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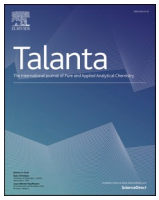

An electrochemical biosensor for the highly sensitive detection of *Staphylococcus aureus* based on SRCA-CRISPR/Cas12a

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ABSTRACT

As one of the major foodborne pathogens, *Staphylococcus aureus* (*S. aureus*) can cause infectious diseases. In the current study, a novel electrochemical biosensor based on saltatory rolling circle amplification (SRCA) combined with CRISPR/Cas12a system was developed for the accurate detection of *S. aureus*. The thio-modified reporter probes (SH-ssDNA-MB) was immobilized on the surface of gold nanoparticle-modified electrode through the Au–S bond. In the presence of *S. aureus,* the target DNA double strands obtained by SRCA can be specifically recognized with Cas12a/crRNA complex. The *trans*-cleavage activity of Cas12a induces SH-ssDNA-MB to be cleaved from the electrode surface, resulting in a decrease in the current signal. Subsequently, the ratio of the current can be calculated as the detection result. Under optimal conditions, the detection limits were 2.51 fg/μL for genomic DNA and 3 CFU/mL for *S. aureus* in pure cultures, respectively. Moreover, the method demonstrated satisfactory specificity, acceptable stability and reproducibility. In comparison with ISO methods, the sensitivity, specificity and accuracy of the developed method were 100%, 97.8% and 98%, respectively. In conclusion, the developed novel electrochemical biosensor provides a potential powerful platform for the accurate detection of *S. aureus*.

Credit author statement

Luqi Huang: Writing - Original Draft, Conceptualization, Methodology, Investigation. Ning Yuan: Validation, Data Curation. Wei Guo: Formal analysis, Writing - Review & Editing. Yunzhe Zhang: Resources, Visualization. Wei Zhang: Conceptualization, Methodology, Supervision, Funding acquisition, Project administration.

1. Introduction

In the past few decades, foodborne diseases caused by the pathogens and/or their toxins pose serious threats to public health [1,2]. *S. aureus* is one of the most common foodborne pathogens that threaten human health with a variety of virulence factors. The consumption of *S. aureus* contaminated food can lead to food poisoning, such as toxic shock syndrome, severe allergic and autoimmune diseases [3]. According to the US Center for Disease Control and Prevention, food poisoning incidents caused by *S. aureus* account for about one-third of bacterial food poisoning [4]. Therefore, to establish a sensitive and efficient detection method is great important for the better control of *S. aureus*.

The conventional methods are considered as the “gold standard” due to their high reliability, however, they are time-consuming and labor-intensive. Recently, nucleic acid-based isothermal amplification technologies have been well studied depending on their outstanding rapidity, simplicity, including loop-mediated isothermal amplification (LAMP) [5–7], rolling circle amplification (RCA) [8,9], and saltatory rolling circle amplification (SRCA). SRCA is a novel isothermal amplification method with a similar amplification process to RCA. Different from RCA, SRCA does not require lock probe, ligase, and avoids complex probe cyclization process with the time shortened at least 3 h. Compared with LAMP, SRCA is simpler only requiring two primers. Relying on the strong DNA synthesis characteristics and wonderful strand displacement activities of *Bst* DNA polymerase, nucleotides can be added to the unclosed circular DNA to synthesize DNA independent of templates

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[10]. Thus, a large number of linear and repeatedly tandem DNA double-stranded products are continuously produced like a rolling circle. This method has been applied to detect various pathogenic bacteria based on the characteristics of the high efficiency, simple operation, strong specificity and high sensitivity [11–14].

Electrochemical sensor as a promising tool has attracted more and more attention. This is attributed to its remarkable features of portability, simple operation, short response time, high sensitivity, low-cost and miniaturization [15,16]. In electrochemical DNA (E-DNA) sensors, the recognition of the target DNA (double or single strand) by the electrochemical sensor changed the conformation of the probe on the electrode surface, which induced the change of the electron transfer distance of the contacting medium, thereby leading to the variation of the electrochemical signal [17–20]. The successful development of electrochemical DNA sensors has made significant contributions to various fields. It is considered particularly suitable for direct and rapid sensing detection [21,22]. In previous research, our group successfully established an SRCA-based electrochemical biosensor to detect *Salmonella enterica* serovar Typhimurium in food [23]. However, the signal readouts relied on the combination between dsDNA and *β*-cyclodextrin fixed on the electrode, which could not avoid the impact caused by the non-specific dsDNA.

Recently, the development of CRISPR/Cas-based nucleic acid detection systems have opened up a new research avenue towards molecular diagnostics [24]. Particularly, Cas12a (cpf1) displays remarkable application potential in nucleic acid-based biosensor detection due to its incidental cleavage activities for non-specific single-stranded nucleic acids (namely *trans*-cleavage or collateral cleavage). Cas12a is an RNA-guided DNA endonuclease, which can form a complex with crRNA and specifically bind to the target DNA. Then Cas12a initiates cleavage of specific targets of dsDNA by a single RuvC catalytic domain while triggering *trans*-cleavage. Such unique recognition and collateral property can achieve single-base specificity and provides the basis for signal readouts [25]. When CRISPR/Cas12a is combined with nucleic acid amplification technology, the amplified products will trigger the collateral cleavage for reporters to achieve additional sensitivity [26]. For example, Wang et al. successfully established a 1-h low-cost multi-purpose Efficient System (HOLMES) based on PCR, which can detect the concentration as low as 10 aM [27]. Subsequently, Doudna et al. created a method termed “DETECTR” by combining Cas12a with recombinase polymerase amplification (RPA), which achieves attomolar sensitivity [28]. Zhang et al. designed a CRISPR-assisted PCR system with the detection limit of 1.02×10^2 copies/μL [29]. These methods are combined with CRISPR to achieve sensitive readout for target amplicons.

To further improve the detection specificity and sensitivity, we intended to introduce SRCA-based CRISPR/Cas12a into an E-DNA biosensor platform for the first time. In the present study, repeated tandem sequences obtained by SRCA can be specifically recognized and combined with multiple Cas12/crRNA complexes by complementary hybridization. The accidental cleavage characteristic of Cas12a will be activated by this combination, which will induce the amplification of the reporting signal. This step will make an important contribution to improve the sensitivity and specificity. We used single-stranded reporter probe modified with methylene blue (MB) as electrical signal reporter, and test the performance of various aspects. The results indicated that the hybridization of SRCA products and Cas12a/crRNA activates non-specific cleavage activity of Cas12a, which made the electrochemical biosensor perform well in specificity, stability and repeatability, and more sensitive than before. It provides a highly specific and ultra-sensitive detection platform for foodborne pathogen.

2. Materials and methods

2.1. Chemicals and materials

All oligonucleotide sequences (Table S1), including primers, crRNA, ssDNA-FQ (FAM-TTATT-BHQ1) reporter probe and MB-ssDNA reporter probe, were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). TIANamp bacteria DNA kit was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). *Bst* DNA polymerase, $MgSO_4$ were purchased from Dalian Bao Biological Engineering Co., Ltd. (Dalian, China). Deoxynucleotide solutions (dNTPs) were purchased from Beijing Bomade Biotechnology Co., Ltd. (Beijing, China). EnGen® Lba Cas12a and NEBuffer™ 2.1 were provided by New England Biolabs Co., Ltd. (Beijing, China). Ethylenediamine tetraacetic acid (EDTA), Potassium chloride were both supplied by Fuchen Chemical Reagent Co., Ltd. (Tianjin, China). Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) was acquired from Yuanye biology Co., Ltd. (Shanghai, China). Potassium ferricyanide (K_3 [$Fe(CN)_6$]) and potassium ferrocyanide (K_4 [$Fe(CN)_6$]) were provided by Tianjin Guangfu Science and Technology Development Co., Ltd. (Tianjin, China). Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Hefei Bomei Biotechnology Co., Ltd. (Hefei, China). 6-Mercaptohexanol (MCH) were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Glassy carbon electrodes (GCEs, 3.0 mm in diameter, 80 mm in length) were provided by Wuhan Corrtest Instrument Corp., Ltd. (Hubei, China). RNase-free water was used as solvent for all tests.

2.2. Apparatus

SRCA products were observed by JY04S-3E electrophoresis analyzer (Beijing JunYi Electrophoresis Co., Ltd, Beijing, China) after agarose gel electrophoresis. The temperature required for the reaction was regulated by DK-8D electric heating thermostatic sink (Shanghai Yiheng Scientific Instrument Co., Ltd. Shanghai, China). Ultrasonic cleaning machine (Kun Shan Ultrasonic Instruments Co., Ltd, Kunshan, China) was used for ultrasonic cleaning of electrodes. Scanning electron microscopy (SEM) image was obtained from ZEISS gemini 300 (ZEISS, Ltd., Germany). All electrochemical measurements including square wave voltammetry (SWV), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were performed by using a CS350H electrochemical work-station (Wuhan Corrtest Instruments Corp., Led, Wuhan, China) with a standard three-electrode configuration. A glassy carbon electrode was modified with gold as working electrode, and a platinum wire was used as counter electrode and an Ag/AgCl as reference electrode, respectively. The UV–Vis absorption spectra was recorded with a F-320 spectro-photometer (Tianjin Gangdong Technology Development Co., Ltd, Tianjin, China).

2.3. Bacteria culture and genomic DNA extraction

In this study, 1 *S. aureus* strain and 7 non-*S. aureus* strains were used to verify the specificity, including *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (CMCC 50041), *Escherichia coli* O157: H7 (CICC 21530), *Shigella flexneri* (CMCC 51107), *Vibrio parahaemolyticus* (ATCC 17802), *Cronobacter* (ATCC 29544), *Listeria monocytogenes* (CMCC 54001), *Pseudomonas aeruginosa* (CICC 21636). All of the strains were inoculated in luria-bertani medium overnight at 37 °C (200 rpm). Then, 1 mL of bacteria solution was taken for genomic DNA extraction. DNA was extracted using the DNA extraction kit according to the operation manual. The purity and concentration of the extracted genomic DNA was determined by Nanodrop 2000 (Thermo Fisher Scientific, America).

2.4. SRCA amplification

A segment with high homology from the *nuc* gene of *S. aureus* was selected as the target sequence through blast alignment in NCBI. A pair

of primers employed for SRCA were designed using DNAMAN software as presented in Table S1 (Version 6.0, Lynnon Biosoft., USA). The SRCA reactions were carried out in a total 40 μL of reaction mixture containing 2 μL forward primer (FWP) (10 μmol/L), 2 μL reverse primer (RVP) (10 μmol/L) , 8 μL dNTP (2.5 mmol/L), 4 μL *Bst* DNA polymerase buffer (10 ×), 6 μL $MgSO_4$ (25 mmol/L), 2 μL DNA template, 4 μL *Bst* DNApolymerase (8000 U) and sterile deionized water. Before the reaction, the template was denatured at 94 °C for 3 min. *Bst* DNA polymerase was added to the reaction mixture to conduct the amplification at 62 °C for 60 min, and finally at 80 °C for 5 min to terminate the reaction. The accuracy of the amplified products was visually evaluated by 1.6% agarose gel.

2.5. Fabrication of electrochemical biosensor modified the SH-MB-ssDNA reporter

Prior to modification, the bare GCE was polished with 1.0, 0.3 and 0.05 μM Al_2O_3 powder in turn to remove particles on the surface. Then they were ultrasonically rinsed with ultrapure water, ethanol and water (1:1), and ultrapure water sequentially for 5 min and dried at room temperature. The sensor was fabricated following the below steps. Firstly, the pretreated GCE was immersed in 1% $HAuCl_4$ solution and gold nanoparticles modified GCE (AuNPs/GCE) was obtained by electrochemical deposition at a potential of −0.2 V for 80 s. Secondly, the SH-MB-ssDNA was treated with 1 mmol/L TCEP and incubated at 37 °C in the dark for 2 h. Then SH-MB-ssDNA solution was diluted to 1 μmol/L with 10 mmol/L Tris buffer (100 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 10 mmol/L EDTA), then it was incubated with TCEP at 37 °C for 40 min in the dark until it became colorless. The fully restored SH-MB-DNA solution can be proved by the disappearance of blue [30]. The step was repeated before each use. Afterwards, 10 μL prepared SH-MB-DNA solution was directly cast onto the AuNPs/GCE at 37 °C in the dark for 4 h. Then SH-MB-ssDNA reporter probe immobilized electrode was flushed with 10 mmol/L Tris buffer to remove the unbound reporter probe. Finally, the modified electrode was immediately immersed in 2 mmol/L MCH to occupy the nonspecific binding site to eliminate non-specific adsorption, and the linear structure of ssDNA probe was maintained to improve the hybridization efficiency. Then the treated electrode was carefully rinsed by ultrapure water and air-dried for further use.

2.6. Cas12a-mediated cleavage assay

CRISPR/Cas12a system for electrochemical analysis contains 1 μmol/L crRNA, 1 μmol/L Cas12a, 1 × NEBuffer 2.1, and 35 μL SRCA products, which was supplemented to 140 μL with enzyme-free water. The prepared electrode was immediately immersed in the reaction mixture and incubated at 37 °C for 60 min. After the non-specific cleavage reaction of CRISPR/Cas12a system was completed, the electrode was carefully rinsed with enzyme-free water and then dried for electrochemical measurement.

For CRISPR/Cas12a fluorescent monitoring, the Cas12a-mediated cleavage system contained 2 μmol/L crRNA, 1 μmol/L Cas12a, 2 μL 1 × NEBuffer 2.1 and 5 μL amplified SRCA products. Additionally, 2 μmol/L ssDNA-FQ was added to the reaction mixture to incubate with above agents together in a water bath at 37 °C for 40 min. After the incubation, the fluorescence signal generated by Cas12a cleavage was measured by F-320 spectro-photometer, with the measurement parameters setting as excitation at 495 nm and emission at 520 nm.

2.7. Electrochemical measurement

Each modification process on the electrode surface was characterized and analyzed by CV and EIS. Both CV and EIS were performed in 0.1 mol/L phosphate buffered saline (containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl, pH 7.0). SWV was applied to detect the current change before and after the cleavage of the MB-DNA reporter probe on the electrode. It was carried out in a 10 mmol/L tris buffer (containing 100 mmol/L NaCl) with a potential window from −0.5 to −0.1 V, a potential increment of 4 mV, a 25 Hz Frequency and 25 mV amplitude. Electrochemical data ΔI% [$\Delta I\% = (I_0 - I)/I_0$] was recorded as an indicator, where I_0 was the current signal of the reporting probe before CRISPR was introduced, and I was the current signal of the reporting probe after CRISPR reaction.

2.8. Analysis of platform performance

To evaluate the sensitivity, gradient diluted DNA extracted from *S. aureus* was used as the template for the test (initial DNA concentration was 41.5 ng/μL), the dilution ratio was 1:10. The pure culture solution of *S. aureus* was diluted 10 times gradient with normal saline, and the initial concentration was calculated as 3.9×10^8 CFU/mL.

The specificity of the developed biosensor was evaluated by using 1 strains of *S. aureus* and 7 strains of non-*S. aureus* in this study. The products of different strains using SRCA were detected by agarose gel electrophoresis and electrochemical test respectively. The specificity of SRCA primers was evaluated by 1.6% agarose gel electrophoresis.

To evaluate the repeatability of the biosensor, 8 GCEs were prepared to detect the same samples under the same conditions. In the stability test, the modified electrodes were placed in a 4 °C for different time, and then the change of the MB report signal on the electrode was detected.

2.9. Analysis of spiked and natural samples

The accuracy of the method was verified by the spiked milk samples. Milk samples, which were confirmed to be *S. aureus*-negative by traditional methods, was used in this study. Twenty-five milliliter of milk sample and 225 mL physiological saline was taken into a sterile bottle and homogenized thoroughly. The bacterial suspension of *S. aureus* cultured overnight (6.2×10^8 CFU/mL) was 10-fold gradient diluted with normal saline solution, and the plate count was performed for each dilution. The dilutions of *S. aureus* were mixed with the milk samples to prepare contaminated *S. aureus* samples in the range of 6.2×10^2–6.2×10^5 CFU/mL. Gegomic DNA was extracted from 1 mL bacterial solution from each sample, and then determined using SRCA-CRISPR/Cas12a based sensor platform assay.

To verify the application capability of SRCA-CRISPR/Cas12a based sensor platform in food samples, a total of 50 samples including meat, milk, eggs, grains and their products were purchased from the market (Hebei, China; Table S2). These samples were used as the actual test objects. For comparison, these samples were tested using the ISO method at the same time. The sensitivity, specificity, and accuracy of these samples were calculated in comparison with ISO method.

$$\text{Sensitivity} = \left(T_{pos}\right)/\left(T_{pos}+F_{neg}\right) \times 100\%$$

$$\text{Specificity} = \left(T_{neg}\right)/\left(T_{neg}+F_{pos}\right) \times 100\%$$

$$\text{Accuracy} = \left(T_{pos}+T_{neg}\right)/\left(T_{pos}+T_{neg}+F_{pos}+F_{neg}\right) \times 100\%$$

The true positive and negative samples detected by both ISO and this method are recorded T_{pos} and T_{neg} respectively; while the samples detected by only one of them are called false positive (F_{pos}) and false negative (F_{neg}) samples.

2.10. Statistical analysis

Each test was repeated 3 times, and the experimental data was expressed as "mean ± SD". The multiple groups of experiments were evaluated using one-way ANOVA analysis of variance, and Duncan's multiple comparison test was used to detect differences through SPSS 19.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered

statistically significant.

3. Results and discussion

3.1. Principle of the SRCA-CRISPR/Cas12a

The principle of electrochemical biosensor based on SRCA-CRISPR/Cas12a for the detection of *S. aureus* was demonstrated in Scheme 1. This method mainly relies on the highly efficient amplification of SRCA and the *trans*-cleavage activity of CRISPR/Cas12a, thus achieving highly sensitive detection of *S. aureus* by inducing changes in the electrical signal of the electrochemical biosensor. In the first stage, we designed a pair of primers based on *nuc* gene. In the presence of target DNA, a large amount of double-stranded DNA will be obtained through SRCA. Notably, the amplified products containing PAM sequence of TTT can be recognized by crRNA and Cas12 protein. To verify the accuracy of the SRCA, the amplified products were confirmed by 1.6% agarose gel electrophoresis and sequencing (Fig. S1). The positive amplification products showed obvious ladder-like bands (Fig. S1B), and the amplification sequence was a repeated tandem unit composed of target base and added bases. The result indicated that the target region can be specifically amplified by SRCA.

In the second stage, to achieve electrochemical transduction of the electrochemical signal, a reporter probe with MB as indicator signal was modified on the electrode. Firstly, in order to increase the electron transfer and obtain a greater specific surface area, the representative gold nanoparticles (AuNPs) were selected to modify on the surface of bare GCE. Then the SH-MB-ssDNA reporter probe was fixed on the surface of AuNPs/GCE electrode through the Au–S bond to form SH-MB-ssDNA/AuNPs/GCE. Meanwhile, a stable MB current signal was obtained on the electrode surface.

Subsequently, the designed crRNA specifically recognized and combined with SRCA-amplified dsDNA, resulting in the activation of the *trans*-cleavage activity of Cas12a and cleavage of the report probe from the electrode. Therefore, the electrochemical signal changed significantly after introducing the target *S. aureus* into the biosensor. SWV is a sensitive and reliable method, which is used as a monitoring method for this strategy. The ΔI% can reflect the cleavage degree of the reporter probe and its positive relationship with the target. By combination of Cas12a with SRCA, an ultrasensitive and highly specific electrochemical biosensor for the detection of *S. aureus* DNA was successfully constructed.

3.2. Characterization of the electrochemical biosensor

The morphology of the electrode was characterized by SEM. After cleaning, a smooth bare electrode surface was obtained, which provided a good basis for the subsequent modification of AuNPs (Fig. S2A). After electrodeposition, AuNPs with uniform distribution and size can be observed on the surface of the GCE, which showed that the modification of AuNPs provided a good platform for oligonucleotide immobilization (Fig. S2B).

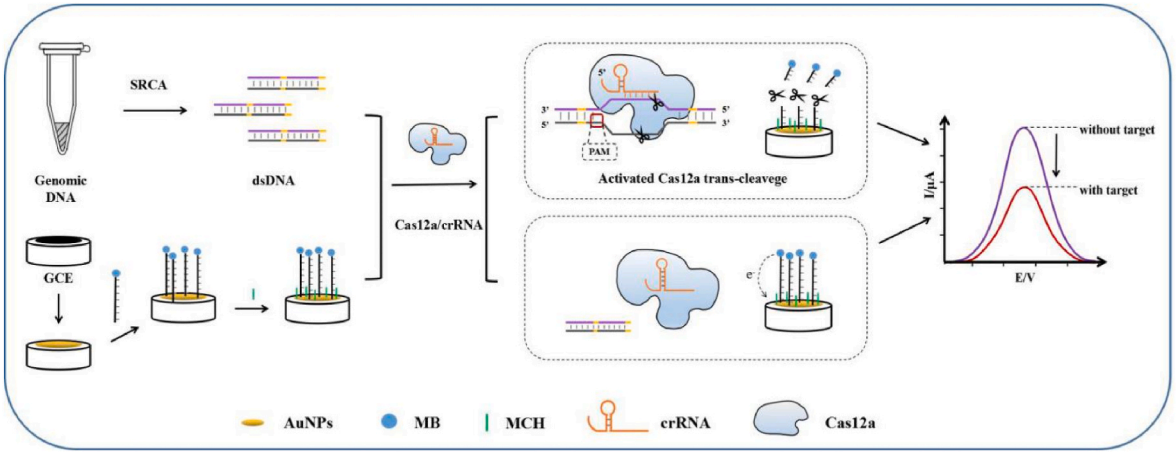
To confirm the fabrication processes, CV and EIS measurements were carried out to characterize the stepwise assemble process in the electrolyte solution including 5 mmol/L $[Fe(CN)6]^{3-/4-}$ (1:1) and 0.1 mol/L KCl (Fig. 1). In a classic EIS, the electron transfer resistance (R_{et}) was the main indicator and corresponded to the semicircle diameter in Nyquist diagram [31].

According to previous reports, the immobilization of DNA on the electrode surface changed the Ret value [32]. A significant semicircle diameter was observed at bare GCE (curve a). After modification of the GCE by AuNPs, the resistance decreased and the curve approximated straight line (b), indicating that AuNPs/GCE had excellent conductive capability and facilitated electron transfer. When the thiolated MB-ssDNA was immobilized on the AuNPs/GCE surface covalently via Au–S bond, the semicircle diameter increased obviously (c), which suggested that there was an electrostatic repulsion between the negatively charged phosphate backbone of DNA molecule and $[Fe(CN)6]^{3-/4-}$. When the electrode was blocked with MCH, the semicircle diameter further increased (curve d). This phenomenon may be explained by upright strands of negatively charged DNA made by MCH and the decrease in steric hindrance on the electrode surface. Through incubation with the SRCA and CRISPR/Cas12a reaction solution, the semicircular diameter of electrode decreased (curve e). These might be contributed to the cleavage of the MB-ssDNA from the electrode surface by activated Cas12a, which leading to the sharply decreased amount of DNA on the electrode surface.

In the CV characterization (Fig. 1B), a pair of obvious redox peaks can be observed through the bare GCE (a). Due to the strong conductivity of gold, a significant increase in peak current is observed when AuNPs were modified (b). Immobilization of MB-ssDNA could inhibit the redox peak currents, which is attributed to a large amount of DNA fixed on the electrode (c). As the MCH was assembled, the peak current further increased (d). The peak current increased significantly after the cleavage reaction (e). These results of CV measurements were in accordance with the results obtained from EIS. Both results verified that the electrochemical biosensor indeed worked as designed.

3.3. Feasibility of the electrochemical biosensor

To verify the accuracy of the designed crRNA and the feasibility of CRISPR/Cas12a, the cleavage activity was investigated through



Scheme 1. The schematic of detection by electrochemical biosensor based on SRCA-CRISPR/Cas12a.

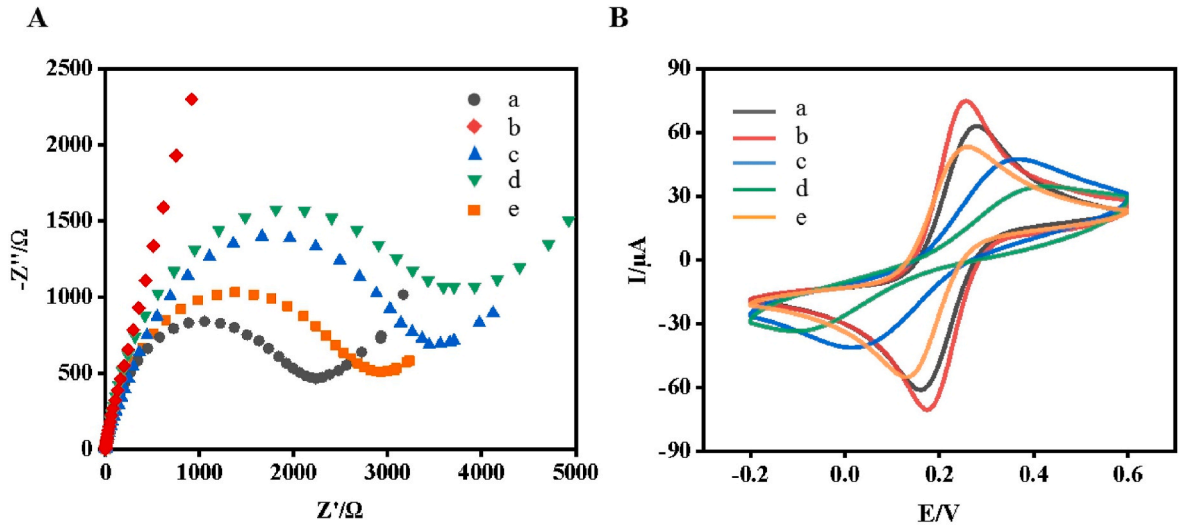


Fig. 1. EIS and CV measurements characterizes the electrode modification process of the electrochemical biosensor based on SRCA-CRISPR/Cas12a: (a) bare GCE, (b) GCE modified with AuNPs, (c) MB-ssDNA/AuNPs/GCE, (d) MCH modified MB-ssDNA/AuNPs/GCE, (e) The *trans*-cleavage of Cas12a is activated. The setting parameters of EIS test were as follow: initial potential 220 mV (relative reference), scanning frequency 10^{-2}–10^{5} Hz, amplitude width 5.0 mV. The setting parameters of CV test were as follow: potential interval −0.2 V–0.6 V, scanning rate 100 mV/s, and sampling frequency 100 Hz.

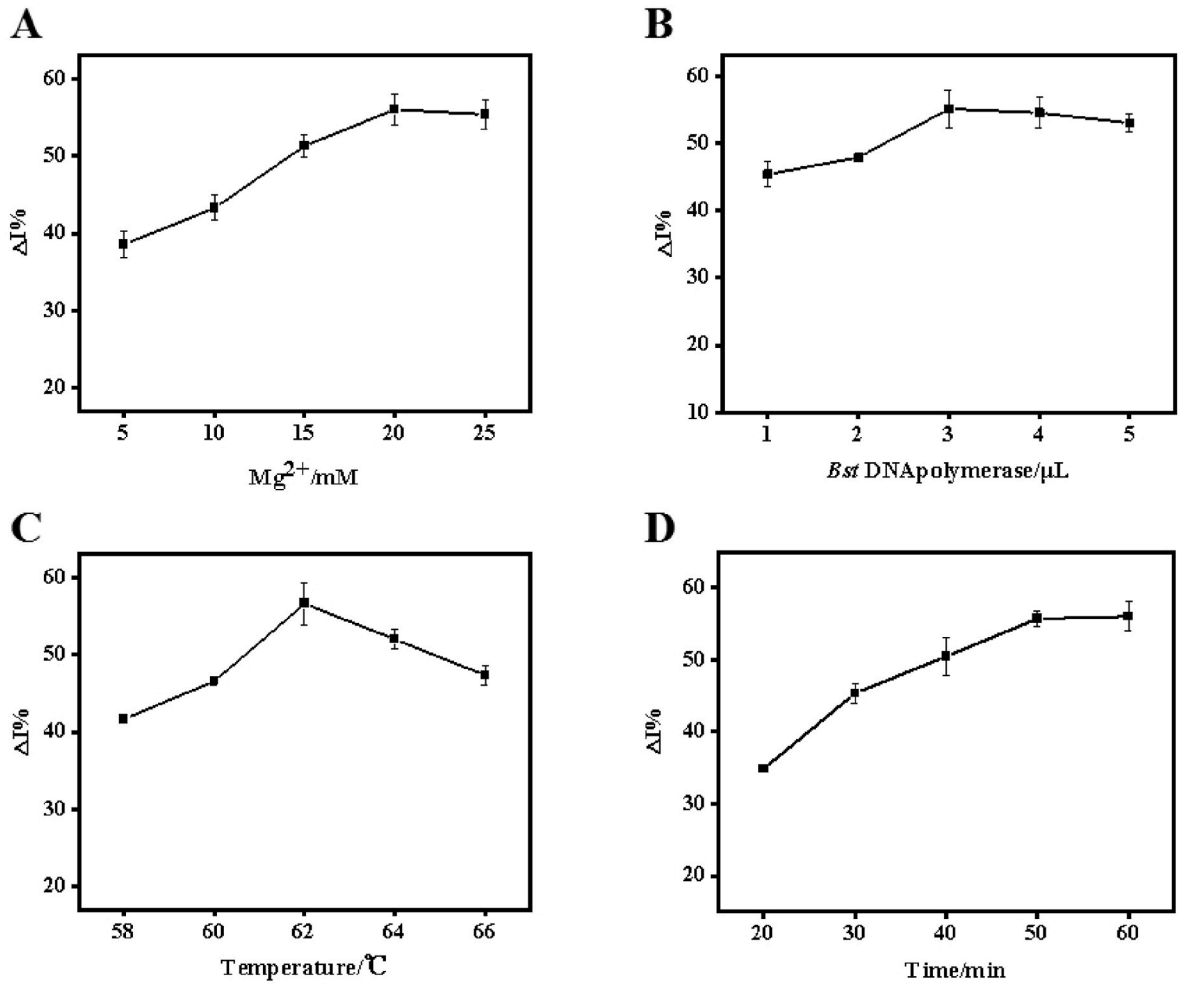


Fig. 2. Optimization of SRCA test parameters. The optimized reaction parameters were as follows: (A) the concentration of added Mg^{2+}, (B) the volumes of *Bst* DNA polymerase, (C) the temperature and (D) the reaction time. Each experiment was repeated three times, error bars represent mean ± SD.

fluorescence assay. Owing to Cas12a's propensity for ssDNA cleavage, we designed ssDNA with two terminals labeled with fluorescence moiety and quenching moiety (ssDNA-FQ). The results of fluorescence experiments were illustrated in Fig. S3A. The significant fluorescence intensity at 520 nm only occurred when the system was complete and the target was present, while no obvious fluorescence intensities were observed when Cas12a, crRNA, or target DNA were absent. These results indicated that the products amplified by SRCA could be accurately recognized by Cas12a/crRNA complex and the *trans*-cleavage activity was activated to cleave the ssDNA-FQ. Subsequently, in order to further examine the feasibility of the constructed electrochemical biosensor for *S. aureus* detection, SWV technique was performed. Specific target dsDNA sequences were obtained by SRCA, then the samples with target and non-target were tested by SWV, respectively. As Fig. S3B showed, the peak current corresponding to the system in the presence of *S. aureus* decreased significantly in comparison to that of non-target. This result was ascribed to *trans*-cleavage activity of Cas12a activated by SRCA products and cleavage of a large amount of MB-ssDNA probes from the electrode. Hence, the above results verified the satisfactory feasibility of electrochemical biosensor based on SRCA-CRISPR/Cas12a for *S. aureus* detection.

3.4. Optimization of conditions

To maximize the electrochemical performance of this proposed electrochemical biosensor, the experimental conditions were optimized. The activity of *Bst* DNA polymerase were significantly affected by Mg^{2+} during the SRCA reaction. Meanwhile, Mg^{2+} not only can induce the conformational coordination between RuvC domain and ssDNA by changing the spatial distribution of ssDNA [33], but also can further enhance the stability of crRNA which substantially promote the overall affinity of Cas12a with its cognate crRNA [34–36]. The results showed that 20 mmol/L Mg^{2+} was the optimal concentration for the SRCA system (Fig. 2A). As shown in Figs. 2B and 3 μL of *Bst* DNA polymerase (24 U) was determined as the optimal condition. Due to the amplification temperature is an important parameter that related to the efficiency of *Bst* DNA polymerase, the temperature was also studied and 62 °C was the best condition for the subsequent SRCA reaction (Fig. 2C). In addition, 50 min was chosen for the optimum SRCA amplification time (Fig. 2D).

The appropriate ratio of Cas12a to crRNA is essential to generate the best signal readout on Cas12a-mediated *trans*-cleavage. As shown in Fig. S4A, the △I% value reached the maximum when C_{Cas12}/C_{crRNA} was 1:2. The signal of the ssDNA-MB probe on the electrode was gradually decreased with the increasement of the CRISPR *trans*-cleavage time. All things considered, 50 min was used as the optimum *trans*-cleavage digestion time (Fig. S4B).

3.5. Analytical performance

3.5.1. Sensitivity analysis

The performance of the developed electrochemical biosensor was evaluated with the optimized experimental conditions. SWV was used to detect the peak current of MB corresponding to different *S. aureus* dilutions after the reactions. As the DNA concentration of *S. aureus* increased from 0.415 fg/μL ~41.5 ng/μL, the current peak of MB gradually decreased (Fig. 3A). The reason for this phenomenon may be that the targets triggered the *trans*-cleavage activity of Cas12a-crRNA complex, which accelerated the fragmentation of the reporter probe on the electrode, resulting in a continuous signal drop. At the same time, there was a positive linear relationship between ΔI% and the logarithm of the DNA concentration ranging from 4.15 fg/μL~4.15 ng/μL. The corresponding regression equation was Y = 6.4725X+51.3432, with a correlation coefficient R^2 = 0.9983 (Fig. 3B). The limit of detection (LOD) of electrochemical biosensor was 2.51 fg/μL (3 S/K, where S was the standard deviation of detecting 11 blank samples and K was the slope of the regression equation).

Moreover, the sensitivity of the electrochemical sensor based on SRCA-CRISPR/Cas12a for *S. aureus* (3.9×10^1–3.9×10^8 CFU/mL) was further evaluated. As Fig. 4A showed, the MB signal response (I) gradually decreased with the increasing of *S. aureus* concentration. And there was a strong linear relationship between ΔI% and the logarithm of *S. aureus* concentrations in the range of 3.9×10^1–3.9×10^7 CFU/mL (Fig. 4B). The linear regression equation was Y = 5.4774X+6.4957 with a correlation coefficient of 0.9822. The LOD was 3 CFU/mL (3 S/K). To further highlight the detection performance of the biosensor, its analytical characteristics was compared with several reported methods based on electrochemical sensors. As shown in Table 1, the designed biosensor based on SRCA-CRISPR/Cas12a has better sensitivity than other electrochemical biosensors. This is mainly attributed to the high amplification efficiency of SRCA and the unique *trans*-cleavage activity of CRISPR/Cas12a system.

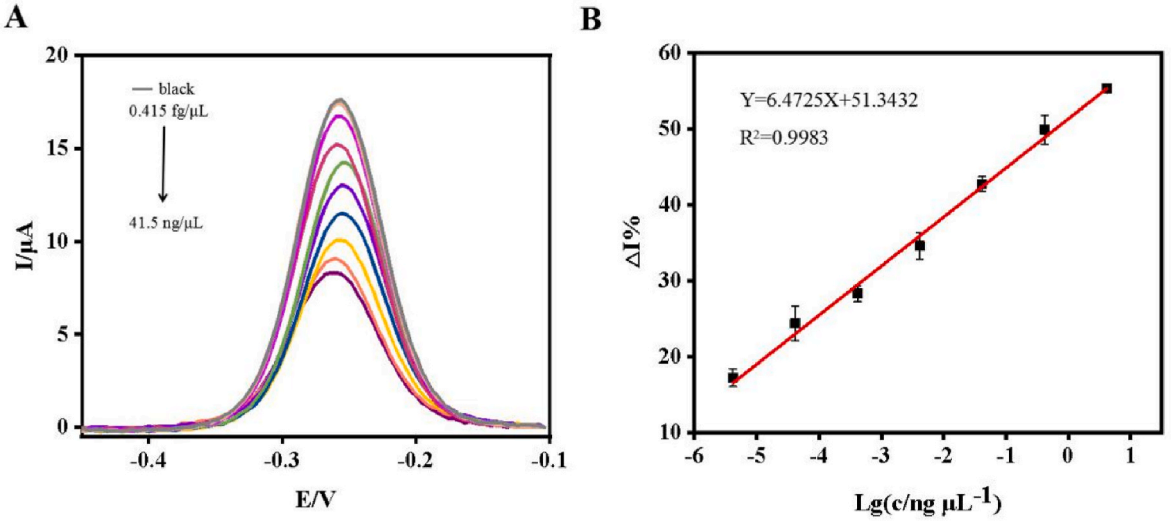


Fig. 3. (A) The proposed electrochemical biosensor detects *S. aureus* at different DNA concentrations (from 0.415 fg/μL to 41.5 ng/μL). (B) Linear relationship between the △I% value and the logarithm of the concentration of genomic DNA of *S. aureus*. Each experiment was repeated three times, error bars represent mean ± SD.

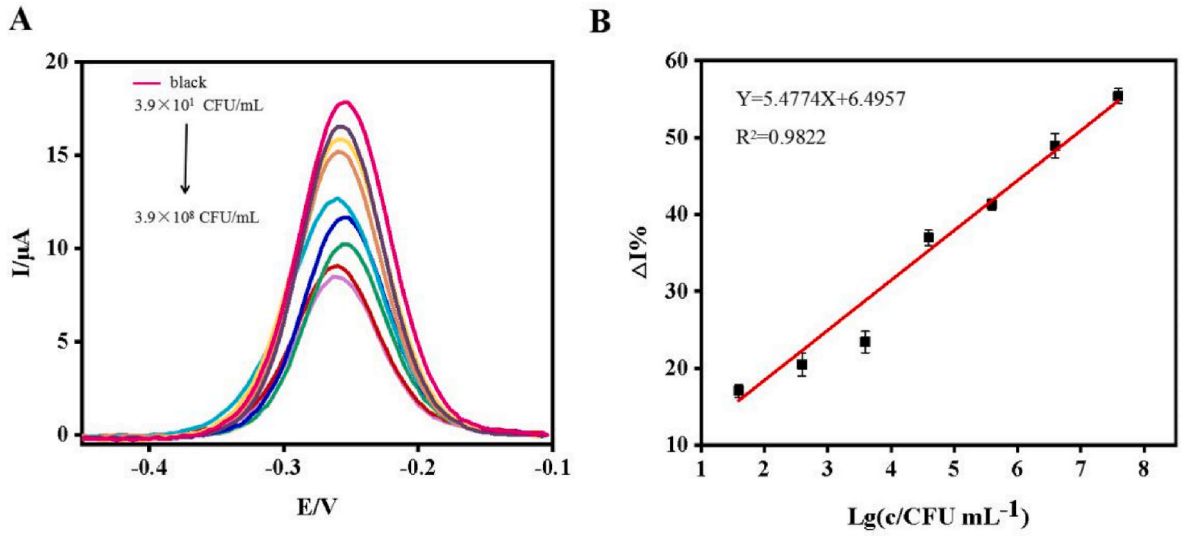


Fig. 4. (A) The proposed electrochemical biosensor detects the *S. aureus* at different concentrations (from 3.9 × 10^1 to 3.9 × 10^8 CFU/mL). (B) Linear relationship between the △I% value and the logarithm of the concentration of *S. aureus*. Each experiment was repeated three times, error bars represent mean ± SD.

Table 1
Comparison of different electrochemistry biosensor-based detection and sensing platforms.

Methods	Targets	Electrochemical technique	LOD	References
–	*Salmonella*	EIS	0.15 pM	[37]
Aptamer-RCA	*S. typhimurium*	DPV	16 cfu/mL	[38]
RCA	*E. coli* O157:H7	DPV	31 cfu/mL	[39]
Aptamer-RCA	*E. coli LPS*	EIS	4.8 fg/mL	[40]
Cas12a	HPV	DPV	30 pM	[41]
Cas12a	HPV-16、TGF-b1	SWV	50 pM、0.2 nM	[42]
Cas12a	PB-19	SWV	10 fM	[18]
Cas12a-SRCA	*Staphylococcus aureus*	SWV	2.51 fg/μL 3 cfu/mL	This work

3.5.2. Specificity analysis

In order to investigate the specificity of SRCA, the interfering strains were selected under the same detection conditions for *S. aureus* detection. As shown in Fig. 5A, the target *S. aureus* showed bright ladder bands, whereas no obvious bands were observed for non-target strains. These results suggested that this pair of primers could be specifically matched with the sequences of *S. aureus* during SRCA reaction. In addition, the SRCA-CRISPR/Cas12a was used to detect the different strains. The *S. aureus* sample produced obvious signal response, whereas the other 7 non-*S. aureus* strains produced weak signals (Fig. 5B). The results indicated that the developed SRCA-CRISPR/Cas12a had superior selectivity in distinguishing *S. aureus* from non- *S. aureus* species.

3.5.3. Reproducibility and stability

The reproducibility and stability of detection are fundamental during the process of development of novel electrochemical biosensors. To evaluate the repeatability of the biosensor, 8 GCEs were fabricated and detected under the same condition. As depicted in Fig. 6A, the electrochemical biosensor showed a relatively stable current response in 8 tests, with relative standard deviation (RSD) of 3.48%, which manifested that the developed biosensor had acceptable reproducibility. To investigate the stability, the electrochemical biosensor was verified by examining the changes of SWV of the MB indicator signal on the electrode. After storage at 4 °C for 1 week and 2 weeks, the electrode signal still remained 91.6% and 84.8% of the initial value, respectively (Fig. 6B). The result reflected that the biosensor had good stability.

3.6. Application in real samples

To verify the reliability of proposed method for *S. aureus* detection, milk samples were spiked with different concentrations of *S. aureus* DNA. According to the regression equation, the recovery (%) ranged from 98.8% to 117.1%, which justified the good accuracy of this biosensor (Table 2). Fifty food samples purchased from several local supermarket were used to further explore the practical performance of the electrochemical biosensor. In this study, 6 samples were positive by the SRCA-CRISPR/Cas12a-based electrochemical biosensor, while only 5 samples were positive by the ISO standard method. This result possibly was attributed to the inability of SRCA to distinguish dead or viable but nonculturable bacteria. It also reflected that this method had high sensitivity. Compared with ISO, the sensitivity, specificity, and accuracy of the method were 100%, 97.8%, and 98%, respectively (Table 3). Therefore, our electrochemical biosensor is promising in practical detection.

4. Conclusions

In summary, a novel SRCA based electrochemical biosensor coupled with CRISPR was first developed for ultrasensitive and efficient detection of *S. aureus* in food. The proposed sensing platform, which is time-saving and laborsaving, improves the detection efficiency. The introduction of CRISPR enables the sensor ultrasensitive and high specific for target detection. The LOD was 2.51 fg/μL and better compared with other SRCA based electrochemical biosensor. Due to the specific recognition of the target by CRISPR/Cas12a, the possibility of false positive results caused by non-specific amplification has also been reduced. Moreover, the newly developed biosensor exhibits acceptable reproducibility and good stability. In the real sample testing, this biosensor shows excellent sensitivity, specificity, and accuracy in comparison with ISO. Furthermore, the biosensor can be conveniently applied to the detection of other pathogens or DNA sequences by selecting specific primers and rationally designing crRNA. In the future, new Cas proteins may be further explored and new electrode materials can be applied to improve detection performance. Overall, the proposed biosensing strategy can provide an alternative detection platform for

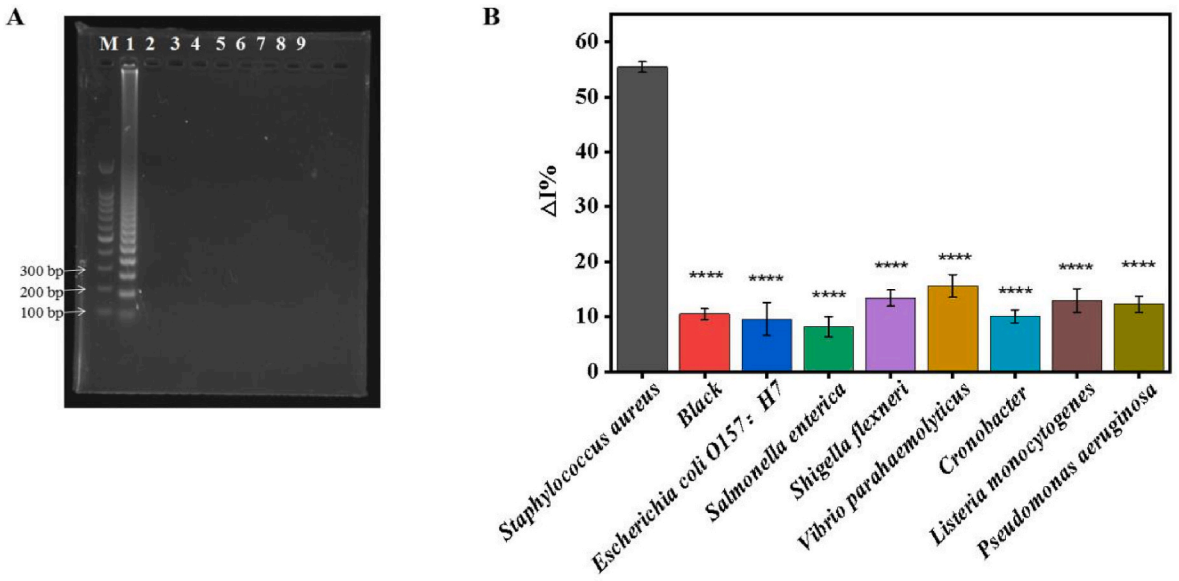


Fig. 5. Specific detection of the proposed method by using 7 strains of non-*S. aureus*, including *Escherichia coli* O157:H7, *Salmonella enterica*, *Shigella flexneri, Vibrio parahaemolyticus, Cronobacter, Listeria monocytogenes and Pseudomonas aeruginosa.* (A) Gel electrophoresis was verified the specificity of SRCA. lane M: 100 bp DNA maker, Lane 1: *Staphylococcus aureus*, Lane 2: blank, lane 3: *Escherichia coli* O157:H7, lane 4: *Salmonella enterica*, lane 5: *Shigella flexneri*, lane 6: *Vibrio parahaemolyticus*, lane 7: *Cronobacter*, lane 8: *Listeria monocytogenes*, lane 9: *Pseudomonas aeruginosa.* (B) The constructed electrochemical biosensor detects different foodborne pathogens. ****$p < 0.0001$. Each experiment was repeated three times, error bars represent mean ± SD.

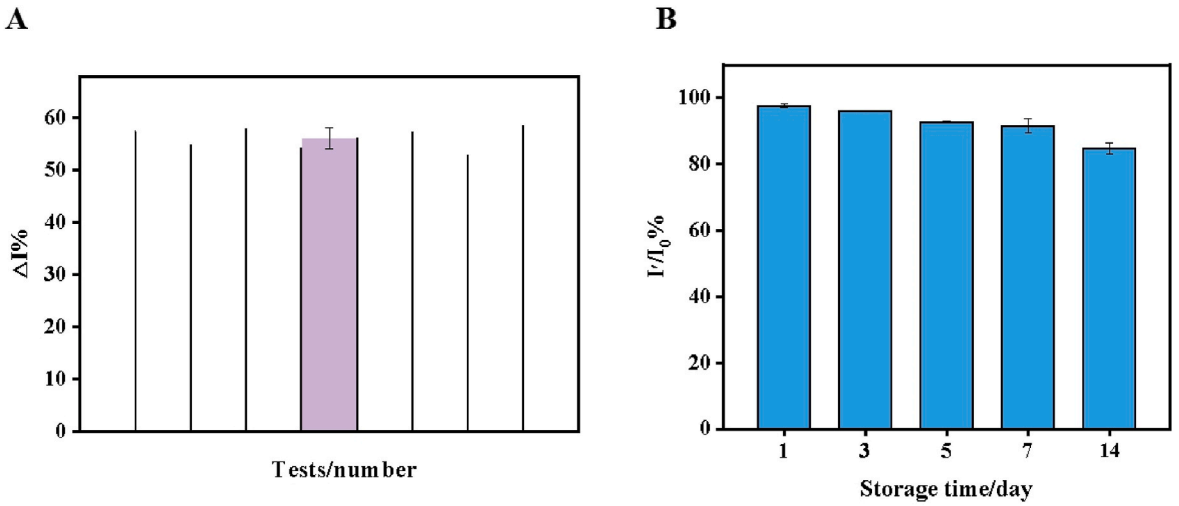


Fig. 6. (A) Reproducibility of electrochemical biosensors for detection of *S. aureus*. Average values are represented by the purple bars. The error bars in the histograms show the standard deviation of 8 individual measurement. (B) Stability of electrochemical biosensors for detection of *S. aureus*. I_0: the initial signal of the MB on the electrode, I': the signal of the MB on the electrode after a period of time. Each experiment was repeated three times, error bars represent mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Test results of artificially contaminated milk samples.

Samples	Added (CFU/mL)	Measured mean ±SD[a] (CFU/mL)	Recovery (%)	RSD (%)[b]	*p* value
1	6.2×10^5	$7.2 \times 10^5 \pm 2.9 \times 10^4$	117.1	2.4	0.2368
2	6.2×10^4	$6.2 \times 10^4 \pm 1.8 \times 10^3$	100.5	2.4	0.7015
3	6.2×10^3	$6.5 \times 10^3 \pm 3.3 \times 10^2$	105.2	3.7	0.5383
4	6.2×10^2	$6.1 \times 10^2 \pm 4.2 \times 10^1$	98.8	6.1	0.6384

[a] SD: standard deviation.
[b] RSD: relative standard deviation.

Table 3
Detection results of *S. aureus* in food by electrochemical biosensor.

Result		ISO method	
		Positive (n = 5)	Negative (n = 45)
This study	Positive (n = 6)	5	1
	Negative (n = 44)	0	44
	Sensitivity	100%	
	Specificity	97.8%	
	Accuracy	98%	

pathogens, which has great application potential in the field of food safety.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123821.

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