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A facile label-free electrochemiluminescent biosensor for specific detection of *Staphylococcus aureus* utilizing the binding between immunoglobulin G and protein A

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ABSTRACT

A facile label-free electrochemiluminescent (ECL) biosensor was developed for detection of *Staphylococcus aureus* (*S. aureus*) based on the specific binding between Fc region of immunoglobulin G (IgG) and *S. aureus* protein A (SPA) in the cell wall. Carboxyl graphene, with large surface and excellent electron transfer ability, was used as the carrier of IgG for fabrication of ECL biosensor. This biosensor was constructed by depositing carboxyl graphene/porcin IgG composite on the surface of a glassy carbon electrode. The specific reaction between SPA and IgG resulted in a decrease of ECL signal because the bound *S. aureus* interrupted the interfacial electron transfer and hindered the diffusion of the ECL active substances. The ECL intensity decreased linearly with *S. aureus* concentrations in the range of 1.0×10^3–1.0×10^9 colony-forming units (CFU) mL^{-1}, with a detection limit of 3.1×10^2 CFU mL^{-1}. The whole assay could be accomplished within 70 min when a ready-for-use biosensor was applied. The recovery test for food, environmental and biological samples showed recoveries between 75.0% and 116.7%. This developed biosensor displayed ideal specificity, high sensitivity, facile manipulation, simple fabrication and short assay time, thus provided a new pathway for pathogenic bacteria rapid screening.

1. Introduction

Staphylococcus aureus (*S. aureus*), as the second most common pathogenic bacterium, has long been threatening public health. It exists in most kinds of environments and disseminates rapidly by such media as water, air, food and biological contact, afflicting people worldwide and mostly in developing and underdeveloped countries [1–4]. Therefore, a reliable, rapid, facile, and low-cost detection technique for *S. aureus* is urgently demanded for control of infectious diseases.

The approach relying on bacterial culture and colony counting is well known to be the gold standard for *S. aureus* detection due to its high reliability [5]. Although it is error-proof, it has been proved to be unsuitable for point-of-care test (POCT) because it demands well-trained personnel and long-time culture consuming typically no less than 1 day [6]. Alternatively, polymerase chain reaction-based methods requiring much shorter time (several hours) are also frequently used for *S. aureus* detection in the recent years [7,8]. Unfortunately, in spite of their short assay time and preferable sensitivity, they require cell destruction and nucleic acid extraction, making bacterial samples prone to contamination and leading to false results. Recently, the molecular recognition mode, which utilizes recognition agents to bind specifically with the whole cells of bacteria, is becoming attractive because it is free of cell destruction. Antibody [9–11], antimicrobial peptide [12–15], aptamer [16–19], phage and its function protein [20–22] have been successfully used as molecular recognition agents for *S. aureus* to achieve rapid screening. Various electrochemical, fluorescent, microfluidic and surface plasmon resonance biosensors have been well developed by using these molecular recognition agents [9–11,17,18,20,21]. These agents are usually expensive, unstable, or sometimes commercially unavailable, thus are not fit to be used in source-limited institute. Most of all, these assay methods are usually based on laborious and time-consuming labeling strategy that utilizes enzyme [9–12], fluorescence dyes [13,14,16,20] or nanomaterials [15,17–19,21] as the signal probes. Consequently, additional efforts are still urgently needed to develop a more facile and low-cost technique based on molecular recognition mode to extend its application.

As a protein highly expressed in the cell wall of *S. aureus*,

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S. aureus protein A (SPA) is known to be capable of binding specifically with Fc region of immunoglobulin G (IgG) [23]. This protein has been conventionally adopted in immunoassays to oriented immobilization of antibody and expose Fab region of IgG to facilitate immunobinding between antibody and antigen [24–26].

Graphene, a two-dimensional carbon sheet with almost one atom thickness, has been proved to be an ideal carrier for protein immobilization due to its powerful mechanical strength, large surface area, and excellent thermal stability [27,28]. Moreover, it can also serve as an electrode material for enhancing electrochemical signal owing to its strong electron transfer ability. Compared with graphene, carboxyl graphene displays much better water dispersibility and much stronger immobilization ability for biomolecules. This material has already been reported to greatly improve the ECL intensity of luminol, thus facilitate fabrication of ECL sensor with high sensitivity [29].

Herein, a label-free ECL biosensor for *S. aureus* detection was developed by immobilizing porcin IgG on carboxyl graphene-deposited electrode. This biosensor emitted very strong ECL signal after it was electrochemically triggered in alkaline electrolyte solution containing luminol. The specific binding of *S. aureus* onto the porcin IgG-immobilized biosensor resulted in a great decrease of the ECL emission. In comparison with the previously reported methods based on probe labeling strategy, this label-free ECL biosensor showed attractive merits such as simple fabrication, facile manipulation and low cost, thus provided a promising sensing platform for POCT of pathogenic bacteria.

2. Experimental

2.1. Materials and instruments

Porcin IgG and fluorescein isothiocyanate (FITC)-labeled porcin IgG were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Aladdin Chemistry Co., Ltd. (China). *N*-Hydroxysuccinimide (NHS) and luminol were obtained from Sigma-Aldrich Chemical Co., Ltd. (USA). Bovine serum albumin (BSA) and 2-(*N*-morpholino) ethanesulfonic acid monohydrate (MES) were provided by Beijing Dingguo Biotechnology Co., Ltd. (China). Carboxyl graphene was purchased from Nanjing XFNANO Materials Tech Co., Ltd. (China). Strains of *S. aureus*, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Salmonella*, *Micrococcus luteus* (*M. luteus*) and *Bacillus subtilis* (*B. subtilis*) were all provided by Chongqing Center for Disease Control and Prevention (China). Phosphate buffer saline (PBS) at 0.01 M was used as dilution buffer for all biomaterials. Ultra-pure water (18.2 MΩ) purified by an ELGA PURELAB classic system (France) was used to prepare all aqueous solutions. Milk was purchased freshly from the local supermarket. Lake water was collected from Chongde Lake inside campus. Human saliva and human urine were all acquired from a healthy volunteer in the lab.

ECL signals were detected on a MPI-A ECL Analyzer (Xi'an Remax Electronic Science & Technology Co., Ltd., China) equipped with a three-electrode system consisting of a glassy carbon working electrode (3 mm in diameter), an Ag/AgCl reference electrode, and a platinum wire auxiliary electrode. Electrochemical impedance spectroscopy (EIS) was measured using an Autolab PGSTAT302 electrochemical workstation (Netherlands). Scanning electron microscopy (SEM) images were obtained with an S-3000N scanning electron microscope (Hitachi Instrument Co., Ltd., Japan). Fluorescence micrographs were obtained from a LSM710 laser confocal fluorescence microscopy (Carl Zeiss Co., Ltd., Germany).

2.2. Bacteria culture, counting and pretreatment

The strains of bacteria were grown in Luria-Bertani broth medium at 37 °C with constant shaking for 11 h. The result of agar plate counting for colony units showed that the concentration of the achieved *S. aureus* solution was 1.2×10^9 colony-forming units (CFU) mL^{-1}. The bacterial culture were centrifuged at 5000 rpm for 3 min to discard the supernatant, and washed twice with PBS. The collected bacteria pellet were then re-suspended and diluted serially with PBS.

2.3. Preparation of carboxyl graphene/porcin IgG composite

Porcin IgG was conjugated to carboxyl graphene by a classic EDC/NHS chemical reaction. Briefly, 0.6 mg of carboxyl graphene was dispersed in 2.0 mL of freshly prepared MES buffer (pH 5.2) containing 800 mmol L^{-1} EDC and 200 mmol L^{-1} NHS to activate the carboxyl groups for 0.5 h. Then the activated carboxyl graphene was centrifugally washed thrice to remove excessive EDC and NHS. Afterward, it was re-dispersed in 2.0 mL of PBS at pH 7.4, and mixed with 20 μL of 1.0 mg mL^{-1} porcin IgG. After overnight reaction at 4 °C, it was centrifugally washed thrice to remove unconjugated porcin IgG. The resulted carboxyl graphene/porcin IgG composite was re-suspended in 2.0 mL of PBS at pH 7.4, and stored at 4 °C until use.

2.4. Confocal microscopy imaging of fluorescence-stained S. aureus

One hundred microliters of *S. aureus* solution at 1.0×10^8 CFU mL^{-1} was mixed with 50 μL of FITC-labeled porcin IgG solution at 1.0 mg mL^{-1}, followed by an incubation of 1 h at 37 °C. The stained *S. aureus* cells was centrifugally washed thrice, and then re-suspended in PBS. Twelve microliters of the obtained solution was dropped onto a microscope slide and covered by a cover slip. Finally, the specimen was observed under the laser confocal fluorescence microscopy with a magnification of 1000. The excitation and emission wavelength for FITC were chosen to be 488 nm and 525 nm, respectively.

2.5. Preparation of ECL biosensor

The glassy carbon working electrode was polished and ultrasonically washed in ethanol and water sequentially, to get a mirror-like surface. Subsequently, 5.0 μL of carboxyl graphene/porcin IgG composite was deposited onto the electrode and dried at room temperature to form film. Then the electrode was blocked with 3.0% BSA for 1.5 h at room temperature to minimize the non-specific binding. After rinsing with PBS, the electrode was stored in PBS at 4 °C until use.

2.6. ECL assay procedure for S. aureus

To conduct a label-free ECL assay, the biosensor was incubated with 30 μL of *S. aureus* sample at 37 °C for 1 h, to achieve biological recognition of *S. aureus*. After a thorough rinsing, the biosensor was inserted into an electrochemical cell filled with 6.0 mL of 0.01 M PBS (pH 8.5) containing 0.10 mmol L^{-1} luminol. With a scan rate of 100 mV s^{-1}, a scan potential from −0.6 V to 0.6 V was applied on the biosensor to trigger the ECL signal. Then the signal was collected for *S. aureus* quantification.

3. Results and discussion

3.1. Binding behavior of porcin IgG to S. aureus

To demonstrate the binding ability of porcin IgG to *S. aureus*, FITC-labeled porcin IgG was incubated with *S. aureus* solution, and the fluorescence-stained cells were observed under the fluorescence microscopy. As seen in Fig. 1, green fluorescence from FITC was observed mainly on the outer layer of bacterial cells, which was consistent with the previous claim that SPA existed in the cell wall of *S. aureus* [25]. The phenomenon also demonstrated the feasibility of label-free detection of *S. aureus* based on the binding of porcin IgG to *S. aureus*.

3.2. Principle of ECL bioassay

As an ECL reagent with low oxidation potential yet high emission efficiency, luminol has drawn increasing interest and been frequently applied in bioassay. Its ECL reaction mechanism is shown in Fig. 2A [30]. Carboxyl graphene was reported to greatly enhance luminol ECL emission due to its strong electron transfer ability [29]. Therefore, this nanomaterial was adopted to modify the glassy carbon electrode in this work. It also displayed strong immobilization ability to porcin IgG for its large surface and abundant carboxyl groups. Since Fc region of porcin IgG bound specifically with SPA, *S. aureus* whole cells were captured on the surface of biosensor. Then the interfacial electron transfer was interrupted and the diffusion of the ECL active molecules was hindered by the as-formed porcin IgG/*S. aureus* biocomplex, which resulted in a decrease of ECL intensity (Fig. 2B). Thus a label-free biosensor for *S. aureus* detection was developed based on the decrease of ECL signal resulting from the bio-recognition event.

3.3. Characterization of ECL biosensor

The surface of the *S. aureus* biosensor was characterized by SEM. Fig. 3A shows the surface morphology of the biosensor before it was incubated with *S. aureus*. After the biosensor was incubated with bacterial solution, a large number of *S. aureus* cells (0.8 μm in diameter) were captured on the surface of the biosensor (Fig. 3B), demonstrating the binding between porcin IgG and *S. aureus*.

The electrode modifying and binding processes were characterized by EIS obtained in 5.0 mmol L^{-1} $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ solution containing 0.1 mol L^{-1} KCl. As seen in Fig. 3C, the bare glassy carbon electrode showed a electron transfer resistance (R_{et}) of 4261 Ω (curve a). After carboxyl graphene was modified on the electrode, a greatly decreased R_{et} value of 1580 Ω was obtained because carboxyl graphene facilitated the interficial electron transfer (curve b). An increased R_{et} value of 2351 Ω was obtained for carboxyl graphene/porcin IgG modified electrode since porcin IgG hindered the electron transfer (curve c). A further increase of R_{et} value (4788 Ω) was observed for the same reason after the modified electrode was blocked with BSA (curve d). After the prepared biosensor was incubated with *S. aureus* solution, an even larger R_{et} value of 5220 Ω was observed owing to the binding of bacterial cells onto its surface (curve e).

3.4. Optimization of experimental conditions

Some factors influencing the performance of the proposed biosensor were investigated in detail using *S. aureus* at 1.0×10^5 CFU mL^{-1}. For this label-free assay strategy, it is known that the ECL response of the biosensor was decided by the amount of the as-formed porcin IgG/*S. aureus* biocomplex on its surface, while the latter was affected by the blocking time, the incubation time and the amount of immobilized porcin IgG. As shown in Fig. 4A and B, the ECL intensity decreased gradually with an increasing blocking and incubation time, and almost tended to a plateau at 90 min and 60 min, respectively, suggesting a binding saturation of *S. aureus* to the biosensor. Additionally, the influence of the amount of immobilized porcin IgG on the ECL response was investigated using *S. aureus* and PBS (as a blank) in parallel. Fig. 4C shows that the signal-to-blank ratio reached the minimum when the concentration of porcin IgG was 1.0 mg mL^{-1}, indicating a strongest inhibition of ECL signal. Thus, the blocking time of 90 min, the incubation time of 60 min and the porcin IgG concentration of 1.0 mg mL^{-1} were selected for further investigation.

3.5. Analytical performance

Under the optimal conditions, ECL signal decreased linearly with the logarithmic value of *S. aureus* concentration in the range of 1.0×10^3–1.0×10^9 CFU mL^{-1} (Fig. 5A and B), since the binding of *S. aureus* on the biosensor depressed the ECL emission of luminol. The linear regression equation was I(a. u.)= 6396.2 – 497.2log c (CFU mL^{-1}), with a coefficient of determination (R^2) of 0.995. The detection limit defined as the concentration resulting in a 10% decrease of the signal from the blank sample was 3.1×10^2 CFU mL^{-1}, which is comparable with that of immunosensor, one of the most popular biosensors for bacteria detection [9–11]. For *S. aureus* at low (1.0×10^3 CFU mL^{-1}), medium

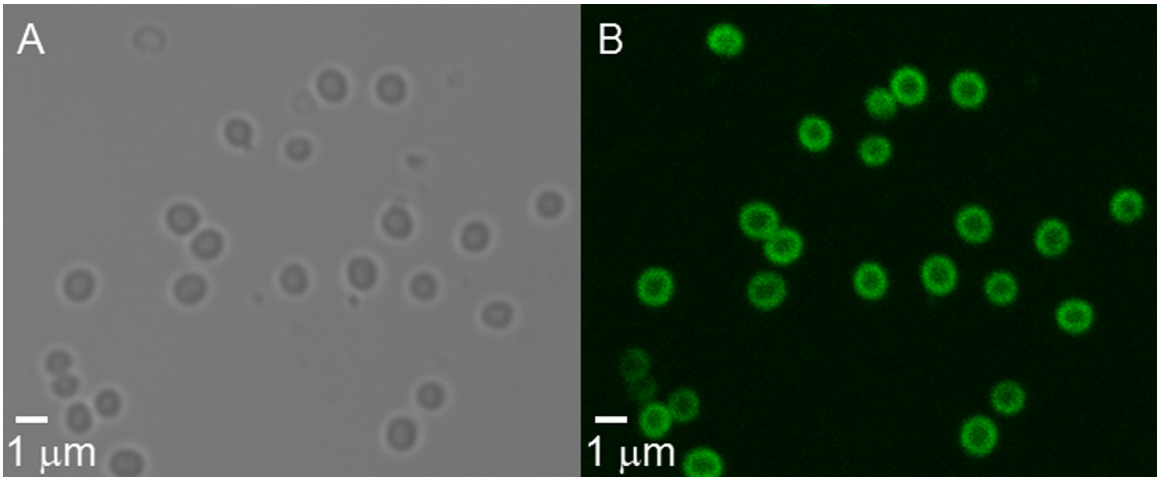

Fig. 1. Fluorescence micrographs of *S. aureus* stained with FITC-labeled porcin IgG. (A) bright field image, (B) image of green fluorescence channel.

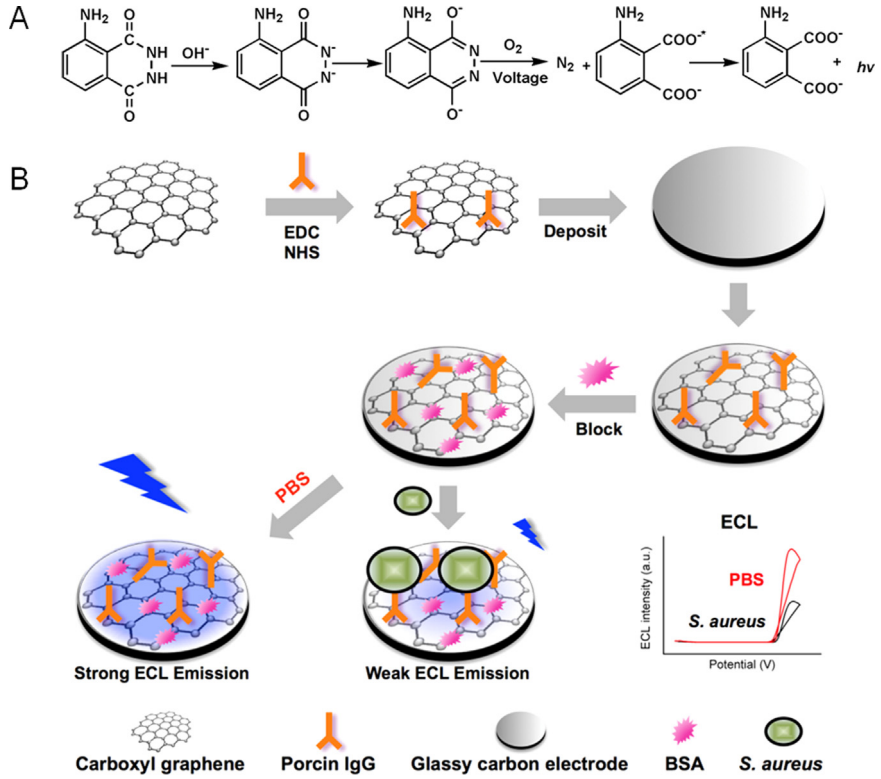

Fig. 2. (A) The ECL reaction mechanism of luminol in alkaline electrolyte solution. (B) Schematic illustration of biosensor fabrication and *S. aureus* assay strategy.

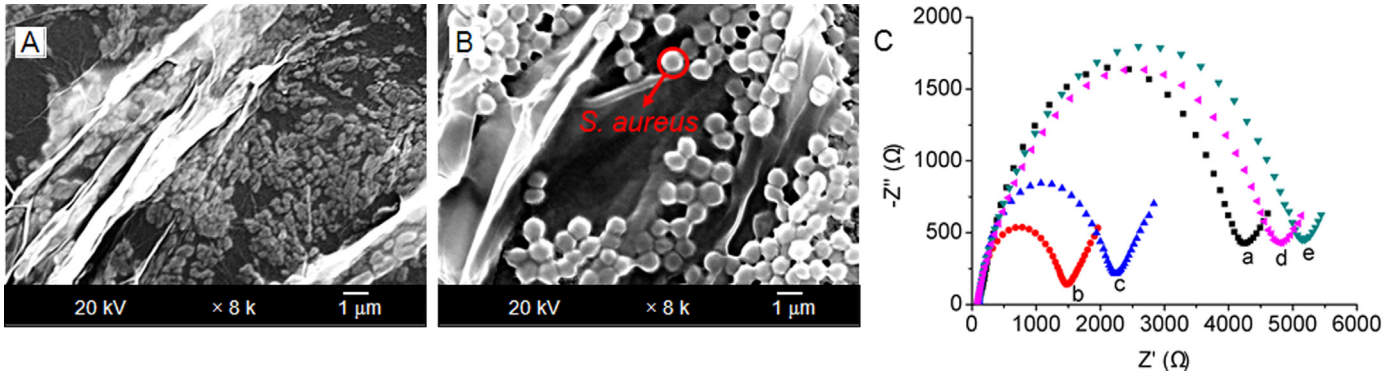

Fig. 3. SEM images of the biosensor surface before (A) and after (B) it was incubated with *S. aureus* solution. (C) EIS spectra of (a) bare glassy carbon electrode, (b) glassy carbon electrode modified with carboxyl graphene, (c) glassy carbon electrode modified with carboxyl graphene/porcin IgG composite, (d) glassy carbon electrode modified with graphene/porcin IgG composite and blocked with BSA, and (e) prepared biosensor incubated with *S. aureus*.

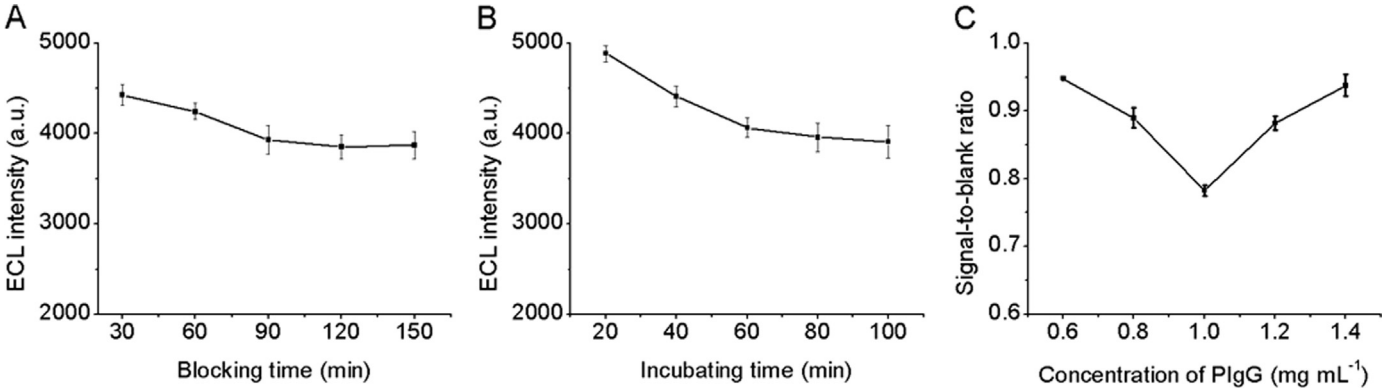

Fig. 4. Effect of (A) blocking time, (B) incubation time and (C) concentration of porcin IgG on the signal-to-blank ratio for detection of *S. aureus* at 1.0×10^5 CFU mL^{-1}. All the tests were performed under the optimal conditions, where $n=5$ for each point.

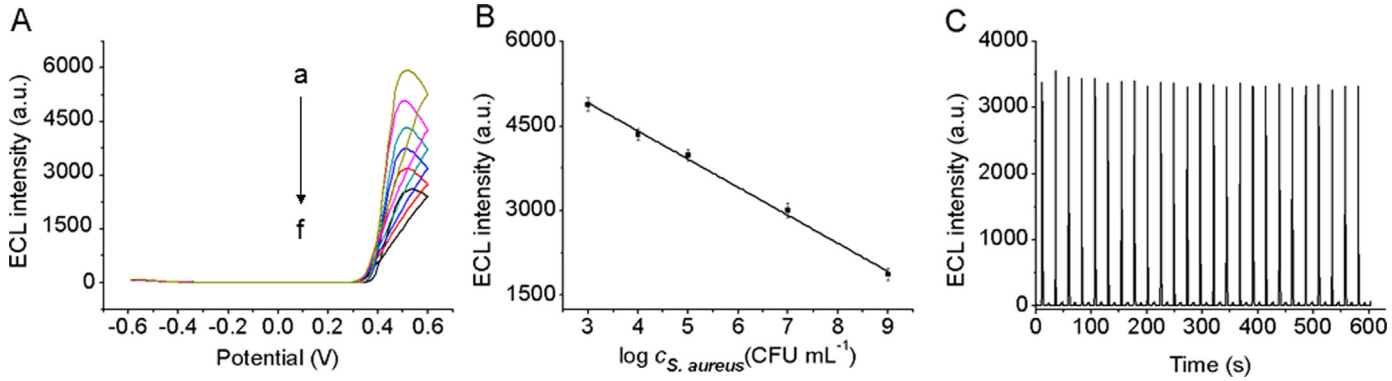

Fig. 5. (A) The ECL responses of *S. aureus* at (a) 0 (blank), (b) 1.0×10^3, (c) 1.0×10^4, (d) 1.0×10^5, (e) 1.0×10^7 and (f) 1.0×10^9 CFU mL^{-1}. (B) Regressive curve for *S. aureus* detection, where $n=5$ for each point. (C) ECL emission under 25 cycles of continuous potential scans for the detection of 1.0×10^6 CFU mL^{-1} *S. aureus*. All the tests were under the optimal conditions.

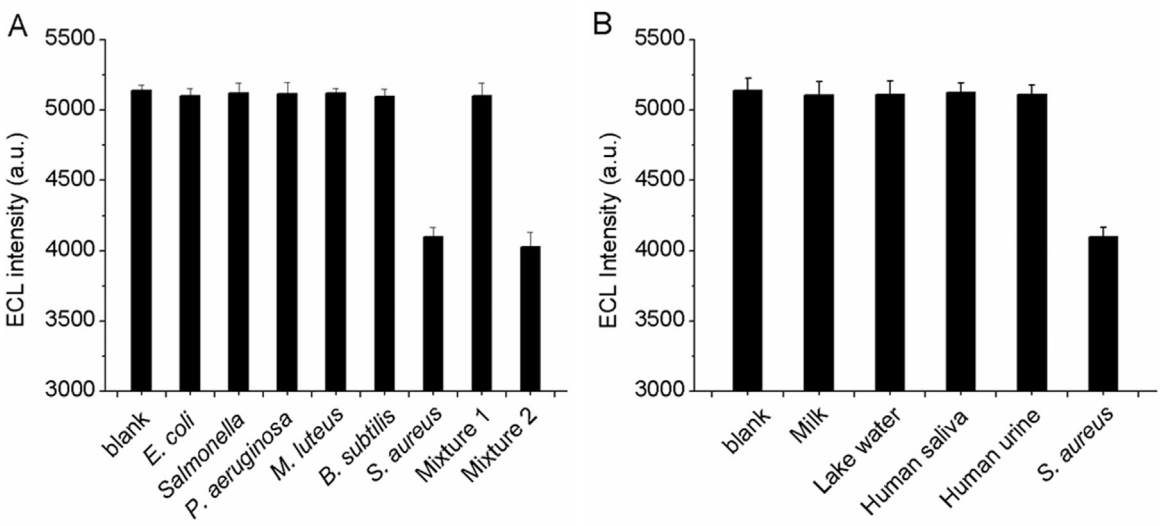

Fig. 6. (A) ECL responses of blank sample (PBS), *S. aureus* and the interfering bacteria all at 1.0×10^5 CFU mL^{-1}. (B) ECL responses of the four un-spiked real samples, blank sample (PBS) and *S. aureus* at 1.0×10^5 CFU mL^{-1}. All assay conditions were the chosen optimal conditions, where $n = 5$ for all points.

Table 1
Recovery test for real samples spiked with *S. aureus* at different concentrations.

Sample	Spiked (CFU mL^{-1})	Found (CFU mL^{-1})	RSD (%)	Recovery (%)
Milk	1.2×10^4	1.1×10^4	2.4	91.7
	1.2×10^5	0.9×10^5	2.2	75.0
	1.2×10^6	1.0×10^6	1.6	83.3
Human saliva	1.2×10^4	1.3×10^4	4.9	108.3
	1.2×10^5	1.0×10^5	1.4	83.3
	1.2×10^6	1.1×10^6	2.4	91.7
Lake water	1.2×10^4	1.0×10^4	3.3	83.3
	1.2×10^5	1.4×10^5	2.4	116.7
	1.2×10^6	1.2×10^6	3.8	100.0
Human urine	1.2×10^4	1.4×10^4	3.8	116.7
	1.2×10^5	1.0×10^5	2.1	83.3
	1.2×10^6	1.4×10^6	5.7	116.7

(1.0×10^5 CFU mL^{-1}) and high (1.0×10^9 CFU mL^{-1}) concentrations, the relative standard deviation (RSD) values for five replicated detections were 2.5%, 2.6% and 5.9%, respectively, showing acceptable repeatability. By using a ready-for-use biosensor that had already been modified and blocked, the whole assay process could be accomplished within 70 min, which is shorter than those of the reported immunosensors [9–11]. Stability was one of the major concerns for practical application of biosensor. Fig. 5C displays the ECL emission of the luminol system under 25 cycles of continuous potential scans between −0.6 and 0.6 V for the detection of 1.0×10^6 CFU mL^{-1} *S. aureus*. Stable ECL signals were observed with a RSD value of 1.8%. The biosensor retained 91.3% of its initial response after it was stored in PBS at 4 °C for 15 days, showing acceptable storage stability. In comparison with the reported immunosensors, this method shows outstanding advantages such as low cost and facile manipulation because a label-free protocol and non-specific IgG were adopted.

3.6. Specificity

The specificity of the developed biosensor was investigated in detail by comparing its ECL responses to PBS (as blank), *S. aureus* and five potential interfering bacteria, including three gram-negative bacteria (*E. coli*, *Salmonella* and *P. aeruginosa*), and two gram-positive bacteria (*M. Luteus* and *B. subtilis*). As seen in Fig. 6A, only *S. aureus* at 1.0×10^5 CFU mL^{-1} resulted in an obvious

decrease of ECL emission of luminol. The ECL responses from the interfering bacteria at the same concentration all showed negligible difference compared to that from blank. The degree of interference value of these interferents was calculated according to the following equation:

$$\text{Degree of interference} = \frac{A - C}{B - C} \times 100\% \quad (1)$$

where *A*, *B* and *C* are the signals from the interfering bacteria, *S. aureus* and the blank, respectively. The values of degree of interference for the five interferents (*E. coli*, *Salmonella*, *P. aeruginosa*, *M. Luteus* and *B. subtilis*) were 4.0%, 2.1%, 2.6%, 1.8% and 4.3%, respectively.

Additionally, mixture 1 composed of the five interfering bacteria (all at 1.0×10^5 CFU mL^{-1}) and mixture 2 composed of *S. aureus* and the five interferents (all at 1.0×10^5 CFU mL^{-1}) were prepared and assayed with the biosensor. It was noted that the degree of interference for mixture 1 was 3.8%. Furthermore, only negligible change (1.7%) of ECL response was observed from the mixture 2 in comparison with that from *S. aureus*. The matrix effects of some real samples including milk, lake water, human saliva and human urine were investigated by comparing the ECL responses to the un-spiked real samples, PBS buffer (as blank) and *S. aureus* at 1.0×10^5 CFU mL^{-1}. As seen in Fig. 6B, the ECL responses from the un-spiked real samples all showed negligible difference compared with that from blank. The influence of the un-spiked milk, lake water, human saliva and human urine were 3.2%, 2.8%, 1.6% and 2.9%, respectively. The above results indicated that these potentially co-existing bacteria did not interfere the assay of *S. aureus*. The developed biosensor showed ideal specificity for *S. aureus* detection.

3.7. Assay of real samples

In order to further assess the application potential of the label-free ECL biosensor for pathogen detection, sterile samples of milk, lake water, human saliva and human urine were spiked with *S. aureus* at different known concentrations and assayed using the proposed method. As seen in Table 1, the recovery values ranged from 75% to 116.7%, with RSD values all below 5.8%, indicating acceptable accuracy of this biosensor for real sample assay.

4. Conclusions

In summary, a novel label-free ECL biosensor for *S. aureus* whole cells detection was developed based on the strong and specific affinity between SPA and the Fc region of IgG. This biosensor was fabricated with a very simple protocol by depositing carboxyl graphene/porcin IgG composite on a glassy carbon electrode. Recognition of *S. aureus* led to an obvious decrease of ECL emission because the bound *S. aureus* interrupted the interfacial electron transfer and hindered the diffusion of the ECL active substances. As a molecular recognition mode-based method, it does not require laborious and time-consuming pretreatments such as pathogenic bacteria culture and nucleic acid extracting. Compared with other reported methods based on molecular recognition mode, such as ELISA and aptasensor, this biosensor does not demand probe labeling process. Therefore it is a very facile and user-friendly method for pathogen screening, and shows great application potential in food safety control, medical diagnosis, environmental monitoring, and even bioterrorism defense.

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