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Accurate and sensitive analysis of *Staphylococcus aureus* through CRISPR-Cas12a based recycling signal amplification cascades for early diagnosis of skin and soft tissue infections

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ABSTRACT

Early-diagnosis and treatments of skin and soft tissue infections remain a huge challenge due to the difficulties in the detection of trace amounts of bacteria. We develop here a novel method through CRISPR-Cas12a based recycling signal amplification cascades and demonstrated its feasibility of *Staphylococcus aureus* detection in a sensitive and accurate way. The highlights of the proposed method are calculated as: i) the designed allosteric probe is responsible for accurate identification of SA through PBP2a-specific aptamer; ii) high sensitivity from three processes, including DNA polymerase-based target SA release, Nb.*Bbv*CI enzyme induced ssDNA generation and attached CRISPR-Cas12a. As a result, the proposed method exhibited a detection range from 10^6 to 10^2 CFU/ml. Eventually, we believe that the proposed method could be expanded for the construction of diverse sensing platforms for detecting different trace bacteria.

1. Introduction

Skin and soft tissue infections (SSTIs) are a broad spectrum of diseases involving microbial invasion to the layers of the skin and underlying soft tissues with variable presentation, cause, and severity (Esposito et al., 2019; Tognetti et al., 2012). During the past few decades, the significantly increased incidence of SSTIs have led to a dramatic increased economic burden for both hospital and community healthcare (Esposito et al., 2017; Grammatikos and Falagas, 2014). A variety of studies have reported that SSTIs occupied a substantial cause of ambulatory and emergency department visits with mild-to-serious life-threatening infections (Fritz et al., 2013; Zehtabchi et al., 2016). Although, infected microbiology of SSTIs changes according to the different severity and clinical features, *Staphylococcus aureus* still compose the leading cause of both uncomplicated infections and complicated infections (Bassetti et al., 2017; Nelson et al., 2017). In recent years, treatments for MRSA *(Methicillin-Resistant Staphylococcus aureus)* related SSTIs became challenging due to the recent emergence of multidrug resistant pathogens. Moreover, early detection of causative MRSA of SSTIs is crucial for prescribing an effective treatment before the disease progresses (Park et al., 2019). Therefore, accurate and sensitive analysis of *Staphylococcus aureus* will contribute to selecting an adequate antimicrobial treatment, limiting broad-spectrum antibiotic therapy and reducing morbidity and mortality.

The most conventional method for bacteria detection both in clinical and experimental laboratory is culture incubation (Zasedatelev et al., 2010). Even though culture incubation is widely applied method for bacteria identification and could provide accurate identification report, it is also criticized for long incubation time (2–4 days), heavy labor requirements. The later-developed PCR based molecular techniques could greatly reduce the detection time and improve detection sensitivity in an effective and specific way due to the attached signal amplification (Wang et al., 2020; Chen et al., 2016; Dave et al., 2019; Wang et al., 2016). However, the PCR based methods are faced with some inevitable shortcomings, such as the requirements of long and laborious protocols which can take several hours, trained personnel, thermal cycle and specialized equipment (Guilini et al., 2015; Wang et al., 2017; Xu et al., 2020). The above-mentioned drawbacks of the bacteria detection methods have raised the concern for novel analytical systems capable of accurate and rapid identification, sensitive quantification, and easy-to-operate detection of bacteria. In recent years, diverse bacteria detection methods have been proposed and successfully employed to the detection of bacteria based on Surface plasmon resonance (SPR), florescence, colorimetrical, electrochemical. These methods have made some progress to culture incubation and PCR-based methods and partially settled the drawbacks. Among them, florescence-based bacteria

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detection methods have attracted abundant attention due to its easy-to-operate characteristics, much improved sensitivity and specificity. For example, Liqi Xu et al. developed a novel MRSA detection strategy through integrating the rolling circle amplification and CRISPR-Cas system after dual aptamer-based target bacteria recognition (Li et al., 2020). In addition, Jesús Maldonado et al. also provided a rapid method for the identification of nosocomial pathogens within the clinical requirements of sensitivity for the diagnosis of infections using a nanophotonic interferometric biosensor (Maldonado et al., 2020). Even though all these methods have been employed to sensitively detection of bacteria, a improve sensitivity sensing system is also in highly demand for bacteria detection, especially for those SSTIs patients in early infectious stage with negligible bacterial infection.

Herein, we propose here a novel MRSA detection strategy that could accurately identify and sensitive quantification of MRSA in both clinical and experimental conditions. In the method, we have designed an allosteric probe to specially identify surface protein of MRSA and thus initiate the following allosterism. Afterwards, released section in the allosteric probe hybridize with the designed hairpin probe and thus unfold it. With the assistance of DNA polymerase and the hybridization section as a primer, a double-strand DNA is generated and the bacteria was relapsed from allosteric probe. In combination with nicking site enzyme based nicking site generation and trans-cleavage activity of CRISPR-Cas system, florescence signals are produced and are correlated with the amounts of SA.

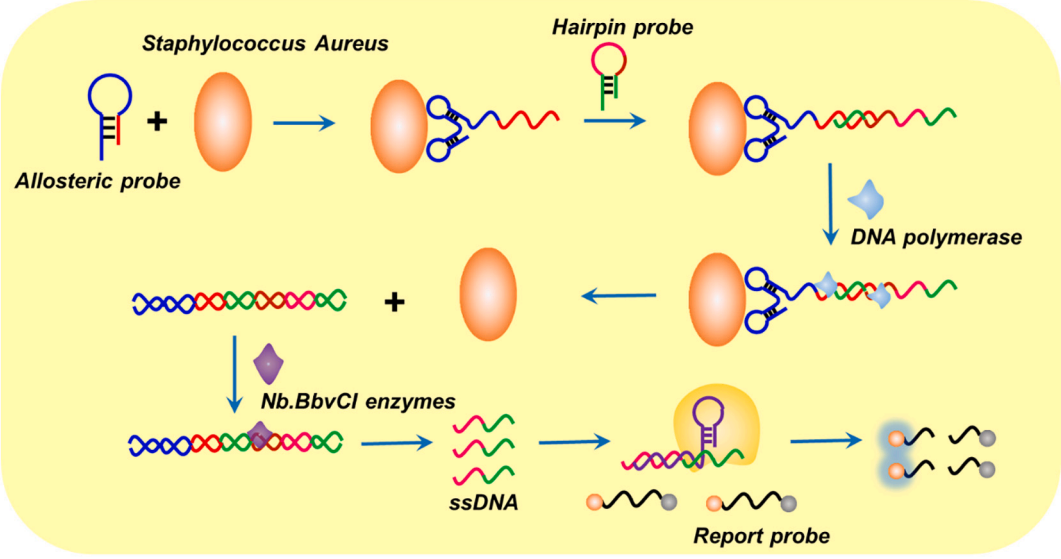
2. Result and discussion

2.1. Principle illustration of the proposed method

The working mechanism of the proposed method for MRSA detection is illustrated in Scheme 1. In the method, we have firstly designed an allosteric probe, which is composed of two active parts: PBP2a-specific aptamer (blued) and initiator (red). Notably, penicillin-binding proteins 2a (PBP2a) are a protein found on the surface of Methicillin-Resistant *Staphylococcus aureus* (MRSA). In the original state, the designed allosteric probe is in a harpin structure through the hybridization section between the two active parts. Furthermore, we have also designed a hairpin structure probe with a nicking enzyme recognition site in loop section (brown) and the probe could be unfold by the initiator in allosteric probe. When target MRSA existed, PBP2a-specific aptamer section in allosteric probe could specially recognize and bind with PBP2a protein on the surface of MRSA, and consequently lead to the release of initiator section. Afterwards, the released initiator section in the probe could then hybridize with the designed hairpin probe and gradually unfolded it. With initiator in allosteric probe and its complementary section in hairpin probe as primer, a dsDNA sequence is generated under the assistance of DNA polymerase and thus lead to the release of MRSA. As a result, the released MRSA could then initiate a next signal cycle. In the obtained dsDNA sequences, the nicking site could be identified by Nb.*Bbv*CI enzymes and the complementary sequence would be cut by the enzyme. Afterwards, the generated nicking site would be extended by DNA polymerase and thus continuously produce single-strand DNA sequences. The produced ssDNA sequences could then be recognized by CRISPR-Cas12a system and initiate the trans-cleavage activity of it. As a result, the surrounding florescence probes, whose two terminals are labeled with florescence moiety and corresponding quenching moiety, would be cut and the obtained florescence signals is positively correlated with the amounts of MRSA.

2.2. Investigation of the designed allosteric probe and hairpin probe

The designed allosteric probe is responsible for accurately identify the MRSA and induce the following signal amplification process. Therefore, we firstly investigate the recognized specificity of the designed allosteric probe through a florescence assay. The florescence experiments details are illustrated in Fig. 1a. As shown in Fig. 1b, the obtained florescence intensity greatly increased when the target MRSA existed, while no obvious florescence enhancements are observed when MRSA absent. Meanwhile, we also monitored the florescence intensity of the allosteric probe when mixed with *Escherichia coli*, which has no PBP2a protein on its surface. The florescence intensity of allosteric probe incubated with *Escherichia coli* showed no significant increasements compared with the control group, indicating that the designed allosteric probe could specially recognize the PBP2a protein on the surface of MRSA and thus initiate its allosterism, while showed no activation with other bacteria. Afterwards, we studied the hybridization between active allosteric probe and hairpin probes through labeling a florescence moiety and corresponding quenching moiety on the two terminals of hairpin probes (Fig. 1c). From the result in Fig. 1d, we have observed a significantly enhanced florescence intensity when MRSA existed while no obvious increasements are observed when MRSA is absent, thus demonstrating the successful unfold of hairpin probe by active allosteric probes. We then studied the feasibility of the proposed method for MRSA



Scheme 1. The working principle of the proposed CRISPR-Cas12a based recycling signal amplification cascades detection methods for MRSA detection.

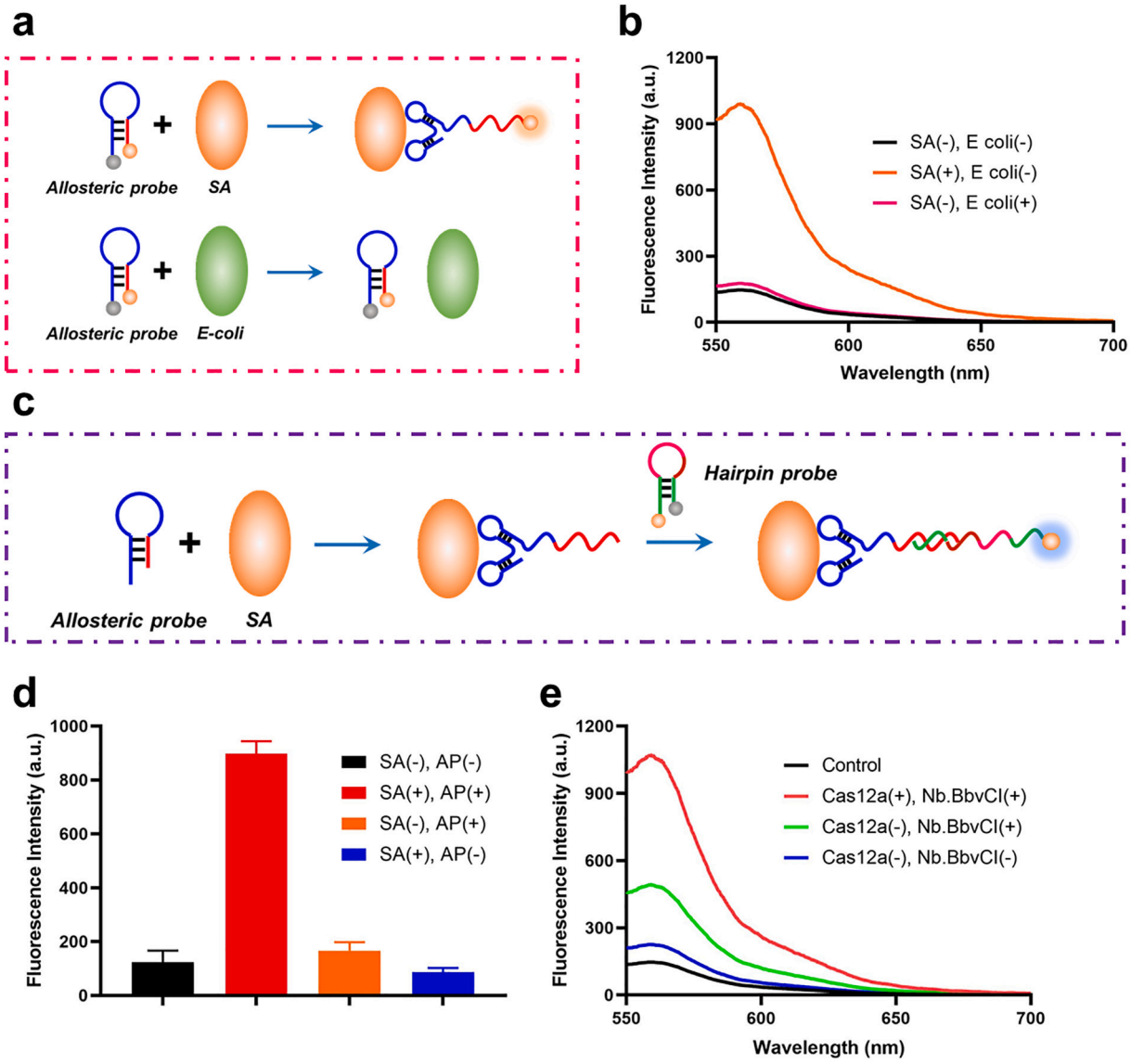


Fig. 1. Investigation of the designed allosteric probe and hairpin probe. (a) illustration of the allosterism process of allosteric probe to recognize MRSA. (b) florescence spectrum of the allosteric probe for MRSA and *E*-coli recognition. (c) illustration of the process to investigate the feasibility of hairpin probe. (d) florescence intensity of the hairpin probe. (e) florescence spectrum of the sensing system.

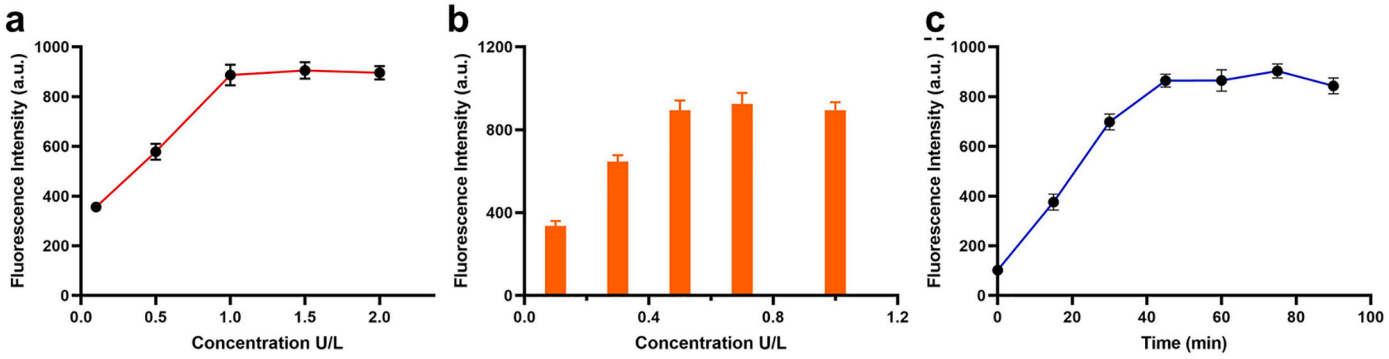


Fig. 2. Experimental conditions optimization of the proposed method. (a) florescence intensity of the proposed method with DNA polymerase of different concentrations. (b) florescence intensity of the proposed method with Nb.*Bbv*CI enzyme of different concentrations. (c) florescence intensity of the proposed method with different incubation time.

detection. It is obvious from the result in Fig. 1e, the florescence intensity of the sensing system greatly increased, which is about 2.5 times higher than that without CRISPR-Cas12a system, suggesting the signal amplification ability of the method. In addition, we have observed no significant florescence enhancements when the Nb.*Bbv*CI enzyme or the DNA polymerase enzymes absent.

2.3. Experimental conditions optimization of the proposed method

We then optimized the experimental conditions of the proposed method, including the concentration of the DNA polymerase enzymes and Nb.*Bbv*CI enzyme, the incubation time of allosteric probes with target MRSA. DNA polymerase plays an important role in the release of MRSA and initiate the following cycles. Therefore, we investigated the concentrations of DNA polymerase in the sensing system through monitoring the florescence intensity with different concentrations of DNA polymerase. From the result in Fig. 2a, the obtained florescence intensity gradually increased with the increasements of DNA polymerase concentration ranging from 0.1 U/L to 1 U/L and no more attached increasements were observed when the concentration more than 1 U/L. Therefore, the optimized DNA polymerase concentration was determined 1 U/L. Meanwhile, the concentration of Nb.*Bbv*CI enzyme is also studied through a florescence assay and determined as 0.5 U/L (Fig. 2b). We then investigated the incubation time of allosteric probes with target MRSA. As shown in Fig. 2c, the obtained florescence intensity gradually increased with the incubation time ranging from 0–45 min and no more increasements were observed when incubated for more than 45 min, indicating that incubated for about 45 min could proved an optimized detection performance.

2.4. Detection performance of the proposed method

Considering the robust signal amplification and output efficiency, we have then applied the proposed method for MRSA detection under optimized experimental conditions. From the result in Fig. 3a, we have observed negligible florescence intensity increasements compared with the control group when MRSA absent. The standard MRSA samples with different concentrations were obtained through dilution of original sample whose concentration was determined through culture incubation. Afterwards, the obtained MRSA samples were applied for the investigation of the proposed method. When the concentrations of MRSA increased from 10^2 CFU/ml to 10^6 CFU/ml, the fluorescence of the sensing system was also gradually increased. In addition, we have also measured linear correlation between the obtained fluorescence intensity and concentrations of MRSA, and eventually obtained positive correlation equation between florescence signals and the logarithm of MRSA concentration (C) ranging from 10^6 to 10^2 CFU/ml (Fig. 3b), spanning over six orders of magnitude. The obtained equation was $Y = 151.3\lg(C) + 58.99$, with a correlation coefficient $R^2 = 0.9965$. From our point of view, the favorable sensitivity of the method is mainly ascribed to the three signal amplification processes, including DNA polymerase induced recycle, Nb.*Bbv*CI enzyme induced ssDNA generation and attached CRISPR-Cas12a. Besides the sensitivity, accurate identification of MRSA from a variety of bacteria also raised a lot of concerns. Thus, we have then studied the specificity of the method for detection of different bacteria, including *E. coli*, *P. aeruginosa*, Kpn, *S. pneumoniae* and their mixture. From the comparison florescence result of result for different bacteria detection in Fig. 3c, only a weak signal was observed when the *E. coli*, *P. aeruginosa*, Kpn and *S. pneumoniae* existed compared with the florescence signals obtained from MRSA detection. Furthermore, we have also employed the proposed method for detection of bacteria mixture (*E. coli*, *P. aeruginosa*, Kpn, *S. pneumoniae* and MRSA) and obtained a significantly enhanced florescence signals compared with the bacteria alone.

3. Conclusion

Accurate and sensitive detection of MRSA play a pivotal role in the early-diagnosis and SSTIs. Besides the traditional colony count method, there have also reported two main methods that could successfully applied for the bacteria detection: i) nucleic acids-based method through integration with bacteria specific aptamer; ii) antibody-based method for specific antigen identification. In details, the nucleic acids-based methods have attracted substantial attention due to its capability to initiate signal amplification processes.

Even though, there are still some drawbacks to be solved: i) many of the former reported methods need a PBS buffer wash process, which lead to complicated procedures and increase labor burdens; ii) a relatively high cost of these methods limited their further amplification, especially for undeveloped countries. Therefore, we proposed here a novel MRSA detection method through combination of three signal amplification processes, including DNA polymerase-based target MRSA release, Nb.*Bbv*CI enzyme induced ssDNA generation and attached CRISPR-Cas12a. Consequently, the proposed method exhibited a detection range from 10^6 to 10^2 CFU/ml. we believe the proposed method would be versatile for the detection of diverse bacteria, with an extraordinary specificity and rapid amplification kinetics.

4. Experimental section

4.1. Chemicals and materials

Details of the oligonucleotides nucleic acid used in the experiment is listed in Table S1. The oligonucleotides nucleic acid in this article was

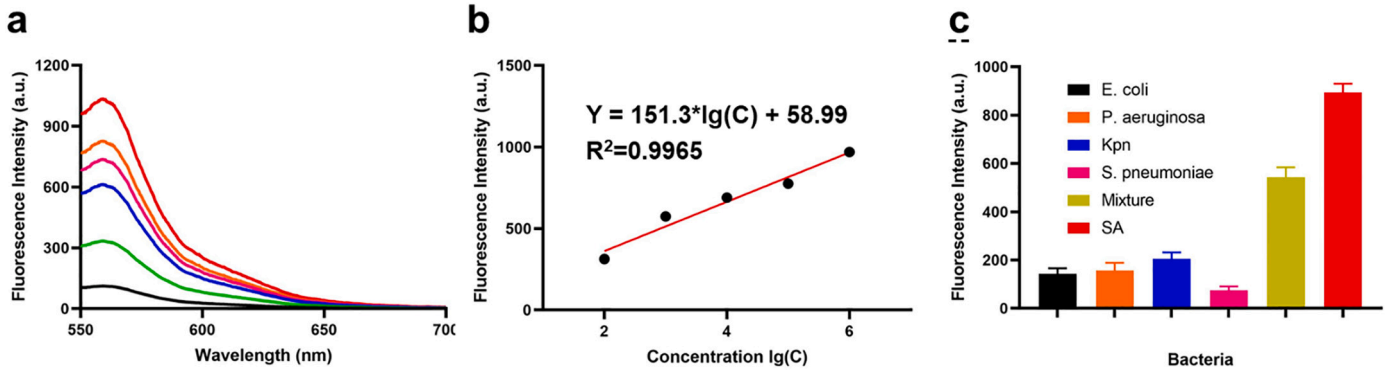


Fig. 3. Feasibility of the proposed method for MRSA detection. (a) florescence spectrum of the proposed method for MRSA detection with different concentrations. (b) equation correlation between the obtained florescence intensity concentrations of MRSA. (c) florescence intensity of the proposed method with different bacteria detection.

brought and purified from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The obtained oligonucleotides nucleic acid was then diluted to 10 μM by HPLC water and stored for the following experiments. The buffer related regents and enzymes, such as bovine serum albumin (BSA), Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), Thermas aquatics (Taq) DNA polymerase and Nb.*Bbv*CI enzyme were purchased from New England BioLabs (MA, U.S.A.). EnGen ® Lba Cas12a enzymes were purchased from BioLabs Tech Co., Ltd. (Beijing, China). *E. coli* (ATCC 25922), MRSA (ATCC 17802), *S. pneumoniae* (ATCC 50761), *P. aeruginosa* (ATCC 19115) and Kpn (ATCC 13313) were obtained from the American Type Culture Collection (ATCC).

4.2. Bacteria culture

We first incubate the target bacteria MRSA with 2.5% NaCl basic protein in water at 37 °C for 18 h. We use *Staphylococcus aureus* to cultivate the obtained strain in lysogenic broth (LB) medium: 37 °C (200 revolutions/min) for 12 h. Preparation of MRSA with different concentration gradients: We first cultured the bacteria in a 3% NaCl alkaline protein aqueous solution medium for 18 h, and then successively diluted the cell suspension with each gradient of 50 μL. Finally, we put the sample in a TCBS petri dish and incubate at 37 °C for 24 h. Then count the colonies and record the average of the three parallel plates.

4.3. Investigation of the designed allosteric probe for target bacteria recognition

We labeled the fluorescent probe and the corresponding quenching group on both ends of the allosteric probe. The obtained purified and diluted allosteric probe was then heat to 95 °C for 5 min and then slowly cooled to room temperature. Then 5ul of the allosteric probe was mixed with *Staphylococcus aureus* and other bacteria for 30 min. Eventually the florescence signals were detected by Fluorescence spectrophotometer Hitachi F-4700 (Beijing, China).

4.4. Working procedure of the method for target S. aureus detection

In order to explore the feasibility of the constructed method, we diluted the cultured bacteria to a concentration of 4×10^4. 10 μl allosteric probe and 10 μl bacteria were mixed and incubated for 30 min, and then 10 μl hairpin probe was added to react for 30 min. Then add 2 μl of DNA polymerase and 2 μl of the corresponding buffer to the system, and react for 1 h. Then add nicking enzyme, 3 μl and corresponding buffer 5 μl, react for 30 min. Finally, 5 μl of Cas12a enzyme, 3 μl of sgRNA, 3 μl of fluorescent probe and 5 μl of corresponding buffer were added, and the fluorescent signal was detected after 30 min of reaction. The experiment to verify the sensitivity of the method used the above steps, except that the bacteria were diluted with 1xPBS buffer to different concentrations. Specificity studies use this procedure to detect several different bacteria.

Data availability

All data associated with this study are available in the paper or the Supplementary Materials.

Declaration of Competing Interest

All authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106167.

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