECL biosensor.

3.2. Principle of label-free ECL bioassay

In this work, luminol was chosen as the ECL reagent due to its low oxidation potential yet high emission efficiency (Cui et al., 2004; Dai et al., 2012; Feng et al., 2015). Fig. 1A illustrates its ECL reaction mechanism. As an ECL nanomaterial with strong electron transfer ability, carboxyl graphene had already been applied in bioassay to enhance luminol ECL emission (Li et al., 2014). Herein this nanomaterial was adopted for phages conjugation and glassy carbon electrode modification. Owing to its abundant carboxyl groups and large surface, it showed great immobilization ability to PaP1. Since phage tail fibers and baseplate could specifically bind with *P. aeruginosa* cell wall, the whole cells of *P. aeruginosa* were captured by the biosensor. Characterizations of the biosensor surface using scanning electron microscopy (SEM) and ESI demonstrated the conjugation of PaP1 onto carboxyl graphene, as well as the capture of *P. aeruginosa* by PaP1 (detailed process and results described in the Supporting Information). Then the diffusion of ECL active molecules was hindered and the interfacial electron transfer was interrupted by the formed phages/bacteria biocomplex, resulting in an obvious decrease of ECL intensity (Fig. 1B). Hence, a label-free ECL protocol was established for *P. aeruginosa* detection.

3.3. Analytical performance

Since the binding of *P. aeruginosa* on the biosensor inhibited the ECL emission of luminol, the ECL signal decreased linearly with the logarithmic value of bacterial concentration in the range of 1.4×10^2

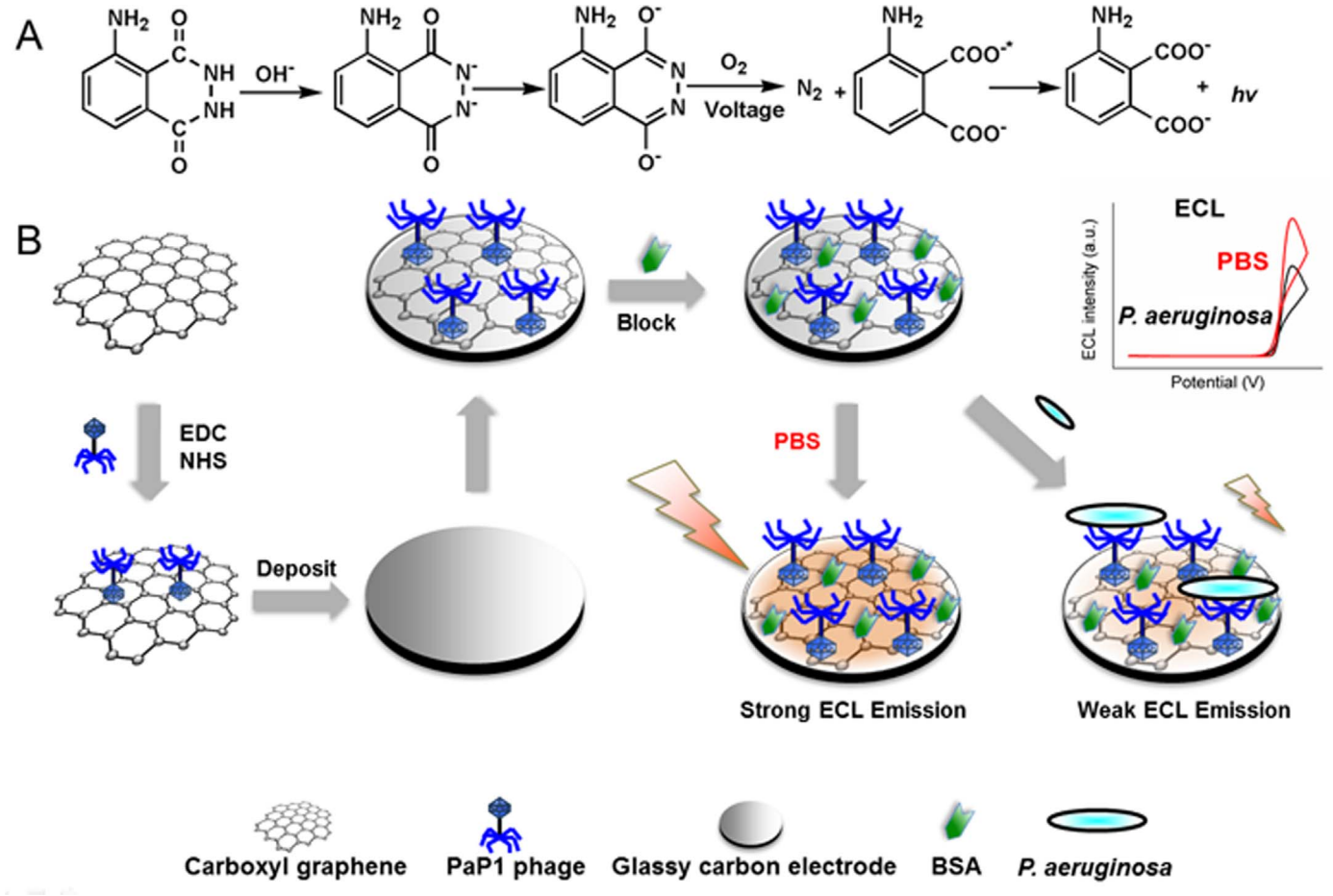

Fig. 1. (A) ECL reaction mechanism of luminol in alkaline electrolyte solution. (B) Schematic illustration of biosensor fabrication and *P. aeruginosa* detection.

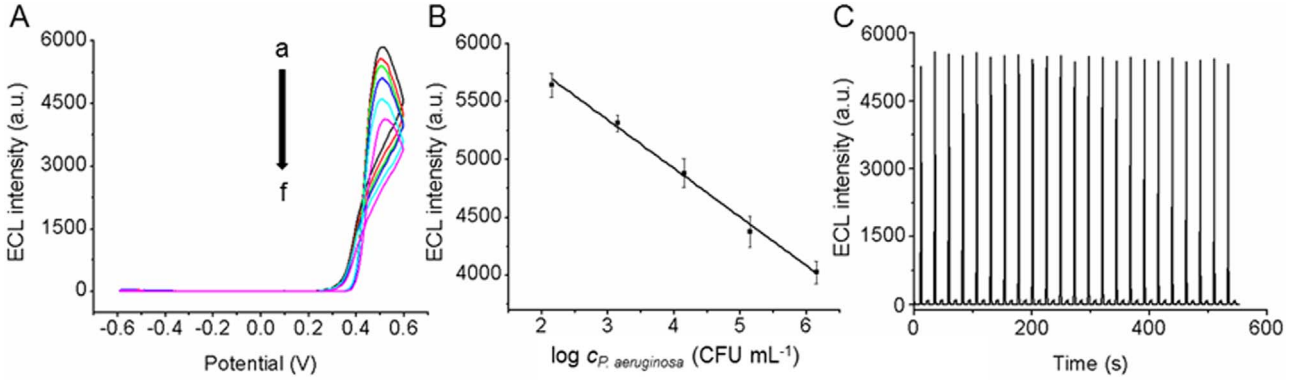

Fig. 2. (A) The ECL responses of *P. aeruginosa* at (a) 0, (b) 1.4×10^2, (c) 1.4×10^3, (d) 1.4×10^4, (e) 1.4×10^5 and (f) 1.4×10^6 CFU mL^{-1}. (B) Regressive curve for *P. aeruginosa* detection, where n =5 for each point. (C) ECL emission under 23 cycles of continuous potential scans for the detection of *P. aeruginosa* at 1.0×10^2 CFU mL^{-1}. All detections were conducted under the optimal conditions.

-1.4×10^6 CFU mL^{-1} (Figs. 2A and B). Under the optimal conditions described in the Supporting Information, the linear regression equation was I (a. u.) =6598.4−419.1 log c (CFU mL^{-1}), with a correlation coefficient (R^2) of 0.994. The detection limit was 56 CFU mL^{-1}, which was defined as the concentration resulting in a 10% decrease of the signal from the blank sample. For *P. aeruginosa* at low (1.4×10^2 CFU mL^{-1}), medium (1.4×10^4 CFU mL^{-1}) and high (1.4×10^6 CFU mL^{-1}) concentrations, the relative standard deviation (RSD) values for five replicated detections were 1.8%, 2.6% and 2.4%, respectively, showing ideal repeatability. The whole detection process could be completed within 30 min as a ready-for-use biosensor was adopted. Fig. 2C shows the luminol ECL emission under 23 cycles of continuous potential scans from −0.6 to 0.6 V for *P. aeruginosa* standard sample at 1.0×10^2 CFU mL^{-1}. Steady ECL emissions were obtained with a RSD value of 1.8%. The storage stability of the prepared biosensor was also investigated. After a storage in PBS at 4 °C for 4 weeks, 92.8% of its initial ECL response still remained. Hence, good repeatability and acceptable durability were achieved for the developed label-free ECL biosensor.

3.4. Specificity

It is well known that phages show high specificity to the host bacteria, thus the biosensor using phages as the recognition agent did

Table 1
Recovery test for real samples spiked with *P. aeruginosa* at different concentrations.

Sample	Spiked (CFU mL^{-1})	Found (CFU mL^{-1})	RSD (%)	Recovery (%)
Milk	1.4×10^2	1.3×10^2	1.5	92.9
	1.4×10^3	1.5×10^3	2.3	107.1
	1.4×10^4	1.6×10^4	4.9	114.3
	1.4×10^5	1.1×10^5	6.7	78.6
Glucose injection	1.4×10^2	1.5×10^2	4.8	107.1
	1.4×10^3	1.4×10^3	7.3	100.0
	1.4×10^4	1.2×10^4	5.5	85.7
	1.4×10^5	1.3×10^5	4.3	92.9
Human urine	1.4×10^2	1.6×10^2	5.3	114.3
	1.4×10^3	1.2×10^3	5.6	85.7
	1.4×10^4	1.2×10^4	2.7	85.7
	1.4×10^5	1.3×10^5	3.6	92.9

not trend to be interfered by other bacteria. In order to assess the specificity of the developed biosensor for *P. aeruginosa* detection, two gram-negative bacteria (*E. coli* and *Salmonella*) and three gram-positive bacteria (*Staphylococcus aureus* (*S. aureus*), *Micrococcus luteus* (*M. luteus*) and *Bacillus subtilis* (*B. subtilis*)), were adopted as the potential interferents. The concentrations of all bacteria in the specificity investigation were 1.0×10^4 CFU mL^{-1}. As seen in Fig. S3, only *P. aeruginosa* led to an obvious decrease of luminol ECL emission, while the five interfering bacteria at the same concentration all showed minor change of ECL responses compared with that from the blank (PBS). The degree of interference (DI) values of these interferents were calculated by the following equation:

$$DI = \frac{B - A}{C - A} \times 100\% \quad (1)$$

Here *A*, *B* and C were the signals from the blank, the interfering bacteria and *P. aeruginosa*, respectively. The DI values for *E. coli*, *Salmonella*, *S. aureus*, *M. luteus* and *B. subtilis* were 6.3%, 6.5%, 3.6%, 2.5% and 3.9%, respectively.

Additionally, mixture 1 composed of all the five interferents and mixture 2 composed of *P. aeruginosa* as well as the five interferents were prepared and detected using this biosensor. A minor DI value of 3.6% was obtained for mixture 1. Furthermore, a negligible change of ECL response (1.8%) was observed from mixture 2 in comparison with that from *P. aeruginosa*. The above results suggested that these investigated potential interfering bacteria did not interfere the detection of *P. aeruginosa* due to the high specificity of bacteria recognition by PaP1.

3.5. Application in real samples

In order to further evaluate the practical application potential of the label-free ECL biosensor for pathogen detection, sterile samples of milk, glucose injection and human urine were spiked with *P. aeruginosa* at various known concentrations and detected using the developed biosensor. As seen in Table 1, the recovery values were from 78.6% to 114.3%, with RSD values all less than 7.3%, indicating acceptable reliability of this biosensor for real sample detection.

4. Conclusions

In conclusion, a phage named as PaP1 was isolated from hospital sewage based on a lambda phage isolation protocol. This phage was used as a high specific recognition agent to fabricate an ECL biosensor for detection of *P. aeruginosa* whole cells based on the strong binding of *P. aeruginosa* cell wall by phage tail fibers and baseplate. The bound bacteria led to a noticeable decrease of ECL signal due to the obstructed interfacial electron transfer and the blocked diffusion of ECL active molecules. As a highly specific molecular recognition agent, phage could be cultured in most microbiological laboratories with very low cost. Thus it is much more available for source-limited institutes in comparison with other recognition agents such as antibody and aptamer. Furthermore, detection of the host bacteria using this biosensor was conducted in a label-free mode. Thus it is a very facile and user-friendly protocol for pathogen detection, and shows great application potential in food safety, medical diagnosis and homeland security. However, *P. aeruginosa* has some heterogeneity in lipopolysaccharides and slime polysaccharides, which may affect the performance of the developed biosensor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.03.033.

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