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CRISPR-Cas13a based bacterial detection platform: Sensing pathogen *Staphylococcus aureus* in food samples

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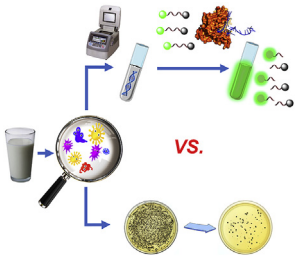
HIGHLIGHTS

- A novel CRISPR-Cas13a based bacterial detection method was developed and named as CCB-Detection.
- The method was capable of *S. aureus* detection in 4.0 h with proved sensitivity and specificity.
- Through the detection of real world samples, it promises to meet needs in practical applications.
- CCB-Detection could be generalised to detect other bacterial targets.

GRAPHICAL ABSTRACT



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ABSTRACT

The severity of foodborne diseases caused by foods contaminated by pathogens or their toxins creates an urgent need for the development of specific and sensitive method for detection of bacteria. In this study, taking advantages of CRISPR-Cas13a system, namely, the crRNA programmability and Cas13a "collateral effect" of promiscuous RNase activity upon target RNA recognition, we developed a bacterial sensing strategy with the name of CCB-Detection (CRISPR-Cas13a based Bacterial Detection). *Staphylococcus aureus* (*S. aureus*) was chosen as a model bacteria for validating the performance of CCB-Detection. Specifically, four steps were carried out: 1) simple extraction of genome DNA; 2) specific gene amplification by PCR; 3) *in vitro* transcription; and 4) the "collateral effect" cleavage of reporter RNA to report the analyte signal. It was observed that CCB-Detection was capable to successfully detect the target genomic DNA (gDNA) as low as 10^0 aM. The limit of detection (LOD) was 1 CFU/mL with a dynamic detection range of *S. aureus* from 10^0 to 10^7 CFU/mL. The entire sample-to-answer time for this biosensor was less than 4 h. CCB-Detection demonstrated satisfactory selectivity for *S. aureus* without interference from other bacteria. Furthermore, CCB-Detection was successfully applied for sensing *S. aureus* in real food samples with both known and unknown amounts bacteria (spiked ones and non-spiked ones) and its performance is comparable to the conventional culture-based counting method but with short assay time and high sensitivity. With desirable reliability, sensitivity, specificity and simplicity, herein proposed CCB-Detection could be extended and generalised for other bacterial detection, and has great potential to

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be used in a wide range of applications such as food safety inspection, disease diagnosis, environment monitoring, *etc*.

1. Introduction

Foodborne diseases, caused by the consumption of foods contaminated by pathogens or their toxins, have been causing huge burdens to our public health [1]. *Staphylococcus aureus* (*S. aureus*) is regarded as one of the most influential foodborne pathogens, which causes food poisoning worldwide due to the production of variety of virulence factors [2,3]. Therefore rapid and sensitive detection of *S. aureus* is essential to food safety. Traditional methods for *S. aureus* detection rely on microbiological culture followed by biomedical tests, though as a "gold standard", it is time-consuming and labor-intense [4].

Microbial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) adaptive immune systems contain programmable endonucleases that can be leveraged for CRISPR-powered diagnostics [5–8]. Cas13a, previously known as C2c2, functions as an RNA-guided RNA-targeting endonuclease [9]. In addition, Cas13a has "collateral effect" of promiscuous RNase activity, which can be activated upon CRISPR RNAs (crRNAs) mediated target RNA recognition [10,11]. Combining the collateral RNase cleavage of Cas13a with isothermal amplification, Zhang group established a CRISPR-based diagnostic platform termed as SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) to successfully detect specific strains of virus with attomolar sensitivity and single-base specificity [5,12], and further developed a multiplexed and portable nucleic acid detection platform termed SHERLOCK v2 [13]. Additionally, CRISPR-Cas13a also has been applied to detect avian influenza A (H7N9) virus [14], Ebola virus [15], Epstein-Barr virus (EBV) [16], porcine reproductive and respiratory syndrome virus [17], African swine fever virus [18], *Salmonella* Enteritidis [19], microRNAs [20], plant genes [21], nucleobase methylation [22] and proteins [23].

However, it is rare to see the detection of prokaryotic bacteria using CRISPR-Cas systems. Herein, we exploited the capability of CRISPR-Cas13a to directly detect *S. aureus* with high sensitivity and selectivity. The *nuc* gene is *S. aureus* species specific (GenBank accession No. CP007447.1). Molecular methods for detecting this gene allow a rapid identification of *S. aureus* in real samples [24]. We chose *nuc* gene as a molecular marker, and with the aid of combined crRNA programmability and Cas13a promiscuous RNase activity, the target genomic DNA (gDNA) was successfully detected as low as 10^0 aM. The dynamic detection range of *S. aureus* was 10^0–10^7 CFU/mL. Furthermore, herein proposed method was successfully applied for detecting *S. aureus* in real food samples with superior performance to the gold-standard culture-based counting method.

2. Experimental

2.1. Materials and reagents

The pC013-Twinstrep-SUMO-huLwCas13a was obtained from Addgene repository (http://www.addgene.org) with plasmid number 90097. *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Salmonella spp.*, *Pichia pastoris*, *Agrobacterium tumefaciens* and HeLa cervical carcinoma cells were stored within the group. Primers, RNA and DNA oligonucleotides (listed in Table 1) were synthesized by Synbio Technologies (China). Baird-Parker agar base was purchased from Hopebio-Technology Co., Ltd. (China). Common chemicals were obtained from Sigma-Aldrich (USA) or Aladdin Biochemical Technology Co., Ltd. (China) unless otherwise specified. They were analytical grade and used without further purification. Distilled deionized water (ddH_2O) was used throughout the investigation. The DNA and RNA concentrations were quantified using 260 nm absorbance by a UV–Vis spectrophotometer with the extinction coefficients calculated using online IDT OligoAnalyzer 3.1 (http://www.idtdna.com/calc/analyzer#).

2.2. Bacteria culture and colony-forming unit (CFU) test

Luria-Bertani (LB) broth medium was used to culture *S. aureus* at 37 °C for 12 h where necessary. All the liquid cultures were shaken at the speed of 160 r/min. The spread plate technique was used for enumerating *S. aureus*. Simply, several serial dilutions were prepared and 100 μL aliquots from each dilution were spread onto Baird-Parker agar base. Bacterial plates were incubated at 37 °C for 48 h. Then colonies were counted and the average numbers of three parallel dishes were recorded.

2.3. Bacteria genomic DNA extraction

Bacterial genomic DNA was extracted with MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0 (TaKaRa Biotechnology) according to the manufacturer's guidelines.

2.4. LwCas13a protein purification

Leptotrichia wadei (Lw) Cas13a bacterial expression vectors were transformed into Rosetta™ 2 (DE3) pLysS competent cells using heat shock method. After inoculation, the LB growth media culture containing 100 mg/mL ampicillin was shaken at 37 °C until an absorbance of 0.6 at 600 nm was reached. At this time, protein expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and the cells were cooled to 16 °C for 24 h for protein expression. Cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C. The cell pellets were collected and stored at −20 °C for later purification. All subsequent steps of the protein purification were performed at 4 °C. Cell pellets were crushed and resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 2 mM DTT, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mg/mL lysozyme followed by sonication (XO-650D, Atpio, Nanjing, China). Lysate was centrifugated for 1 h at 12000 rpm and the supernatant was filtered through a 0.22 μm filter. Filtered supernatant was applied to column containing Ni Sepharose™ 6 Fast Flow (GE) and incubated with rotation for 2 h followed by washing of the protein-bound resin three times using lysis buffer. The target protein was eluted with a stepwise gradient of 50, 100 and 200 mM imidazole. The eluted protein was dialysed against 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and concentrated using Millipore concentrators. For further cation exchange and gel filtration purification, protein was loaded onto a 5 mL SOURCE™ 15S 4.6/100 PE cation exchange column (GE Healthcare Life Sciences) via FPLC (AKTA PURE, GE Healthcare Life Sciences) and eluted over a salt gradient from 0.15 M to 1.5 M NaCl in elution buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). The resulting

Table 1
DNA and RNA oligonucleotides used in this study.

Name	Complete sequence (5′-3′)
T1 crRNA	GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC**UAGAUUGCUGUUCUACCAAGUAAUCCAU**
Non-related T1 crRNA	GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACAACUUGUGGCCGUUUACGUCGCCGUCCA
T1 target RNA	GGGGGCCAGUGAAUUCGAGCUCGGUACCCGGGGAUCCUCUAGAAAU**AUGGAUUACUUGGUAGAACAG CAAUCUA**CUCGACCUGCAGGCAUGCAAGCUUGGCGUAAUCAUGGUCAUAGCUGUUUCCUGUGUU UAUCCGCUCACAAUUCCACACAACAUACGAGCCGGAAGCAUAAAG
S. aureus crRNA	GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC**UAAAUGCACUUGCUUCAGGACCAUAUUU**
S. aureus target RNA	GGGCACCUGAAACAAAGCAUCCUAAAAAAGGUGUAGAG**AAAUAUGGUCCUGAAGCAAGUGCAUUUA** CGAAAAAAAUGGUAGAAAAUGCAAAGAAAAUUGAAGUCGAGUUUGACAAAGGUCAAAGAACU GAUAAAUAUGGACGUGGCUUAGCG
S. aureus target F (for *nuc* gene amplification)	TAATACGACTCACTATAgggCACCTGAAACAAAGCATCCTAAA
S. aureus target R (for *nuc* gene amplification)	CGCTAAGCCACGTCCATATT
S. aureus qPCR F	CACCTGAAACAAAGCATCCTAAA
S. aureus qPCR R	CGCTAAGCCACGTCCATATT
Reporter RNA	6-FAM-UUGGCGUAAUCAUGGUCAUA-BHQ1

Note: F = forward primer; R = reverse primer. The bolded part is the hybridisation part between target RNA and its respective crRNA; The T7 promoter is underlined; ggg is required for T7 transcription.

fractions were tested for presence of LwCas13a by SDS-PAGE, and fractions containing the protein were pooled and concentrated via Millipore concentrator. The concentrated protein was loaded onto a gel filtration column (Superdex® 200 Increase 10/300 GL, GE Healthcare Life Sciences) via FPLC. The eluted protein was dialysed against buffer (50 mM Tris-HCl, 600 mM NaCl, 10% glycerol, 2 mM DTT, pH 7.5) and concentrated. The protein concentration was measured based on Lambert-Beer law and frozen at −80 °C for further usage.

2.5. Preparation of crRNA and target RNA

For preparation of crRNAs for *S. aureus* detection, a single-stranded cognate DNA oligonucleotide with T7 promoter sequence was annealed with a primer sequence having a complementary T7 primer sequence (final concentrations 10 μM), which was then transcribed to crRNA using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). This transcription reaction was carried out at 37 °C overnight. Subsequently, the product crRNA was purified using Monarch® RNA Cleanup Kit (New England Biolabs). *Nuc* gene amplification from gDNA of *S. aureus* was carried out by conventional PCR using Taq PCR MasterMix II (Tiangen Biotech.), which was subsequently collected for target RNA preparation using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). This transcription reaction contained 4 μL PCR product, 1 μL T7 polymerase, 1 mM NTP Mix to reach a 30 μL reaction, which was incubated for 1 h at 37 °C followed by a 10 min heating at 75 °C to denature the RNA polymerase.

2.6. Feasibility analysis of LwCas13a for nucleic detection

A target, named as T1, was synthesized as DNA form and transcribed into RNA. crRNA was designed and transcribed. The sequences of T1 target RNA and crRNA were shown in Table 1. The RNA reporter was labelled by 5′ 6-FAM (Fluorescein) fluorophore and 3' BHQ1. The detection assays were performed with 45 nM purified LwCas13a, 25 nM crRNA, 100 nM quenched fluorescent RNA reporter, 2 μL murine RNase inhibitor, and 1 mM target RNA, unless otherwise indicated, in a 100 μL reaction buffer with 40 mM Tris-HCl, 60 mM NaCl, 6 mM MgCl2, pH 7.3. Assays were carried out at 37 °C and kinetic data were recorded in a Tecan Infinite® 200 Pro plate reader (Tecan, Switzerland) with excitation and emission wavelengths at 484 nm and 529 nm respectively and fluorescent intensities were measured every 30 s.

2.7. Standard curves, sensitivity and specificity of CCB-Detection for sensing S. aureus

The extracted genomic DNA was used as a template for setting up the standard curve for CCB-Detection for sensing *S. aureus*. Assays were performed by using 45 nM purified LwCas13a, 25 nM crRNA, 100 nM quenched fluorescent RNA reporter, 2 μL murine RNase inhibitor (New England Biolabs), and varying concentrations of input nucleic acid target, in a 100 μL reaction buffered with 40 mM Tris-HCl, 60 mM NaCl, 6 mM $MgCl_2$, pH 7.3. Assays were carried out at 37 °C and kinetic data were recorded in a Tecan Infinite® 200 Pro plate reader (Tecan, Switzerland) with excitation and emission wavelengths at 484 nm and 529 nm respectively and fluorescent intensities were measured every 30 s. The different bacterial concentrations expressed as ($\log^{CFU/mL}$) were plotted against the corresponding fluorescent values [25]. To test the specificity of the system, Genome DNA, extracted from six different organisms including bacteria, fungi and human cells separately, was amplified and transcribed using *S. aureus* primers, followed by CCB-Detection. Each data point was tested in triplicate. The LOD was defined as the lowest detectable quantities of bacteria.

2.8. Detection of S. aureus in food samples using proposed CCB-Detection

Each sample of milk, juice, beer, and water was made a 10 times dilution using double distilled H_2O without other pre-treatment. Then, these confirmed *S. aureus* negative samples were spiked with different amount of *S. aureus* cells (density calculated using traditional plate counting method). The spiked samples were centrifuged at 12000 rpm for 2 min and the bacteria pellets were firstly resuspended in TEX buffer (10 mM Tris-HCl, 1 mM EDTA and 1% TritonX-100, pH 8.0) with 200 μg/mL of lysostaphin and lysozyme. The mixture was incubated at 37 °C for 0.5 h. Proteinase K (final concentration 0.5 mg/mL) and RNase A (final concentration 0.1 mg/mL) were added to each sample mixture and incubated at 56 °C for 30 min. Finally, each sample was incubated at 95 °C for 10 min to denature the enzyme, which was used subsequently for the CCB-Detection. Commercial milk samples were exposed to routine environment condition and 2 mL samples were taken every 24 h in a 5-day time slot. A part of each sample was diluted with saline, and the appropriate dilution was applied to the plate with

Baird-Parker agar base. The plates were incubated at 37 °C for 48 h. Then the typical *S. aureus* colonies were counted and the average numbers of three parallel dishes were recorded. The rest of each sample was tested using the CCB-detection, and the detection process was identical to the above-mentioned CCB-detection for testing *S. aureus* spiked samples.

2.9. Comparison with conventional culture-based counting, chromogenic petrifilm test, qPCR and fluorescent visualisation method for S. aureus detection

For conventional culture-based counting of *S. aureus*, the bacteria were cultured in LB (Luria-Bertani) broth medium for 12 h. The cell suspension was subjected to gradient dilution. 100 μL of each dilution was plated on Baird-Parker agar base in duplicate, followed by incubation at 37 °C for 48 h to allow the bacteria to grow. Colonies were counted and the average numbers of three parallel dishes were recorded. For MicroFast®2 SA petrifilm detection, 1 mL of each dilution was plated on the petrifilm (Longrun Bio-Tech, China) according to the manufacturer's protocols, followed by incubation at 37 °C for 24 h to allow the bacteria to grow. For qPCR experiments, the genomic DNA extraction step was exactly the same as that used for CCB-Detection in the above section (2.3 Bacteria genomic DNA extraction). Each reaction was performed in a 20 μL mixture containing 10 μL 2 × SYBR Green qPCR Master Mix (Bimake.com, USA), 0.5 μL gDNA of *S. aureus* (template), 0.4 μL 50 × ROX reference dye and 200 nM of each primer. The cycling protocol was 95 °C for 20 min, 40 cycles of denaturation (95 °C, 3 s), and annealing and extension (60 °C, 30 s), using an Applied Biosystems StepOne™ Real-Time PCR System. The different bacterial concentrations (expressed as $\log^{CFU}$) were plotted against the corresponding Cq values. A negative control using water to replace DNA template was included.

2.10. Statistical analysis

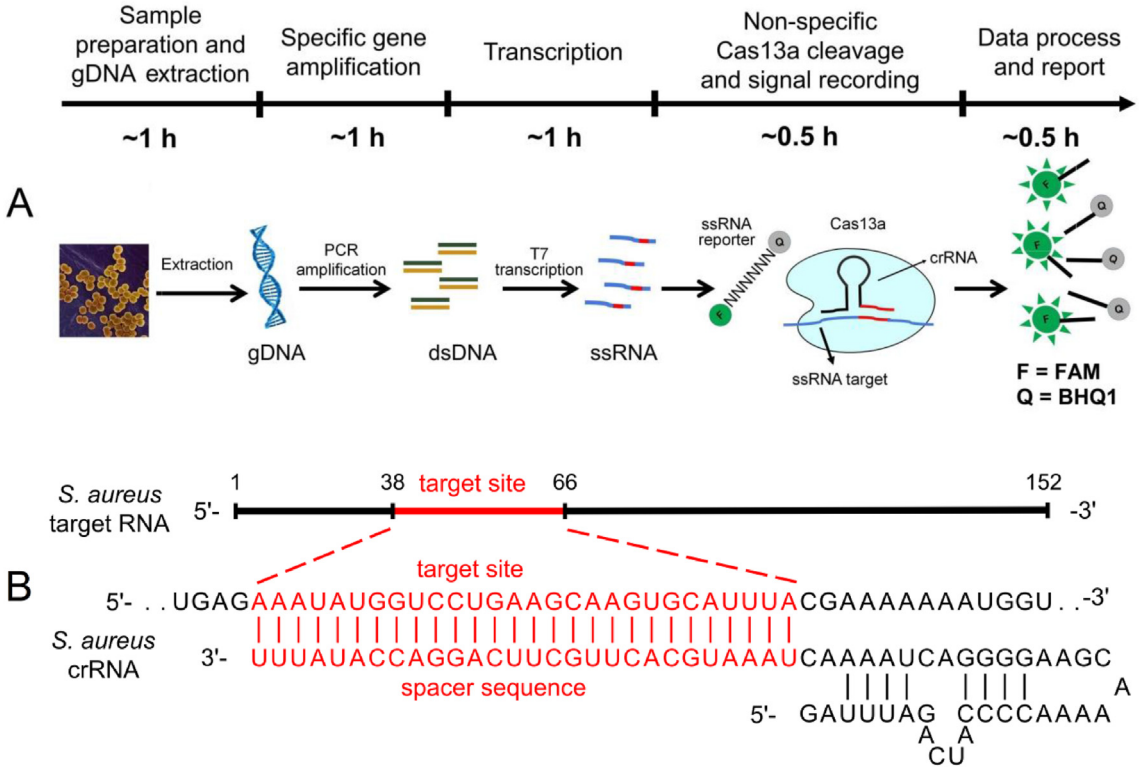
The values obtained in the triplicated experiments were expressed as the mean ± standard deviation (SD) and analysed by Student's t-test if necessary. All statistical analyses were performed using SPSS 17.0 software and $P < 0.05$ was considered statistically significant.

3. Results and discussions

3.1. Application of the proposed CCB-Detection for bacterial detection

In the first place, we proposed a sensing strategy using CRISPR-Cas13a biosensing system for detection of the model analyte *S. aureus*. Scheme 1 depicted the steps and estimated timing for this method. The species-specific *S. aureus nuc* gene (whole length 694 bp) was chosen as the target gene sequence because it encodes thermonuclease and widely used as a specific target for the identification of *S. aureus*. T7 transcription was then carried out to transcribe double-stranded DNA (dsDNA) amplicon to single-stranded RNA (ssRNA); the latter was then subjected to Cas13a recognition and the collateral RNase cleavage of a pre-added doubly labelled ssRNA was finally achieved resulting in dequenching of FAM fluorophore from BHQ1 (black hole quencher 1) to generate significantly enhanced fluorescence. The change of fluorescence intensity could be readily recorded and processed for quantification of the analyte. The whole detection process was done within 4.0 h. The purified Cas13a protein was shown in Figure SI-1. The extraction of gDNA of *S. aureus* and the preparation of crRNA were shown in Figure SI-2.

In order to verify this, we firstly tested the feasibility and specificity of LwCas13a. A target, named as T1, was synthesized as



Scheme 1. Proposed CCB-Detection for *S. aureus* sensing (A) and the schematic of ssRNA target and crRNA used in this study (B). The target site is highlighted in red.

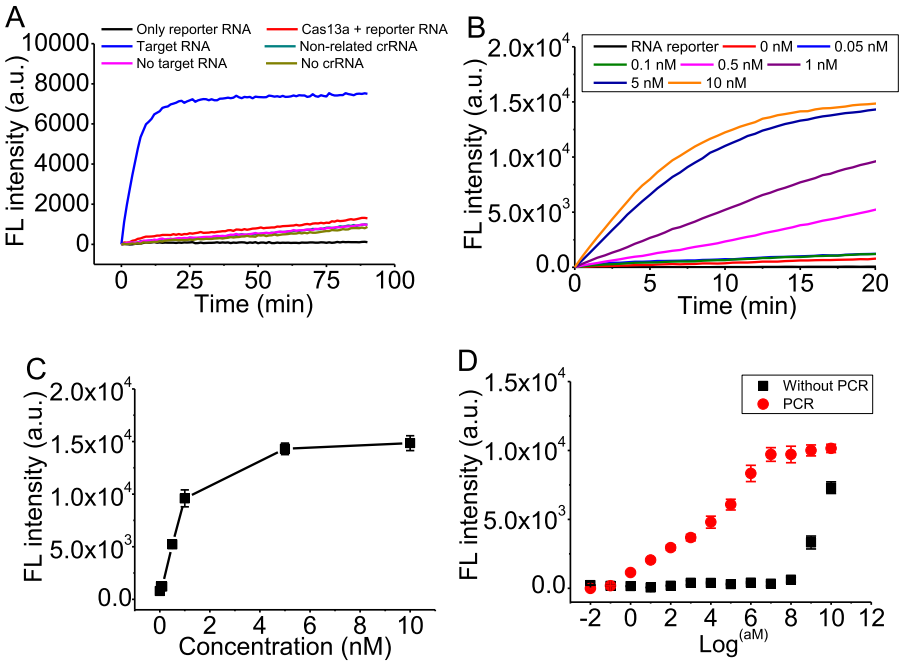


Fig. 1. Proof-of-principle for CCB-Detection for *S. aureus* sensing. A: Feasibility assessment of CCB-Detection. The RNA reporter was labelled by 5′ 6-FAM (Fluorescein) fluorophore and 3′ BHQ1. The detection assays were performed with 45 nM purified LwCas13a, 25 nM crRNA, 100 nM quenched fluorescent RNA reporter, 2 μL murine RNase inhibitor, and 1 mM target RNA, unless otherwise indicated, in a 100 μL reaction buffer. B: The real time fluorescence recording for CRISPR-Cas13a cleavage upon different concentrations of input target RNA. C: The fluorescence intensities of FAM dye under different concentrations of target input RNA (data were extracted from Fig. 1B). D: The fluorescent intensities for CRISPR-Cas13a cleavage with and without initial PCR amplification. n = 3 technical replicates; error bars represent mean ± SD.

DNA form and transcribed into RNA. crRNA was designed and transcribed. The sequences of T1 target RNA and crRNA were shown in Table 1. The RNA reporter was labelled by 5′ 6-FAM (Fluorescein) fluorophore and 3' BHQ1. As shown in Fig. 1A, in the absence of correct crRNA or target RNA, the readout stayed almost unchanged at the time of investigation; in contrast, only upon the right target RNA and crRNA, the signals of Cas13a mediated cleavage was triggered, generating significantly increased fluorescent signal (FL). It was also confirmed that there was a target RNA concentration-dependent signal increment for Cas13a collateral detection, indicative of capability for sensing (Fig. 1B and C). Additionally, the conventional PCR method could be used for initial gene amplification for sensing *nuc* gene of *S. aureus*. However, when Cas13a was integrated, it enabled a highly sensitive detection of *nuc* gene. As shown in Fig. 1D, with the aid of PCR, the LOD would reach 10^0 aM DNA; while the detection devoid of PCR amplification only allowed a LOD of 10^9 aM DNA. This remarkable difference further emphasized the necessity of initial gene amplification by PCR. Thus CRISPR-Cas13a biosensing system integrated with PCR amplification is more sensitive than CRISPR-Cas13a alone for detection of RNA.

3.2. Specific and sensitive detection of foodborne bacterial pathogen S. aureus

Next, we endeavored to use this CCB-Detection for detecting *S. aureus*. The relationship between fluorescence intensity and the concentrations of *S. aureus* was tested (Fig. 2). With incremental amounts of *S. aureus*, more ssRNA can be eventually obtained and thus more Cas13a ssRNA collateral cleavage was activated resulting in a significant increase in fluorescence, evidenced by the 529 nm emission fluorescence of FAM labels (excited at 484 nm). Fig. 2A showed that changes in fluorescence upon different concentrations of *S. aureus*. When plotting the fluorescence intensities against the

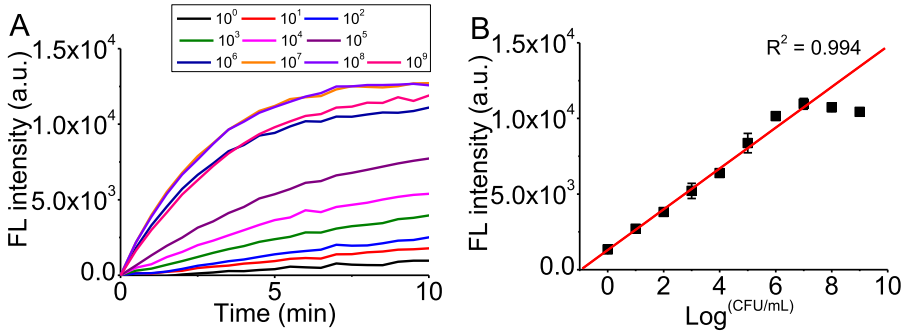


Fig. 2. The detection utility of the proposed CCB-Detection for *S. aureus* sensing. A: Fluorescence recording for *S. aureus* samples with different concentrations. B: The dependence of fluorescence (t = 10 min) on *S. aureus* concentrations and the linear relationship between fluorescence intensity and *S. aureus* concentration (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6,10^7 CFU/mL). n = 3 technical replicates; error bars represent mean ± SD.

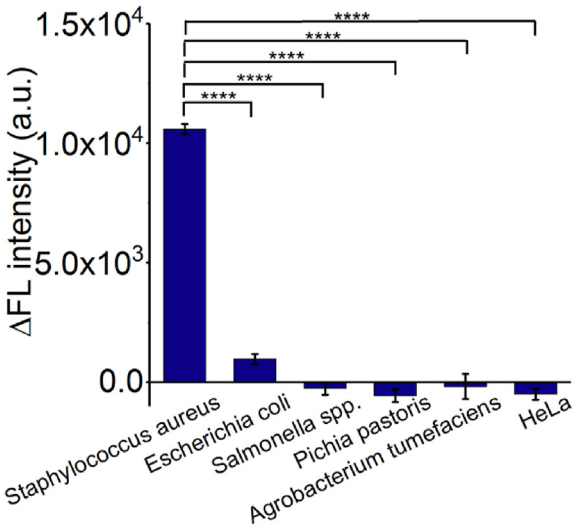


Fig. 3. Selectivity of proposed CCB-Detection for *S. aureus* sensing over other common cells. The data were obtained by triplicated experiments. ΔFL intensity = FL of each sample tested by CCB-Detection - FL of sample without target RNA. Please note that 10^4 CFU/mL concentration was adopted for each type of cells in this test. n = 3 technical replicates; two-tailed Student's t-test; ****p < 0.0001; bars represent mean ± SD.

concentrations of added *S. aureus*, a linear relationship ($R^2 = 0.994$) was obtained over the range from 10^0 to 10^7 CFU/mL (Fig. 2B). Based on the method adopted by Zhang group [5], the fluorescence intensities between 1 CFU/mL and 0 CFU/mL were compared, and a significantly statistical difference ($p < 0.0001$) was observed (Figure SI-3), and thus a 1 CFU/mL limit of detection was obtained.

The selectivity for *S. aureus* over some other common prokaryotic and eukaryotic cells was investigated. Fig. 3 presented the histograms of fluorescence intensity in solutions of *S. aureus* and other cell culture samples. It was observed that other interference (both prokaryotic and eukaryotic cells) at a five-fold higher concentration than *S. aureus*, did not show any influence to the assay based on the almost unchanged fluorescence intensities. Thus these competing cells only had a negligible effect on our detection system. This approach exhibited high selectivity for *S. aureus,* which enabled its scope for future use.

There is a difference of the analytical signal obtained for a concentration of 10^4 CFU/mL presented in Figs. 2 and 3. It may be caused by the different amplification cycles when preparing the target. In order to get enough template, PCR amplification cycles in Fig. 3 were more than those in other experiments. So, there is a higher fluorescence intensity in Fig. 3 for a concentration of 10^4 CFU/mL.

Table 2
Results of CCB-Detection for detection of *S. aureus* in some spiked diary food samples.

Samples (CFU/mL) (Mean ± SD)				
Traditional culture method		$5.0 \times 10^5 \pm 2.0 \times 10^4$	$5.0 \times 10^3 \pm 2.0 \times 10^2$	$5.0 \times 10^1 \pm 2.0 \times 10^0$
CCB-Detection	beer	$4.7 \times 10^5 \pm 5.7 \times 10^4$ (p = 0.6014)	$4.9 \times 10^3 \pm 7.4 \times 10^2$ (p = 0.8647)	$4.3 \times 10^1 \pm 9.3 \times 10^0$ (p = 0.4146)
	juice	$4.1 \times 10^5 \pm 3.8 \times 10^4$ (p = 0.1241)	$4.7 \times 10^3 \pm 6.3 \times 10^1$ (p = 0.3389)	$4.1 \times 10^1 \pm 0.2 \times 10^0$ (p = 0.0576)
	milk	$4.2 \times 10^5 \pm 5.6 \times 10^3$ (p = 0.0596)	$4.5 \times 10^3 \pm 1.3 \times 10^2$ (p = 0.1615)	$4.3 \times 10^1 \pm 8.1 \times 10^0$ (p = 0.3764)
	water	$4.6 \times 10^5 \pm 2.0 \times 10^4$ (p = 0.2789)	$4.8 \times 10^3 \pm 1.7 \times 10^2$ (p = 0.4376)	$4.8 \times 10^1 \pm 0.3 \times 10^0$ (p = 0.4609)

A

	Samples (CFU/mL) (Mean ± SD)			
	1st	3rd	4th	5th
Traditional culture method	ND	$9.3\times10^2 \pm 1.4\times10^1$	$1.2\times10^3 \pm 1.1\times10^2$	$1.8\times10^3 \pm 5.6\times10^1$
CCB-Detection	ND	$9.2\times10^2 \pm 4.1\times10^1$ (p=0.4667)	$1.1\times10^3 \pm 1.1\times10^2$ (p=0.5487)	$1.6\times10^3 \pm 1.9\times10^2$ (p=0.3097)

B

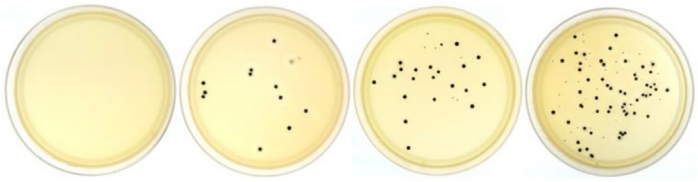


Fig. 4. Detection of *S. aureus* in contaminated sour milk samples (with unknown bacteria) by using CCB-Detection. A: the results of CCB-Detection. B: sour milk samples subject to conventional culture-based counting method. ND = not determined. For Fig. 4B and 100 μL bacterial culture was spread onto each plate. The calculation of concentration of S. aureus would be colonies numbers/100 μL, which equals to 10 × colonies numbers/mL. The day 1 to day 5 denotes that how many days the contaminated milk has been kept.

3.3. The use of proposed CCB-Detection for detection of S. aureus in real food samples

On the basis of the success of using CCB-Detection for sensing *S. aureus* contaminated samples in ddH_2O, the application of this system was further explored for detection of *S. aureus* in real food samples. The beer, juice, milk and water samples were used for testing. All these *S. aureus* negative samples were spiked with *S. aureus* at a series of concentrations. For comparison, concentrations of added *S. aureus* were obtained using traditional plate counting method which is a "gold standard" method. As shown in Table 2, CCB-Detection was competent in detection of *S. aureus* in sour milk samples with unknown amount of bacteria (Fig. 4), and the data obtained by CCB-Detection were not significantly different ($P > 0.05$) from the data derived from conventional culture-based counting method, suggesting our developed method was successful to detect *S. aureus* in real food samples.

3.4. Comparison of CCB-Detection with other routine methods

Finally, a side-by-side comparison was performed in order to compare the proposed CCB-Detection with other well-proved ones. The *S. aureus* contaminated water samples were used in this study. Parallel prepared water samples with *S. aureus* concentrations from 10^5 CFU/mL to 10^0 CFU/mL were subjected to analysis. As shown in Fig. 5A and B, numbers of colonies were formed on the bacteria positive samples, which agreed well with the initial concentrations of the pathogen *S. aureus*. The real time quantitative PCR (qPCR) was also applied, which demonstrated a detection limit of 10^4 CFU/mL and the linear range spanning from 10^5 to 10^9 CFU/mL (Fig. 5C). Bacteria positive samples were detected by CCB-Detection and the levels of *S. aureus* infection were directly obtained based on the fluorescence readout (Fig. 5D). The total assay time of CCB-Detection was within 4.0 h, which is another advantage since the operation of culture-based counting requires at least 48 h. Even if the qPCR method was able to provide results in hours, the sensitivity was less satisfactory. Additionally, the proposed method was compared with previously reported assays (Tables SI–1), and it shows CDC-Detection provided better performance in terms of LOD and detection linear range. In summary, this novel CCB-Detection demonstrated superior performance in sensitivity, specificity, detection time and ease of use.

For bacterial pathogen detection, plate counting and PCR are both classic methods [26,27]; and also serve as national food inspection standards worldwide. Despite being highly reliable and robust, the time-consuming operation process, the requirement of specialised equipment and trained professionals usually restrict their applications in rapid and on-site detection. In this regard, our proposed CCB-Detection could be a fine complementary or alternative method to the above traditional methods. Moreover, as a novel core of detection, this strategy could be put to the "tool box" as a "module" for analysts, which can be further coupled with other well established methods in a "plugged in" manner. For example, CRISPR-Cas13a could be integrated with other nucleic acid amplification techniques such as RPA (recombinase polymerase amplification), LAMP (loop-mediated isothermal amplification) and RCA (rolling circle amplification) as the versatile replacement to PCR, which could facilitate instrument-free on-site detection [28,29]. Besides, CCB-Detection could be assembled with some fast and/or visual detection techniques to translate "bacterial signals" to colorimetric [30], electrochemical signals [31,32], lateral flow strip readouts [21] or integrated with microfluidic biosensor [33].

3.5. The rational choice of Cas13a instead of Cas12a

We then ask why we chose Cas13a instead of Cas12a in CDC-Detection? Cas12a is another CRISPR effector, which takes dsDNA or ssDNA as substrate and has been developed for biosensing previously without the need of transcription process [38]. Reasons to select Cas13 are 1) based on our experimental data, we found that in terms of *trans*-cleavage/collateral cleavage, Cas13a was more active than Cas12a, suggesting that it resulted in more fluorescence enhancement than Cas12a. This Cas13a has a larger linear range (Δ fluorescence) and a better signal-to-background ratio (S/BG) for detection. 2) adding the transcription process in herein study using Cas13, was not seemingly a tedious step, but had its advantage. During this transcription process, the PCR amplicon were amplified again (the *nuc* gene was amplified twice by PCR and transcription steps) resulting in a more sensitive detection and a lower LOD. 3) for comparison, Cas12a was used to detect *S. aureus*. The LOD (limit

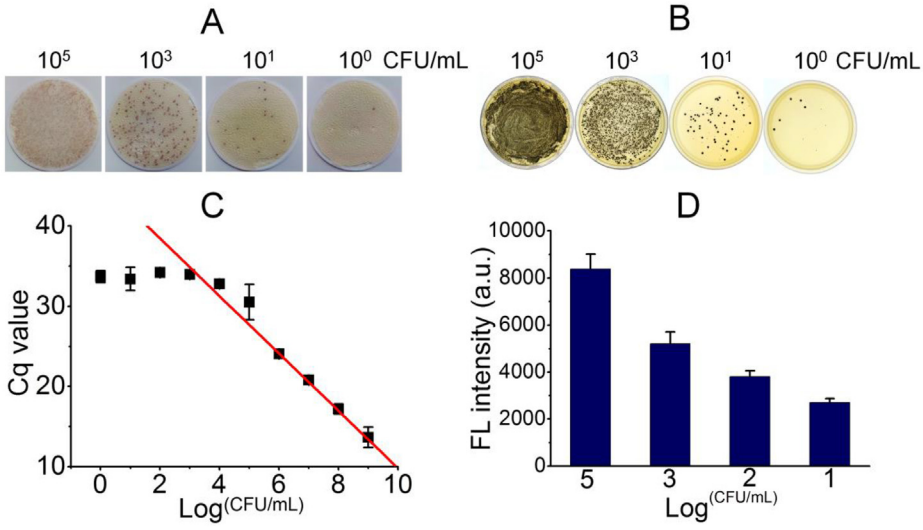


Fig. 5. The comparison of proposed CCB-Detection with other well-established methods for detection of *S. aureus*. A: chromogenic petrifilm test. The cell suspension was subjected to gradient dilution. 1 mL of each dilution was plated on the petrifilm followed by incubation at 37 °C for 48 h. B: conventional culture-based counting method. Each dilution (100 μL) was spread onto Baird-Parker agar base followed by incubation at 37 °C for 48 h. C: qPCR method. The different bacterial concentrations (expressed as $\log^{(CFU/mL)}$) were plotted against the corresponding Cq values. n = 3 technical replicates; bars represent mean ± SD. D: CCB-Detection. n = 3 technical replicates; bars represent mean ± SD.

of detection) of *S. aureus* for Cas13a based method was 1 CFU/mL, while the LOD for Cas12a based method was 10^3 CFU/mL, which will be reported in a separate study. Cas13a was far better than Cas12a in terms of detection sensitivity. The sensitivity is crucial for *S. aureus* detection since the range for *S. aureus* is usually below from 10 to 10^4 CFU/mL [39] although different countries have different regulations in term of the concentrations of *S. aureus*. This reinforces the needs to develop highly sensitive detection methods. To meet this sensitivity requirement, a Cas13a based method with a 1 CFU/mL LOD was selected in herein study. 4) we have noticed that the CRISPR type III effector nuclease Csm6 could be leveraged, which is activated by cyclic adenylate molecules or linear adenine homopolymers terminated with a 2′,3′-cyclic phosphate. According to the work done by Zhang group, by combing the Cas13a with Csm6, the *trans*-cleavage signals were enhanced by more than 8 folds, leaving a larger increase for detection [13]. Therefore, if using Cas13a, the *trans*-cleavage signals could be further enlarged with Csm6, which could not be achieved by Cas12a. Thus by sacrificing one additional step of transcription, Cas13a was desirable for this study in order to achieve the critical sensitivity. Therefore, our selection of Cas13a for herein CDC-Detection is the result of careful consideration of all factors in terms of sensitivity, selectivity, assay time et al.

4. Conclusions

In summary, we have successfully developed the CCB-Detection for sensing bacterial evidenced by specific and sensitive detection of *S. aureus.* This method benefits from the striking sequence recognition capability of Cas13a-crRNA complex and the subsequent incurs promiscuous RNase activity of Cas13a nuclease. The former can be utilised for gene sequence identification and the latter can be adopted for generating readout using a fluorophore-quencher doubly labelled ssRNA molecule. This enables the highly specific and sensitive detection of DNA. As we know, genetic information is contained in the nucleotide sequence of DNA (and sometimes RNA). Therefore the detection of DNA is crucial for identification and quantification of microorganisms of interest. With the help of conventional nucleic acid amplification technology such as PCR, this method could be used for bacterial sensing in a wide concentration range with a low limit of detection. Though there is intensive research in nano-, aptamer-based biosensors [34,35], the pursuit of novel detection strategy has always been underway. CRISPR-Cas biology promises rapid, accurate, portable and multiplexed diagnostic tools [36,37]. In conclusion, our current work would enrich the reach of CRISPR-Cas biology based detection [40–46]. Although there are many other methods for bacterial sensing [47–50], our Cas13a based method has demonstrated its advantages. Also, with proved reliability, sensitivity, specificity and simplicity, our proposed method could be extended and generalised for other bacterial detection, promising to win applications in a wide range of areas such as food safety inspection, disease diagnosis, environment monitoring, *etc.*

CRediT authorship contribution statement

Jin Zhou: Methodology, Investigation, Data curation, Writing - original draft. **Lijuan Yin:** Investigation, Methodology, Formal analysis, Validation. **Yanan Dong:** Conceptualization, Writing - review & editing. **Lei Peng:** Software. **Guozhen Liu:** Writing - review & editing. **Shuli Man:** Supervision, Project administration, Methodology. **Long Ma:** Conceptualization, Project administration, Supervision, Writing - original draft, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.06.041.

References

[1] T. Martinović, U. Andjelković, M.Š. Gajdošik, D. Rešetar, D. Josić, Foodborne pathogens and their toxins, J. Proteomics 147 (2016) 226–235.
[2] M.M. Dinges, P.M. Orwin, P.M. Schlievert, Exotoxins of Staphylococcus aureus, Clin. Microbiol. Rev. 13 (2000) 16–34.
[3] D. Oliveira, A. Borges, M. Simões, Staphylococcus aureus toxins and their molecular activity in infectious diseases, Toxins 10 (2018) 252.
[4] B. Priyanka, R.K. Patil, S. Dwarakanath, A review on detection methods used for foodborne pathogens, Indian J. Med. Res. 144 (2016) 327–338.
[5] J.S. Gootenberg, O.O. Abudayyeh, J.W. Lee, P. Essletzbichler, A.J. Dy, J. Joung, V. Verdine, N. Donghia, N.M. Daringer, C.A. Freije, C. Myhrvold, R.P. Bhattacharyya, J. Livny, A. Regev, E.V. Koonin, D.T. Hung, P.C. Sabeti, J.J. Collins, F. Zhang, Nucleic acid detection with CRISPR-Cas13a/C2c2, Science 356 (2017) 438–442.
[6] Y. Li, S. Li, J. Wang, G. Liu, CRISPR/Cas systems towards next-generation biosensing, Trends Biotechnol. 37 (2019) 730–743.
[7] A. Pickar-Oliver, C.A. Gersbach, The next generation of CRISPR–Cas technologies and applications, Nat. Rev. Mol. Cell Biol. 20 (2019) 490–507.
[8] X. Zuo, C. Fan, H.-Y. Chen, Biosensing: CRISPR-powered diagnostics, Nat. Biomed. Eng. 1 (2017), 0091.
[9] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, J. Joung, I.M. Slaymaker, D.B.T. Cox, S. Shmakov, K.S. Makarova, E. Semenova, L. Minakhin, K. Severinov, A. Regev, E.S. Lander, E.V. Koonin, F. Zhang, C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector, Science 353 (2016) aaf5573.
[10] A. East-Seletsky, M.R. O'Connell, S.C. Knight, D. Burstein, J.H. Cate, R. Tjian, J.A. Doudna, Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection, Nature 538 (2016) 270–273.
[11] Q. Wang, X. Liu, J. Zhou, C. Yang, G. Wang, Y. Tan, Y. Wu, S. Zhang, K. Yi, C. Kang, The CRISPR-cas13a gene-editing system induces collateral cleavage of RNA in glioma cells, Adv. Sci. 6 (2019) 1901299.
[12] M.J. Kellner, J.G. Koob, J.S. Gootenberg, O.O. Abudayyeh, F. Zhang, SHERLOCK: nucleic acid detection with CRISPR nucleases, Nat. Protoc. 14 (2019) 2986–3012.
[13] J.S. Gootenberg, O.O. Abudayyeh, M.J. Kellner, J. Joung, J.J. Collins, F. Zhang, Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6, Science 360 (2018) 439–444.
[14] Y. Liu, H. Xu, C. Liu, L. Peng, H. Khan, L. Cui, R. Huang, C. Wu, S. Shen, S. Wang, CRISPR-Cas13a nanomachine based simple technology for avian influenza A (H7N9) virus on-site detection, J. Biomed. Nanotechnol. 15 (2019) 790–798.
[15] P. Qin, M. Park, K.J. Alfson, M. Tamhankar, R. Carrion, J.L. Patterson, A. Griffiths, Q. He, A. Yildiz, R. Mathies, K. Du, Rapid and fully microfluidic Ebola virus detection with CRISPR-cas13a, ACS Sens. 4 (2019) 1048–1054.
[16] Y. Wu, S.-X. Liu, F. Wang, M.-S. Zeng, Room temperature detection of plasma epstein–barr virus DNA with CRISPR–cas13, Clin. Chem. 65 (2019) 591–592.
[17] Y. Chang, Y. Deng, T. Li, J. Wang, T. Wang, F. Tan, X. Li, K. Tian, Visual detection of porcine reproductive and respiratory syndrome virus using CRISPR-Cas13a, Transbound. Emerg. Dis. (2019) 1–8, 00.
[18] Q. He, D. Yu, M. Bao, G. Korensky, J. Chen, M. Shin, J. Kim, M. Park, P. Qin, K. Du, High-throughput and all-solution phase African Swine Fever Virus (ASFV) detection using CRISPR-Cas12a and fluorescence based point-of-care system, Biosens. Bioelectron. 154 (2020) 112068.
[19] J. Shen, X. Zhou, Y. Shan, H. Yue, R. Huang, J. Hu, D. Xing, Sensitive detection of a bacterial pathogen using allosteric probe-initiated catalysis and CRISPR-Cas13a amplification reaction, Nat. Commun. 11 (2020) 267.
[20] Y. Shan, X. Zhou, R. Huang, D. Xing, High-fidelity and rapid quantification of

miRNA combining crRNA programmability and CRISPR/Cas13a trans-cleavage activity, Anal. Chem. 91 (2019) 5278–5285.
[21] O.O. Abudayyeh, J.S. Gootenberg, M.J. Kellner, F. Zhang, Nucleic acid detection of plant genes using CRISPR-cas13, CRISPR J 2 (2019) 165–171.
[22] Y. Chen, S. Yang, S. Peng, W. Li, F. Wu, Q. Yao, F. Wang, X. Weng, X. Zhou, N1-Methyladenosine detection with CRISPR-Cas13a/C2c2, Chem. Sci. 10 (2019) 2975–2979.
[23] Q. Chen, T. Tian, E. Xiong, P. Wang, X. Zhou, CRISPR/Cas13a signal amplification linked immunosorbent assay for femtomolar protein detection, Anal. Chem. 92 (2020) 573–577.
[24] O. Brakstad, K. Aasbakk, J. Maeland, Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene, J. Clin. Microbiol. 30 (1992) 1654–1660.
[25] L. Ma, G. Wu, Y. Li, P. Qin, L. Meng, H. Liu, Y. Li, A. Diao, A reversible metal ion fueled DNA three-way junction molecular device for "turn-on and-off" fluorescence detection of mercury ions (II) and biothiols respectively with high selectivity and sensitivity, Nanoscale 7 (2015) 18044–18048.
[26] H. Li, H. Xin, S.F.Y. Li, Multiplex PMA–qPCR Assay with internal amplification control for simultaneous detection of viable Legionella pneumophila, Salmonella typhimurium, and Staphylococcus aureus in environmental waters, Environ. Sci. Technol. 49 (2015) 14249–14256.
[27] S. Song, X. Wang, K. Xu, G. Xia, X. Yang, Visualized detection of Vibrio parahaemolyticus in food samples using dual-functional aptamers and cut-assisted rolling circle amplification, J. Agric. Food Chem. 67 (2019) 1244–1253.
[28] K. Pardee, A.A. Green, M.K. Takahashi, D. Braff, G. Lambert, J.W. Lee, T. Ferrante, D. Ma, N. Donghia, M. Fan, N.M. Daringer, I. Bosch, D.M. Dudley, D.H. O'Connor, L. Gehrke, J.J. Collins, Rapid, low-cost detection of zika virus using programmable biomolecular components, Cell 165 (2016) 1255–1266.
[29] W. Zhou, L. Hu, L. Ying, Z. Zhao, P.K. Chu, X.-F. Yu, A CRISPR–Cas9-triggered strand displacement amplification method for ultrasensitive DNA detection, Nat. Commun. 9 (2018) 5012.
[30] K. Tram, P. Kanda, B.J. Salena, S. Huan, Y. Li, Translating bacterial detection by DNAzymes into a litmus test, Angew. Chem. Int. Ed. 53 (2014) 12799–12802.
[31] Y. Dai, R.A. Somoza, L. Wang, J.F. Welter, Y. Li, A.I. Caplan, C.C. Liu, Exploring the trans-cleavage activity of CRISPR Cas12a (cpf1) for the development of a universal electrochemical biosensor, Angew. Chem. Int. Ed. 58 (2019) 17399–17405.
[32] Y. Xiang, Y. Lu, Using personal glucose meters and functional DNA sensors to quantify a variety of analytical targets, Nat. Chem. 3 (2011) 697–703.
[33] R. Bruch, J. Baaske, C. Chatelle, M. Meirich, S. Madlener, W. Weber, C. Dincer, G. Urban, CRISPR/Cas13a-Powered electrochemical microfluidic biosensor for nucleic acid amplification-free miRNA diagnostics, Adv. Mater. 31 (2019) 1905311.
[34] L. Ma, N. Sun, C. Tu, Q. Zhang, A. Diao, Design of an aptamer – based fluorescence displacement biosensor for selective and sensitive detection of kanamycin in aqueous samples, RSC Adv. 7 (2017) 38512–38518.
[35] L. Ma, N. Sun, J. Zhang, C. Tu, X. Cao, D. Duan, A. Diao, S. Man, Polyethylenimine-coated Fe_3O_4 nanoparticles effectively quench fluorescent DNA, which can be developed as a novel platform for protein detection, Nanoscale 9 (2017) 17699–17703.
[36] D.S. Chertow, Next-generation diagnostics with CRISPR, Science 360 (2018) 381–382.
[37] Y. Li, L. Liu, G. Liu, CRISPR/Cas multiplexed biosensing: a challenge or an insurmountable obstacle? Trends Biotechnol. 37 (2019) 792–795.
[38] J.S. Chen, E. Ma, L.B. Harrington, M. Da Costa, X. Tian, J.M. Palefsky, J.A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (2018) 436–439.
[39] N.H. Kim, N.Y. Lee, M.G. Kim, H.W. Kim, T.J. Cho, I.S. Joo, E.J. Heo, M.S. Rhee, Microbiological criteria and ecology of commercially available processed cheeses according to the product specification and physicochemical characteristics, Food Res. Int. 106 (2018) 468–474.
[40] K. Guk, J.O. Keem, S.G. Hwang, H. Kim, T. Kang, E.-K. Lim, J. Jung, A facile, rapid and sensitive detection of MRSA using a CRISPR-mediated DNA FISH method, antibody-like dCas9/sgRNA complex, Biosens. Bioelectron. 95 (2017) 67–71.
[41] S.-Y. Li, Q.-X. Cheng, J.-M. Wang, X.-Y. Li, Z.-L. Zhang, S. Gao, R.-B. Cao, G.-P. Zhao, J. Wang, CRISPR-Cas12a-assisted nucleic acid detection, Cell Discov 4 (2018) 20.
[42] M. Liang, Z. Li, W. Wang, J. Liu, L. Liu, G. Zhu, L. Karthik, M. Wang, K.-F. Wang, Z. Wang, J. Yu, Y. Shuai, J. Yu, L. Zhang, Z. Yang, C. Li, Q. Zhang, T. Shi, L. Zhou, F. Xie, H. Dai, X. Liu, J. Zhang, G. Liu, Y. Zhuo, B. Zhang, C. Liu, S. Li, X. Xia, Y. Tong, Y. Liu, G. Alterovitz, G.-Y. Tan, L.-X. Zhang, A CRISPR-Cas12a-derived biosensing platform for the highly sensitive detection of diverse small molecules, Nat. Commun. 10 (2019) 3672.
[43] F. Teng, L. Guo, T. Cui, X.-G. Wang, K. Xu, Q. Gao, Q. Zhou, W. Li, CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity, Genome Biol. 20 (2019) 132.
[44] X. Sun, Y. Wang, L. Zhang, S. Liu, M. Zhang, J. Wang, B. Ning, Y. Peng, J. He, Y. Hu, Z. Gao, CRISPR-Cas9 triggered two-step isothermal amplification method for E. coli O157:H7 detection based on a metal–organic framework platform, Anal. Chem. 92 (2020) 3032–3041.
[45] B. Tian, A. Minero, J. Fock, M. Dufva, M. Hansen, CRISPR-Cas12a based internal negative control for nonspecific products of exponential rolling circle amplification, Nucleic Acids Res. 48 (2020) e30.
[46] Y. Li, X. Teng, K. Zhang, R. Deng, J. Li, RNA strand displacement responsive CRISPR/Cas9 system for mRNA sensing, Anal. Chem. 91 (2019) 3989–3996.
[47] S. Wu, N. Duan, Z. Shi, C. Fang, Z. Wang, Simultaneous aptasensor for multiplex pathogenic bacteria detection based on multicolor upconversion nanoparticles labels, Anal. Chem. 86 (2014) 3100–3107.
[48] R. Gao, Z. Zhong, X. Gao, L. Jia, Graphene oxide quantum dots assisted construction of fluorescent aptasensor for rapid detection of Pseudomonas aeruginosa in food samples, J. Agric. Food Chem. 66 (2018) 10898–10905.
[49] T. Bu, Q. Huang, L. Yan, W. Zhang, L. Dou, L. Huang, Q. Yang, B. Zhao, B. Yang, T. Li, J. Wang, D. Zhang, Applicability of biological dye tracer in strip biosensor for ultrasensitive detection of pathogenic bacteria, Food Chem. 274 (2019) 816–821.
[50] D. Alexandrakis, G. Downey, A.G.M. Scannell, Detection and identification of bacteria in an isolated system with near-infrared spectroscopy and multivariate analysis, J. Agric. Food Chem. 56 (2008) 3431–3437.