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CRISPR/Cas12a-Based APE1 Enzyme Cleavage Assay for Drug Resistance Analysis of *Staphylococcus aureus*-Related Pneumonia

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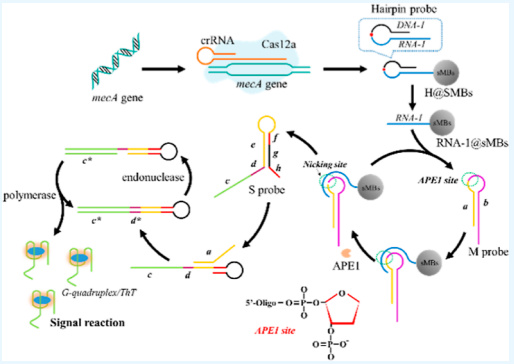
ABSTRACT: Drug resistance analysis of *Staphylococcus aureus* is responsible for generating significant mortality and morbidity in numerous diseases. However, sensitive and accurate analysis of drug resistance of *S. aureus* remains a huge challenge. In this study, we present the development of a fluorescence biosensor based on the CRISPR/Cas12a system that enables label-free and ultrasensitive detection of the *mecA* gene in methicillin-resistant *S. aureus* (MRSA). The biosensor identified the *mecA* gene in MRSA using Cas12a/crRNA. This recognition triggered the *trans*-cleavage activity of Cas12a and the release of RNA1, which subsequently induced Apurinic/apyrimidinic endonuclease 1 (APE1) enzyme-assisted target recycling and G-quadruplexes/Thioflavin T-based signal reaction. Based on this, the biosensor effectively detects the *mecA* gene with a low limit of detection of 212 aM and a high degree of selectivity, even toward single base mutations. Compared with the traditional CRISPR-Cas12a system-based methods, in which the signal amplification process is prone to generate nucleic acid sequence mismatch, which causes errors, the biosensor used APE1 to improve nucleic acid sequence recognition specificity to ensure that the RNA1 sequence released after Cas12a/crRNA cleavage can specifically guide the signal cycle. In addition to enhancing the CRISPR toolkit, the developed biosensor offers a novel method for the precise and sensitive identification of drug-resistant microbes that cause infections.



1. INTRODUCTION

Pneumonia is a common lung disease in the clinic, and it is an important cause of death in children. Severe bacterial pneumonia in children poses a great threat to the life safety of children, and with the progression of the disease, it will affect the prognosis of children to a greater extent and increase the economic burden and psychological pressure of family members. Taking *Staphylococcus aureus* caused pneumonia in children as an example, whether it has methicillin resistance has a great influence on drug selection and treatment effect. The emergence of drug-resistant microorganisms has posed a significant threat to public health, which causes the death of around 700,000 individuals annually on a global scale.[1,2] If essential actions are not taken to reduce drug resistance, it is projected that this number would escalate to 10 million by the year 2050. Methicillin-resistant *S. aureus* (MRSA) is a prevalent and highly drug-resistant bacterium that is responsible for generating significant mortality and morbidity in numerous diseases,[3,4] including pneumonia in child and hospital-acquired infections.[5,6] The sensitive and precise identification of MRSA is essential to preventing its detrimental effects and monitoring its spread within a population. Currently, MRSA detection is accomplished by the utilization of culture-based assays,[7,8] aptamer-mediated signal amplification tests,[9,10] and mass spectrometry-based approaches.[11] Nevertheless, these techniques typically necessitate a lengthy period for identification, significant manipulation of samples, and the use of costly apparatus. The *mecA* gene encodes a novel penicillin-binding protein that is responsible for the antibiotic resistance observed in MRSA.[12,13] There is an urgent need to develop fast, inexpensive, and highly sensitive methods for detecting the *mecA* gene in order to discover drug resistance in MRSA.

The CRISPR-associated nuclease 12a (CRISPR/Cas12a) system, which belongs to class 2 type V-A adaptive immunity, has been widely used in biosensor development due to its capability in specifically recognizing target sequences and cleave surrounding single-stranded DNA (ssDNA).[14,15] The CRISPR/Cas12a-based biosensor has been effectively established for nucleic acids, metal ions, proteins, and other substances. Although the CRISPR/Cas12a method has several turnovers, it can only detect DNA at a limit of 0.1 nM, which is not enough to detect the extremely low levels of the target.[16] In order to enhance the ability to identify small amounts of

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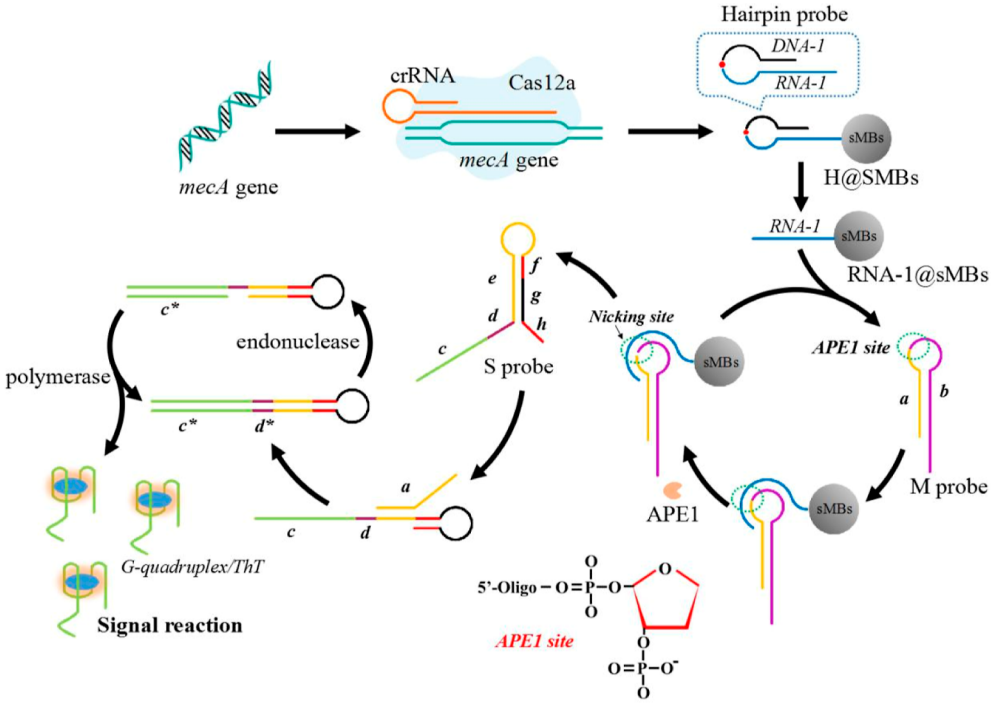

Figure 1. Schematic illustration of the working mechanism of the Cas12a/crRNA-based mecA analysis.

genetic material, some techniques for amplifying nucleic acids, including as polymerase chain reaction,[17] rolling circle amplification,[18,19] and enzyme assisted chain replacement,[20] were incorporated into the CRISPR/Cas12a system. Nevertheless, the stages after target identification using Cas12a/crRNA, which facilitate successive signal cycles, are susceptible to nucleic acid sequence discrepancies, resulting in inaccurate positive outcomes.

Apurinic/apyrimidinic endonuclease 1 (APE1) is a crucial endogenous multifunctional enzyme that exhibits strong enzymatic activity in cleaving specific apurinic/apyrimidinic (AP) sites within random double-stranded DNA (dsDNA) through hydrolyzing DNA phosphodiester backbone.[21] Based on the unique properties of the APE1 enzyme, a variety of highly specific nucleic acid detection methods have been constructed through the signal cycle mediated by integrase cleavage.[22] Due to its robust capability in distinguishing mismatches, it is feasible to leverage its enzyme cleavage capability, designing a strategy for ensuring the specificity of post-Cas12a/crRNA signal amplification.

Herein, we depict a novel sensing strategy that incorporated the CRISPR/Cas12a system-based specific target recognition, APE1 assured initiation of signal recycling, and polymerase/endonuclease-assisted chain displacement into the fluorescent biosensor and achieved label-free and ultrasensitive *mecA* gene analysis in MRSA. In this CRISPR/Cas12a-based biosensor, the *mecA* gene within MRSA was selected as the target sequence for the recognition of CRISPR/Cas12a, and G-quadruplexes/Thioflavin T (ThT) dye was used for the label-free signal output. The final fluorescence signal intensity that corresponds to the amplified *mecA* gene was maximized by triple signal transformation and amplification. This ultrasensitive and label-free fluorescent biosensor can also be expanded to assay other resistant bacteria, providing a promising toolkit to prevent their spread.

2. EXPERIMENTAL SECTION

2.1. Materials. Prior to the experiments, every DNA and RNA oligonucleotide was synthesized and purified by PAGE. Table S1 contains a list of the oligonucleotide sequences. The Bst polymerase, Nb.BbvCI, 10× NEBuffer 2 (comprising 100H mM Tris−HCl, pH 7.9, 500HM NaCl, 100HM $MgCl_2$, 10HM DTT), and 10× CutSmart buffer (comprising 200 × M Tris-Ac, pH 7.9, 500HM KAc, 100 mM $Mg(Ac)_2$, 1 mg/mL BSA) were all obtained from New England Biolabs Inc. (Beverly, MA, United States). Cas12a protein was purchased from Invitrogen (Carlsbad, CA, United States). APE1 were obtained from NEB. All other substances utilized were of analytical grade and did not require additional purification. At room temperature, the fluorescence spectrum was acquired by utilizing an F-7000 fluorescence spectrophotometer (Hitachi). The instrument operated with an excitation wavelength of 440 nm and a collection range of 460−640 nm for fluorescence.

2.2. Feasibility of Cas12a/crRNA. Initially, a mixture of 200 nM Cas12a protein and 250 nM crRNA was created in 1 × NEBbuffer at a temperature of 37 °C for a duration of 15 min. Simultaneously, a reaction solution was made, consisting of 20 μL of H@sMBs, 5 μL of various quantities of the target (dsDNA mecA), and 0.3 μL of the RNAase inhibitor. The above two solutions were mixed and placed in an incubator at a temperature of 37 °C for a duration of 30 min. Following this, the liquid portion above the sediment was extracted, and the ultimate measurement of optical density was documented. The procedures for culturing bacteria and extracting the *mecA* gene are included in the Supporting Information.

2.3. *mecA* Gene Detection. The experimental process consists of three sequential steps: (1) A mixture of 200 nM Cas12a protein and 250 nM crRNA was prepared in 1 × NEBbuffer at a temperature of 37 °C for a duration of 15 min. Simultaneously, a signal solution was made consisting of 20 μL of H@sMBs, 5 μL of various quantities of the target, and 0.3 μL of the RNAase inhibitor. The two solutions were combined

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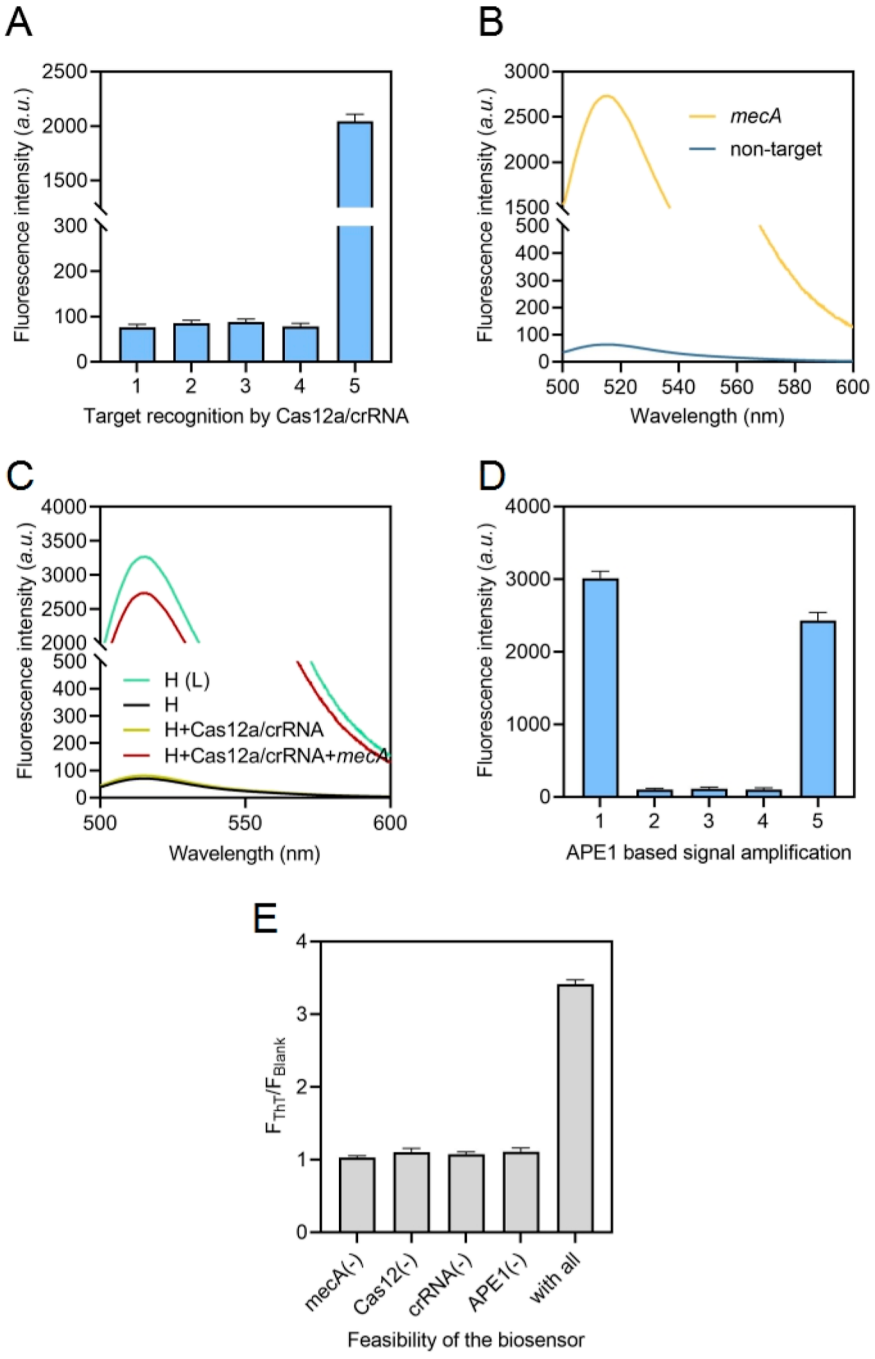

Figure 2. Feasibility analysis of the established fluorescent biosensor. (A) Fluorescence intensities of the R sequence during the target recognition process of Cas12a/crRNA. Column 1: blank control; Column 2: R sequence; Column 3: R sequence+ Cas12a; Column 4: R sequence + Cas12a + crRNA; and Column 5: R sequence + Cas12a + crRNA + mecA. (B) Fluorescence intensity of the Cas12a/crRNA when mecA or nontarget sequences existed. (C) Fluorescence intensities of the hairpin probe (H) during the target recognition process by Cas12a/crRNA. H(L), H probe in the linear state. (D) Fluorescence intensity of the M probe during the APE1-based signal amplification process. Column 1: M probe in the linear state; Column 2: M probe; Column 3: M probe + RNA-1; and Column 4: M probe + RNA-1+ APE1. (E) Fluorescence ratio (F_{ThT}/F_{Blank}) of the approach whether essential experimental components existed or not.

and placed in an incubator at a temperature of 37 °C for a duration of 30 min. Afterward, the liquid portion above the sediment was extracted. The reaction mixture consists of 200 nM of M probe and 0.01 U/μL APE1 enzyme in a buffer solution comprising 20 mM Tris HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 12 mM $MgSO_4$, 0.1% Triton X-100, with a pH of 8.8. The solution was thereafter placed in an incubator set at 37 °C for a duration of 0.5 h. Subsequently, deoxyribonucleotide triphosphates with a concentration of 0.5 mM, polymerase, and Nb.BbvCI were introduced into the system, resulting in a final reaction volume of 50 μL. The reaction was conducted at a temperature of 37 °C for a duration of 0.5 h, followed by a temperature of 85 °C for a duration of 10 min, in order to deactivate the enzymes. (3) ThT (40 μM) and KCl (10 mM) were introduced into the reaction mixture, and the fluorescence signal was measured using a fluorescence spectrophotometer with an excitation wavelength of 445 nm.

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3. RESULTS AND DISCUSSION

3.1. Working Procedures of the CRISPR-Cas12a-Based Method for Sensitive *mecA* Analysis. The fluorescent biosensor utilized the *mecA* gene, which is prevalent among numerous MRSA strains and comprises a protospacer adjacent motif for the Cas12a protein, to detect MRSA. The biosensor's detection procedure comprises three distinct components (Figure 1). MRSA genomic DNA was initially isolated from samples by utilizing a rapid bacterial genomic DNA isolation reagent in order to serve as the signal input. The genomic DNA was utilized without undergoing purification or amplification, which facilitated the direct identification of MRSA. During the phase of signal transduction and amplification, when the crRNA specifically targets the *mecA* gene, the *trans*-cleavage activity of Cas12a is triggered. This action cleaves the ssDNA segment of the hairpin probe, resulting in the release of RNA-1@sMBs. The released RNA-1@sMBs induced an APE1-dependent second signal amplification. Specifically, RNA1@sMBs can form a hybrid complex with the loop of the M probe. Subsequently, the APE1 enzyme cleaves the AP site on the hybridized duplex, which divides the M probe into "a″ and "b″ fragments; RNA1@sMBs are liberated for the subsequent cycle. Therefore, recycling of RNA1@sMBs aided by the APE1 enzyme was formed.

When "a″ is present, the S probe is activated because "a″ attaches to the "e″ region in S probe, causing the self-priming of S probe which allows the "h" domain to function as a primer at the 3′ end of the S probe. The DNA polymerase extension generates an intermediate product ("c″ and "d″) that includes the recognition site for a nicking endonuclease. The location experiences repeated cycles of cleavage, extension, and strand displacement response due to the collaborative actions of DNA polymerase and nicking endonuclease, resulting in the generation of a significant quantity of "c*″. The nucleotide sequence containing the letter "c*″ has the ability to form a structure called the G-quadruplex. This G-quadruplex structure may be particularly detected by the ThT dye, resulting in a notable increase in the ThT fluorescence. This increase in fluorescence serves as the final step in generating the signal.

3.2. Selectivity of the Cas12a/crRNA Complex to *mecA* and Feasibility of the Signal Amplification Process. The synthesized *mecA* gene was employed to verify the target recognition specificity of Cas12a/crRNA and its capacity to induce the *trans*-cleavage activity. Specifically, a ssDNA sequence (R sequence), which was marked with fluorescent dyes and quenched groups at both ends, was employed. The purpose was to analyze and describe the activity of the Cas12a enzyme. Figure 2A demonstrates a notable increase in fluorescence when *mecA* and crRNA are present, indicating that Cas12a cleaves the ssDNA sequences. However, the absence of *mecA* or crRNA resulted in no notable increase in fluorescence, indicating that the *trans*-cleavage characteristic was not activated.

The specificity of Cas12a/crRNA in recognizing *mecA* was further confirmed by the disparity in fluorescence intensity observed when detecting *mecA* compared to other sequences (Figure 2B). The fluorescence signal of the hairpin probe exhibited alterations that confirmed the activation of Cas12a/crRNA in the presence of the *mecA* gene. This activation primarily resulted in the cleavage of DNA1 within the hairpin probe, as shown in Figure 2C. Furthermore, the APE1-based signal cycle was validated through labeling a fluorophore and the quenching group at both ends of the M probe. Figure 2D displays the results that the restoration of the quenched fluorescence signal only occurs in the presence of APE1, demonstrating the cleavage of the M probe and the separation of its "a″ and "b″ portions.

After the production of the G-quadruplex by the polymerase/endonuclease-assisted chain displacement, KCl/ThT were added into the sensing system, resulting in an amplified ThT fluorescence signal. As shown in Figure 2E, the fluorescence signal is significantly elevated when both the target and APE1 enzyme are present, but the fluorescence signal is considerably low when either the *mecA* gene or APE1 is not present in the system. These findings indicate that the presence of essential experimental components, consisting of the target *mecA* gene and APE1 enzyme, is necessary for the release of the "a″ chain and the initiation of the signal reaction. This leads to the synthesis of G-quadruplex/ThT and an increased fluorescence signal, which is crucial for biosensing purposes.

3.3. Optimization of Assay Conditions. The experimental procedure consists primarily of three stages, and we optimized the experimental conditions for each stage to improve the detection performance. Therefore, we optimized the concentration and incubation of the APE1. As illustrated in Figure 3A, the M probe remained uncut by the APE1 enzyme

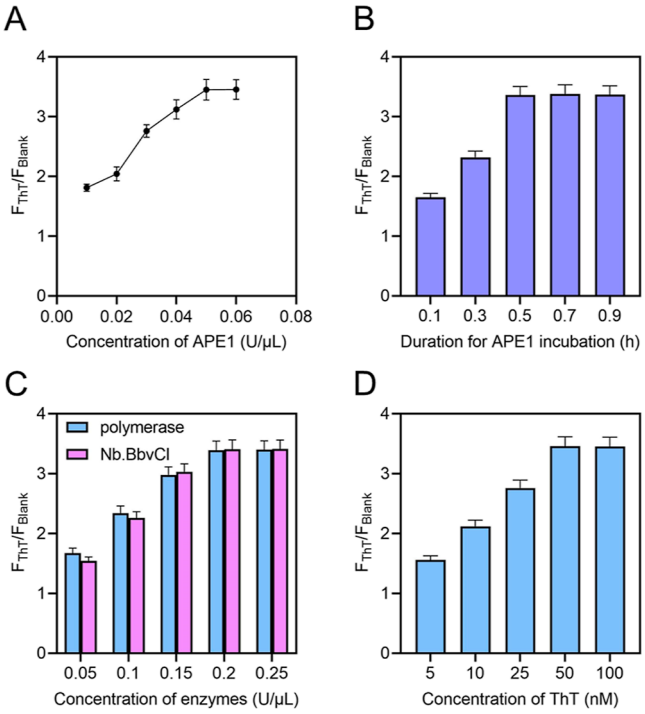

Figure 3. Optimization of experimental parameters. Fluorescence intensity ratio (F_{ThT}/F_{Blank}) of the approach with different APE1 concentrations (A); different incubating duration for APE1 (B); different concentrations of polymerase and Nb.BbvCI (C); and different concentration of ThT (D).

in the absence of RNA1. However, the APE1 was capable of cleaving the AP site and liberating the "a″ chain when RNA1 hybridizes with the M probe. Consequently, an APE1 concentration of 0.05 U/μL was selected in the following experiments. Following that, we continued with optimization of the reaction time of the APE1 enzyme. In Figure 3B, both

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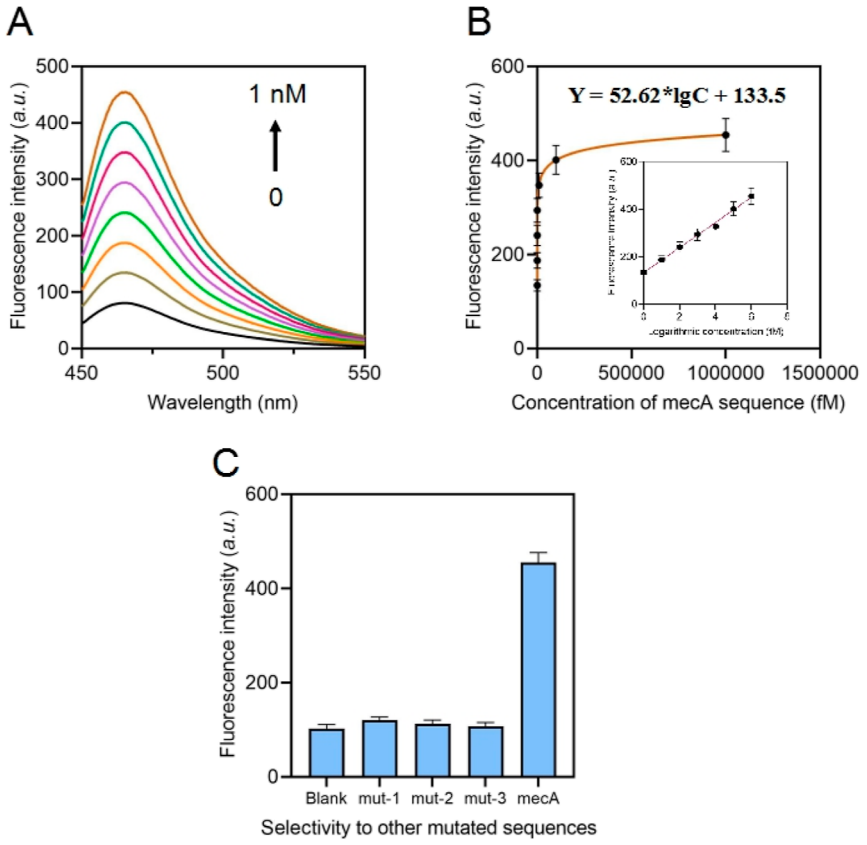

Figure 4. Analytical performance of the approach. (A) Fluorescence spectrum of the approach for different concentrations of mecA analysis. (B) Correlation between the fluorescence intensities and concentration of mecA gene. (C) Fluorescence intensities of the biosensor when detecting mecA gene and its mutations.

the M probe and RNA1 are present at concentrations of 1 μM, while the APE1 enzyme concentration is maintained at 0.05 U/μL. These components were introduced simultaneously into the reaction system for different durations. These experimental components were introduced into the reaction system concurrently for varying durations. After incubation for 05 h, an observable cleavage of the M probe by APE1 was recorded. Therefore, it was determined that the ideal duration for the cleavage of APE1 was 0.5 h. As shown in Figure 3C, the fluorescence intensity elevated progressively with the concentrations of Bst polymerase and Nb.BbvCI increased, and reached equilibrium at 0.2 U/μL, respectively. ThT molecules have the potential to intercalate into G-quadruplex structures, resulting in the emission of fluorescence. The concentrations of ThT were optimized (Figure 3D). A discernible pattern was identified within the concentration range of 5 to 50 μM for ThT. The intensity of the fluorescence increased significantly with the concentration of ThT until it stabilized at 40 μM. We then optimized the complementary bases between domain a and domain b of the M probe. The result in Figure S1 shows that 15 complementary bases between domain 1 and domain 2 provided a most-elevated signal, which was selected for the following experiments.

3.4. Analytical Performance of Cas12a/crRNA-Based Biosensor. The sensitivity and specificity of the biosensor were assessed under ideal conditions. As targets, various concentrations of the *mecA* gene were detected. As the quantity of the *mecA* gene increased from 0 to 1 nM, the fluorescence intensities also escalated (Figure 4A). As predicted, the ThT fluorescence signal exhibits a noticeable increase as the concentration of the target substance rises. The logarithm of the target concentration in Figure 4B provides unambiguous evidence that our biosensor demonstrates a robust linear relationship spanning a broad concentration range of 1 fM to 10 nM. The linear equation that corresponds to the data is $Y = 52.62 \times \mathrm{lgC} + 133.5$ ($R^2 = 0.9952$), where Y denotes the biosensor's fluorescence intensity and lgC signifies the logarithm of the *mecA* gene concentration. The calculated detection limit of this biosensor is 212 aM according to the 3δ rule, indicating that our constructed fluorescent biosensor with a cascade signal amplification strategy can detect the target *mecA* gene with a wide linear range and with high sensitivity. Furthermore, when compared to other recently published methods, its analytical performance is somewhat superior; therefore, it offers a promising avenue for the clinical diagnosis of drug resistance.

Attaining both high sensitivity and robust specificity is necessary for the successful clinical application of biological detection methods. Therefore, we performed a thorough evaluation of the ability of our proposed biosensor to accurately distinguish the target *mecA* gene from other gene sequences that may have mutations. During our assessment, we analyzed all sequences, including the target *mecA*, as well as mut-1, mut-2, and mut-3, at the same concentration of 1 nM. The sequences were evaluated both separately and in combinations with the *mecA* gene. Figure 4C demonstrates that a strong fluorescence signal was produced only when the target was present. However, the fluorescence signal showed no significant difference from that of the control sample when

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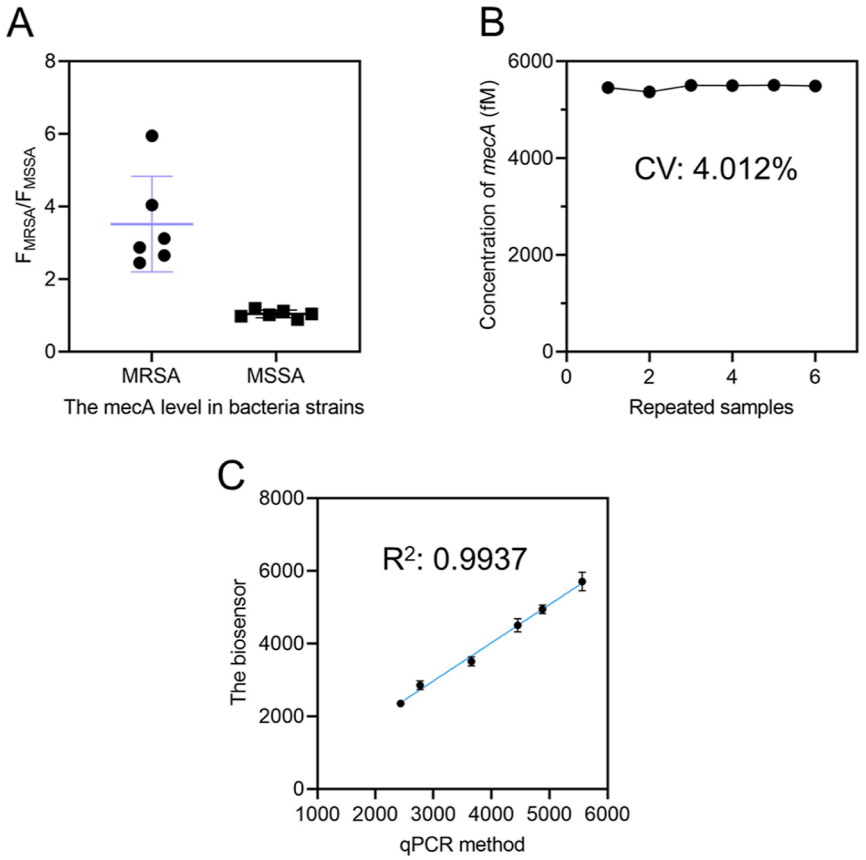

Figure 5. Real sample analysis. (A) Fluorescence ratio of the approach for mecA gene analysis from MRSA and MSSA samples. (B) Concentration of mecA gene from six repeated samples calculated by the biosensor. (C) Correlation between the calculated mecA gene concentration by the method and bu qPCR method.

interfering sequences, such as mut-1, mut-2, and mut-3, were detected.

3.5. Drug Resistance Analysis by the Biosensor. In order to ascertain the effectiveness of the constructed method in assessing bacterial methicillin resistance, the constructed method was employed to detect MRSA, which possesses robust resistance to methicillin, and MSSA, which is sensitive to methicillin and beta-lactam antibiotics. The findings (Figure 5A) indicated that the level of *mecA* gene expression observed in the six MRSA samples was notably greater than that in MSSA samples of the same concentration. This finding suggests that MRSA exhibited substantial resistance to methicillin. Subsequently, the constructed biosensor's repeatability was assessed through the continuous detection of numerous duplicate samples. The coefficient of variation among the test results of the six groups of repeated samples was 4.012%, as illustrated in Figure 5B. This value is deemed satisfactory for clinical application purposes. For comparison, the conventional gold-standard qPCR method and established biosensors were simultaneously applied to analyze these spiked samples. The excellent agreement between the biosensor and the qPCR method, as shown in Figure 5C, provided additional evidence that this biosensor detected genuine samples with remarkable precision.

4. CONCLUSIONS

To summarize, we have created an extremely sensitive biosensor that does not require labeling of the fluorochrome to detect the *mecA* gene. This was achieved by combining three-stage signal amplification with a signal readout based on ThT. This biosensor utilizes Cas12a/crRNA to selectively detect the extracted *mecA* gene and initiate the *trans*-cleavage to facilitate the following signaling cycles. In the conventional method utilizing the CRISPR-Cas12a system, the process of signal amplification after Cas12a/crRNA-based target recognition is susceptible to producing discrepancies in the nucleic acid sequence, resulting in detective errors. In this study, APE1 was utilized to enhance the specificity of M probe recognition by the released RNA1 sequence produced following Cas12a/crRNA cleavage. The biosensor utilizes the excellent specificity of Cas12a/crRNA and APE1-assisted signal amplification to detect the *mecA* gene. It has a linear detection range of 0.5 fM to 10 nM and a limit of detection of 212 aM. These findings highlight the possible practical uses of this study in the diagnosis of diseases. While the mentioned advantages are indeed valid, it is important to note that the current process still requires manual operation. Therefore, there is growing interest in developing a more advanced and automated approach, such as the integration of a microfluidic chip, for future research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c04790.

Sequences of the oligonucleotides used in present study and fluorescence intensities ratio (F_{ThT}/F_{Blank}) of the

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approach with different complementary bases between domain 1 and domain 2 in M probe (PDF)

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Author Contributions

†F.L. and K.X. contributed equally. The study was conceived by Hua Yang. Fuhuang Lai and Kang Xia, as the co-first author, conducted the lab work and wrote the manuscript. Wei Lin and Fanghua Jian assisted data analysis. All authors approved the submission of the manuscript.

Notes

The authors declare no competing financial interest.

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