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CRISPR/Cas12a-based technology: A powerful tool for biosensing in food safety

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ABSTRACT

Background: In the context of the current pandemic caused by the novel coronavirus, molecular detection is not limited to the clinical laboratory, but also faces the challenge of the complex and variable real-time detection fields. A series of novel coronavirus events were detected in the process of food cold chain packaging and transportation, making the application of molecular diagnosis in food processing, packaging, transportation, and other links urgent. There is an urgent need for a rapid detection technology that can adapt to the diversity and complexity of food safety.

Scope and approach: This review introduces a new molecular diagnostic technology-biosensor analysis technology based on CRISPR-Cas12a. Systematic clarification of its development process and detection principles. It summarizes and systematically organizes its applications in viruses, food-borne pathogenic bacteria, small molecule detection, etc. In the past four years, which provides a brand-new and comprehensive solution for food detection. Finally, this article puts forward the challenges and the prospects for food safety.

Key findings and conclusions: The novel coronavirus hazards infiltrated every step of the food industry, from processing to packaging to transportation. The biosensor analytical technology based on CRISPR-Cas12a has great potential in the qualitative and quantitative analysis of infectious pathogens. CRISPR-Cas12a can effectively identify the presence of the specific nucleic acid targets and the small changes in sequences, which is particularly important for nucleic acid identification and pathogen detection. In addition, the CRISPR-Cas12a method can be adjusted and reconfigured within days to detect other viruses, providing equipment for nucleic acid diagnostics in the field of food safety. The future work will focus on the development of portable microfluidic devices for multiple detection. Shao et al. employed physical separation methods to separate Cas proteins in different microfluidic channels to achieve multiple detection, and each channel simultaneously detected different targets by adding crRNA with different spacer sequences. Although CRISPR-Cas12a technology has outstanding advantages in detection, there are several technical barriers in the transformation from emerging technologies to practical applications. The newly developed CRISPR-Cas12a-based applications and methods promote the development of numerous diagnostic and detection solutions, and have great potential in medical diagnosis, environmental monitoring, and especially food detection.

1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR) system originated from the adaptive immune system of the bacteria and archaea, acting as a “gene weapon” against foreign genetic

material, such as phages and plasmids (Koonin et al., 2017; Watson et al., 2021; O’Meara & Nunny, 2019). The basic operation mode of the immune system is that the CRISPR-associated proteins (Cas protein) utilize special sequences on the guide RNA (gRNA) to target the specific sites of the exogenous nucleic acid under the action of gRNA to cut and

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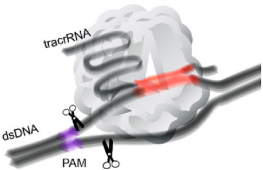
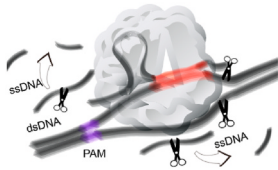
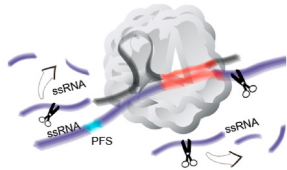
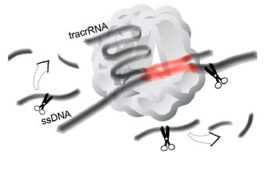
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Table 1

Summarizes the properties of the CRISPR types corresponding to the two CRISPR systems commonly used in CRISPR-Cas sensing.

Cas effector	Cas9	Cas12	Cas13	Cas14
Illustrations				
Guide RNA	tracrRNA:crRNA	crRNA	crRNA	tracrRNA:crRNA
Nuclease domains	RuvC and HNH	RuvC	HEPN	RuvC
cis-cleavage	dsDNA	DsDNA or ssDNA	ssRNA	ssDNA
trans-cleavage	without	ssDNA	ssDNA	ssDNA
PAM/PFS required	NGG	(T)TTN	Non-G	without
Spacer length	18-24 nt	18-25 nt	22-30 nt	20-40 nt

silence the external expression of the source gene. In 1987, Ishino et al. first found an unusual repetitive DNA sequence in *E. coli*, and a series of subsequent discoveries revealed the mechanism of the CRISPR-Cas system (Kozovska et al., 2021), which opened a new era of CRISPR-Cas mediated adaptive immune applications (Araldi et al., 2020; Hendriks et al., 2020; Murugan et al., 2017; Xu et al., 2020a). In 2012, Jennifer Doudna and Emmanuelle Charpentier jointly discovered the CRISPR-Cas9 gene magic clip (Jinek et al., 2012), and the Cas9 protein cleaved double-stranded DNA under the guidance of gRNA, thereby elucidating the potential of the CRISPR-Cas technology in gene editing under the guidance of RNA. The revolutionary use of the CRISPR-Cas9 technology in gene editing was pioneered in 2013 (Cong et al., 2013), paving the way for the subsequent CRISPR-Cas9 applications in clinical medicine (Memi et al., 2018), and biosensors (Sun et al.,

2020). Subsequently, RNA-guided and RNA-targeted CRISPR effectors (Cas12 (Zetsche et al., 2015), Cas13 (Gootenberg et al., 2017) and Cas14 (Harrington et al., 2018)) were successively discovered.

In the CRISPR/Cas system, CRISPR sequences are transcribed pre-CRISPR RNA (pre-crRNA), and further processed into mature CRISPR RNA (crRNA), which serve as the navigation of the Cas effector. Cas effector is formed from a single protein or multiple protein complexes, which is an enzyme unit with targeted cleavage activity. There are combinations of different components in the established CRISPR-Cas-based nucleic acid biosensors, but the fundamental difference still lies in the realization of different Cas effectors. The current reported CRISPR-Cas biosensor systems are divided into four categories according to the different effectors of Cas. Table 1 summarizes the properties of the CRISPR types corresponding to two CRISPR systems commonly used in

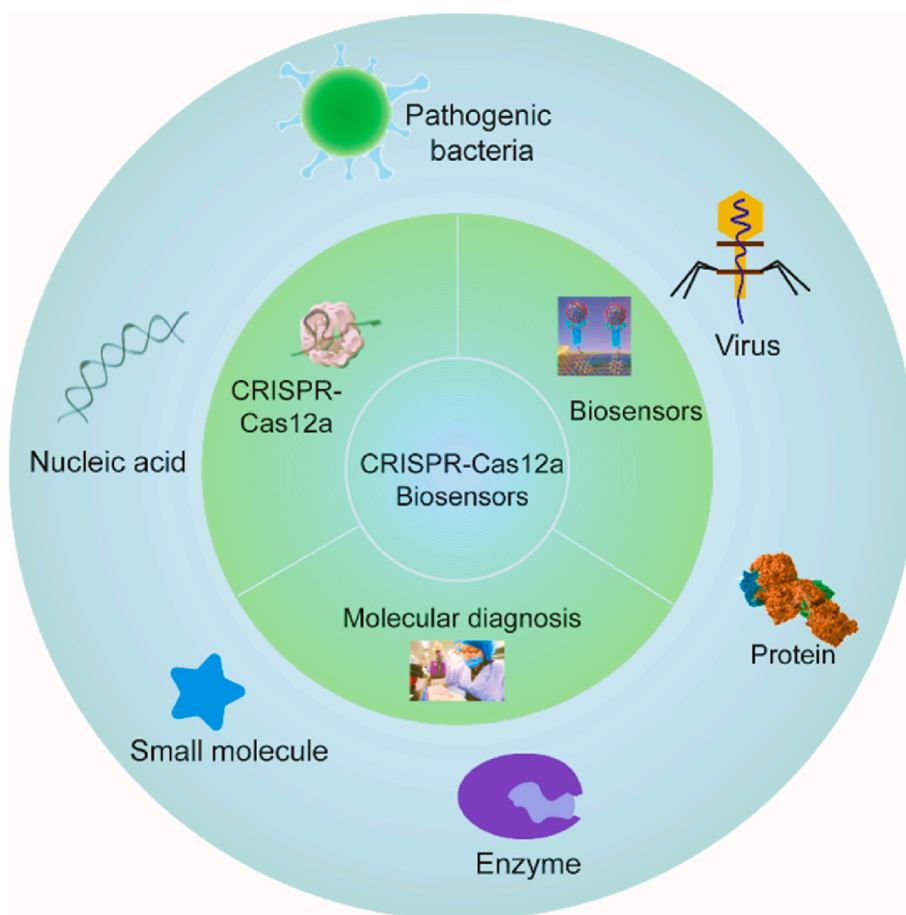


Fig. 1. Schematic representation of CRISPR-Cas12a-based systems for biosensors.

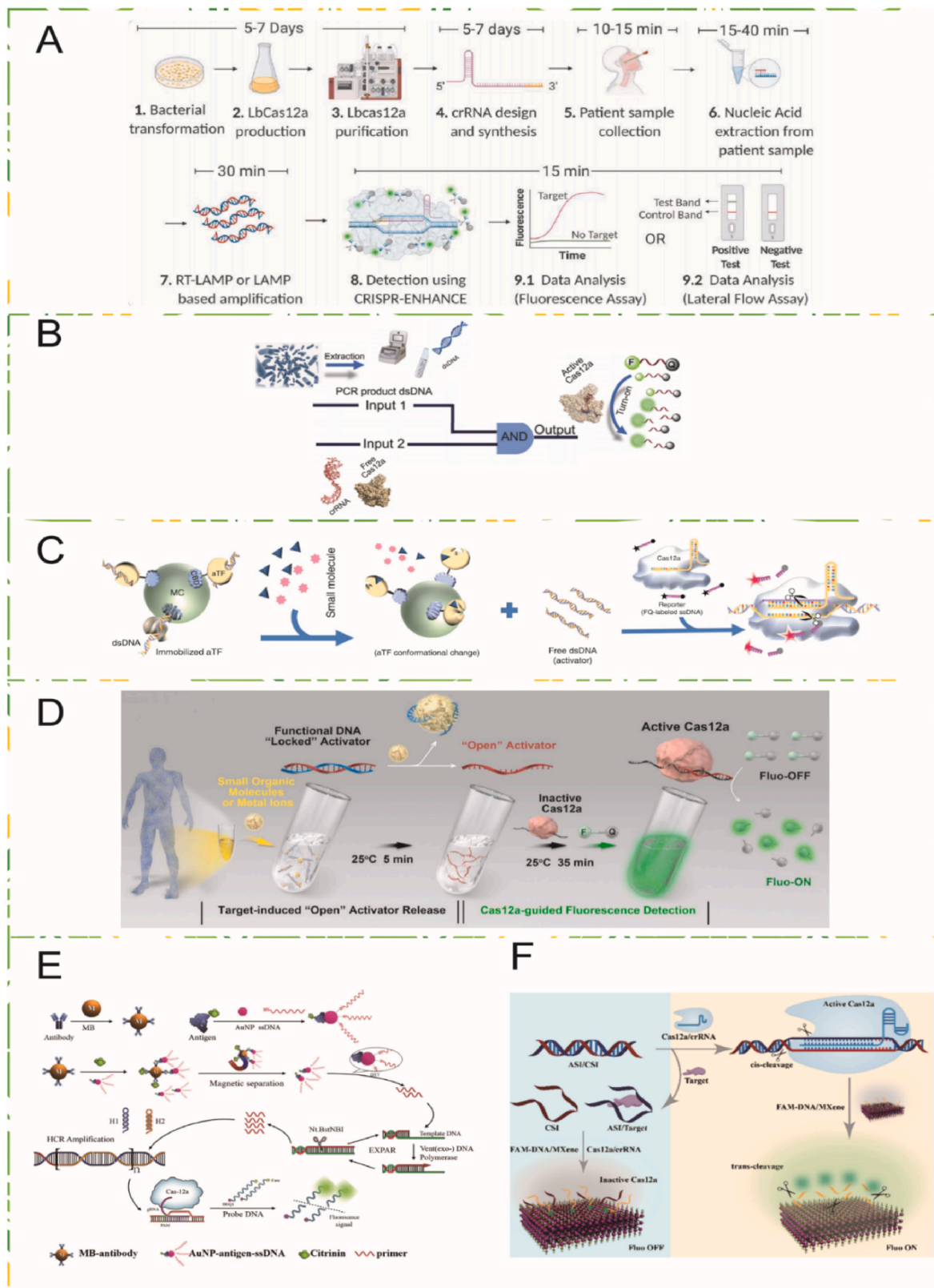


Fig. 2. Fluorescence sensor based on CRISPR-Cas12a (A) The CRISPR-Enhance System work flow (Nguyen et al., 2021). (B) CRISPR/Cas12a system integrated logic gate, rapid and sensitive detection of pathogenic gene schematic (Peng et al., 2020). (C) Schematic diagram of CaT-SMELor detection based on CRISPR-Cas12a (Liang et al., 2019). (D) Detection mechanism of functional DNA-regulated CRISPR-Cas12a sensor for detecting non-nucleic acid targets (Xiong et al., 2020). (E) The principle of citrinin analysis based on CRISPR-Cas12a (Zhang et al., 2021a) (F) Principle of endotoxin analysis based on CRISPR-Cas12a and MXene (Sheng et al., 2021).

the CRISPR-Cas sensing, and the subtypes of the CRISPR-Class II effector systems for CRISPR-based bioengineering and sensing. Herein, the biosensor strategy based on CRISPR-cas12a was summarized.

At present, only CRISPR-Cas12a and CRISPR-Cas12b in the CRISPR-Cas12 family are used for gene editing and nucleic acid diagnosis simultaneously (Kleinstiver et al., 2019; Li et al., 2018, 2019a; Teng et al., 2018). Difference between Cas12a and Cas12b; Similar to Cas9, Cas12b is a dual-RNA-guided endonuclease, in contrast to the single-RNA-guided Cas12a. Due to its thermophilic properties (Shmakov et al., 2015), Cas12b was not overcome and applied to human gene editing until 2019 (Strecker et al., 2019), which is one of the reasons why its research process is half a beat slower than its twin brother Cas12a. Therefore, CRISPR-Cas12a is the most widely used in the CRISPR-Cas12 family. CRISPR-Cas12a is a V-RNA-guided CRISPR-Cas endonuclease with about 1200–1500 amino acids. Zhang Feng et al. studied 16 Cpf1 (Cas12a) which family proteins from different bacteria, and determined that two Cpf1 enzymes (one each from *Acidaminococcus* sp. BV3L6 and Lachnospiraceae bacterium ND2006) efficiently edited the human genome (Zetsche et al., 2015). Compared with the Cas9 protein, the Cas12a protein-mediated CRISPR system has unique features. First, the CRISPR-Cas12a system is directly activated by mature crRNA, which does not require *trans*-activating crRNA (tracrRNA) to participate in the synthesis of guide RNA. Second, the CRISPR-Cas12a binary complex containing a RuvC nuclease domain activates and effectively cuts the target double-stranded DNA (dsDNA) by recognizing the 5'-T-rich short sequence PAM (Protospacer Adjacent Motif), solving the CRISPR-Cas9 preference and recognizing the limitations of the guanine-rich PAM sequences. Third, the CRISPR-Cas12a enzyme digestion system introduces 4 or 5 nucleotide sticky ends after the dsDNA cleavage. The sticky ends increase the probability of the homologous recombination repair, which is beneficial to the introduction of the foreign genes. The studies showed that the off-target effect of Cas12a is significantly lower than that of Cas9 (Kim et al., 2016).

Dounda et al. found that Lachnospiraceae bacterium ND2006 Cas12a (LbCas12a) quickly recognized and cleave single-stranded M13 DNA phage under the guidance of their respective gRNAs, while Cas9 did not (Chen et al., 2018). Thus, the LbCas12a protein was considered to have a powerful non-specific cleavage activity (accessory cleavage activity) to single-stranded DNA (ssDNA) after binding to the target DNA. The research team also found that the CRISPR-LbCas12a binary complex recognized the target dsDNA containing the PAM sequence, as well as targeted ssDNA without dependence on the PAM sequence, thus triggering a powerful non-specific cleavage of ssDNA. The stoichiometric titration experiments showed that the catalytic efficiency (k_{cat}/K_M) of the *trans*-cleavage (non-specific single-stranded DNA cleavage) triggered by the binding of CRISPR-LbCas12a to the single-stranded DNA activating molecule was $5.1 \times 10^8 \text{ s}^{-1}\text{M}^{-1}$, while the catalytic efficiency (k_{cat}/K_M) of its binding to double-stranded DNA activator molecules was $1.7 \times 10^9 \text{ s}^{-1}\text{M}^{-1}$. Therefore, most targets used to activate the Cas12a protein based on CRISPR-Cas12a detection were designed as dsDNA.

CRISPR-Cas12a with unique targeting ability was used for human genome editing (Liu et al., 2021; Wierson et al., 2019). The accessory cleavage activity of the Cas12a protein provided a novel direction for the development of biosensing technology. The article summarizes the latest research progress in the detection, diagnosis, and typing of various pathogenic nucleic and non-nucleic acid molecules based on CRISPR-Cas12a biosensing technology, laying a foundation for the application and expansion of CRISPR-Cas12a (Fig. 1). Finally, the challenges facing the field were summarized, and the prospects of the emerging new directions were proposed.

2. Biosensing technology based on CRISPR-Cas12a

Biosensing is an emerging technology integrating physics, chemistry, biology, medicine, and other disciplines (Xiao et al., 2019), which achieves the rapid identification and analysis of the test substances and

has the advantages of strong specificity, high sensitivity, and low cost. In the past 10 years, biosensor analytical technology was widely used in clinical diagnosis, food and drug analysis and testing, environmental testing, immunology, and other fields. The bioactive materials used in biosensing mainly include enzymes, proteins, DNA, antigens, antibodies, and tissue cells as the molecular recognition elements of the biosensors, which are equipped with specificity and sensitivity. CRISPR-Cas12a protein specifically recognized and cut double-stranded DNA containing the PAM motif under the guidance of crRNA. When the homologous sequences were selectively targeted, the Cas12a protein underwent conformational changes and expressed the cleavage-assisting activity of single-strand DNA near arbitrary cleavage., while the Cas9 protein became inactive after recognition and cleavage (Chen et al., 2018). The unexpected cleavage activity enabled the Cas12a effector play a signal amplification role in the nucleic acid detection, which converted specific sequence signals into highly efficient nuclease ssDNA cleavage activity, thereby significantly improving the sensitivity of detection.

2.1. Fluorescence biosensing

2.1.1. Detection of virus

Food-mediated virus transmission is a persistent problem in food safety and public health. Studies have shown that the virus can survive in the environment, such as doorknobs or stair railings, and infect more people through prolonged contact (Ong et al., 2020). In the past few years, the new crown has swept the world and caused immeasurable casualties and economic losses. The World Health Organization and the Centers for Disease Control and Prevention (CDC) announced that there is no evidence that SARS-CoV-2 is spread and directly contaminated through food and water (CD, 2021), but through consumption of food served on contaminated surfaces, the possibility of spreading the virus during packaging of contaminated rooms, or during processing or sharing food with infected persons cannot be ignored (Galanakis, 2020). In fact, there have been reports in the literature that physical contact and sharing food can cause infections (Pung et al., 2020); SARS-CoV-2 contamination has been detected in imported frozen salmon and even on cutting boards used to process fish (Han et al., 2020a). These facts have triggered a renewed understanding of food safety. If a controllable food-related disease is not detected and prevented early, it may quickly escalate into a global disaster. Therefore, we need to be more vigilant in the prevention and control of viruses, which is even more inseparable from fast and sensitive detection technology. The current diagnostic methods for coronavirus include the detection of the virus using quantitative reverse transcription PCR (RT-qPCR) or sequencing genomic techniques (Corman et al., 2020; Li et al., 2020a; Lu et al., 2020), which have high sensitivity and low detection limits, but are not suitable for screening in large populations. Zahir Ali et al. designed amplification primers and gRNA based on the SARS-CoV-2 gene, and the reverse transcription loop-mediated isothermal amplification (RT-LAMP) was employed to process samples extracted from nasopharyngeal or oropharyngeal swabs before CRISPR-Cas12 detection. The cleavage of the reporter molecule, i.e., the expected coronavirus sequence, was present in the solution, confirming the detection of the virus (Ali et al., 2020). The limit of detection (LOD) was 10 copies/ μL , which was equivalent to the minimum level required to detect the virus in the clinical samples via qRT-PCR (Ali et al., 2020; Broughton et al., 2020; Wang et al., 2020). The one-pot detection (LOD = 100 copies/reaction) established for CRISPR-Cas12b (Joung et al., 2020) increased the sensitivity by an order of magnitude.

Long T. Nguyen et al. used engineering techniques to extend the 3'-or 5'-ends of crRNA through different lengths of ssDNA, ssRNA and ssDNA thiophosphates. The autocatalytic behavior and enhancement rate of the LbCas12a-mediated lateral cleavage activity was 3.5 times that of the non-extended crRNA, and the specificity of the target recognition was significantly improved. In particular, the sensitivity and specificity of

the 7-mer DNA extension for LbCas12a-mediated nucleic acid detection were the most prevalent (Nguyen et al., 2020). Long T. Nguyen et al. suggested that the 7-mer extension of crRNA changed the conformation of LbCas12a after the *cis*-cleavage targets to expose the endonuclease domain in the solvent, which favored *trans*-cleavage. RT-LAMP was used for isothermal amplification of SARS-CoV-2 RNA, and the modified crRNAs were included in the paper-based transverse flow analysis, which detected targets with a sensitivity of up to 23 times within 40–60 min. Long T. Nguyen et al. proposed a CRISPR-Enhance System (Fig. 2A), and the working principle was as follows: BL21 (DE3) competent *Escherichia coli* cells were transformed by LbCas12a plasmid labeled with CL7, and cultured them overnight after induction by IPTG. The cells were collected and purified using CL7 affinity FPLC after 14–18 h of growth. The crRNA was designed and synthesized through the integration of DNA technology (IDT) for specific target DNA sequences. The target nucleic acid was extracted from patient samples, and the extracted nucleic acid was amplified by RT-LAMP, and the amplified products were added to the CRISPR reaction (Nguyen et al., 2021).

2.1.2. Detection of foodborne pathogens

According to the statistics of the World Health Organization (WHO), there are about 600 million people worldwide who are sickened by eating contaminated food, resulting in 420,000 deaths per year, 33 million years of healthy life loss, and huge economic costs associated with food recalls (Chen et al., 2017). Bacterial contamination in food and environment varies in degrees (Yang et al., 2016; Mills & Lee, 2019), such as *Escherichia coli* (*E. coli*) (Yang et al., 2017), *Listeria monocytogenes* (*L. monocytogenes*) (Kyere et al., 2020), *Staphylococcus aureus* (*S. aureus*) (Papadopoulos et al., 2018), *Salmonella* species (spp.) (Jra et al., 2021), *Vibrio parahaemolyticus* (Mok et al., 2021), poses a continuing threat to food safety, which is a worldwide public health problem. Recently, the fluorescence sensing technology based on the CRISPR-Cas12 system expanded from disease diagnosis to the field of food safety monitoring, providing a new strategy for food safety testing. For example, *Vibrio parahaemolyticus*, which is common in aquatic products, is the main cause of seafood-related food poisoning (Li et al., 2019b). Zhang et al. designed the corresponding crRNA by selecting a segment of the special heat-labile hemolysin gene of *Vibrio parahaemolyticus* as the target sequence, pre-installed the CRISPR-Cas12a system in the lid of the test tube, installed it on the micro-thermal cycler that opened the lid, and then performed PCR amplification on the extracted shrimp samples. The mixed reagents were centrifuged and incubated at 37 °C for 5–10 min, the results were read by the naked eye under ultraviolet light, and the detection limit was 1.02×10^2 copies/ μ L (Zhang et al., 2020). Chen et al. designed the CRISPR-Cas12 system for rapid identification of bacterial genotypes for urinary tract infections, which detected Ampicillin-resistant (AmpR) *E. coli* concentration in urine samples as low as 10^3 CFU/ml within 1 h, thereby allowing precise antibiotic treatment decisions in antibiotic susceptibility experiments (Chen et al., 2020). Lei Peng et al. introduced the CRISPR-Cas12a system and nucleic acid amplification technology based on three 2-input elementary AND, OR, and INHIBIT logic gates (Fig. 2B). The detection of the *Staphylococcus aureus* with high sensitivity and specific rapid response was realized. The method took about 2 h from the sample to the result, the dynamic range was 10^3 – 10^7 CFU/mL, and the detection limit was 10^3 CFU/mL (Peng et al., 2020).

2.1.3. Detection of small molecules

In addition to the above nucleic acid targets, researchers are constantly exploring the detection of the non-nucleic acid molecules based on CRISPR-Cas12. The simple, rapid, efficient, and sensitive detection of the non-nucleic acid molecules is of great significance in environmental monitoring, food and drug analysis, and disease diagnosis.

Bacterial allosteric transcription factors (aTFs) are a wide range of transcriptional regulatory proteins, and many bacteria evolved to sense

a variety of small molecules (heavy metals, metabolites, and chemicals) over the course of evolution (Koch et al., 2018). For example, Hucr, Hosa, BGAR and Tetr were produced naturally by some bacteria, which were used to detect small molecules (Li et al., 2016; Yao et al., 2018a, 2018b; Cao et al., 2018; Caron & Trowell, 2018). Liang et al. developed a small molecule detection platform (CaT-SMelor) that immobilized allosteric transcription factors on microcrystalline cellulose (Fig. 2C). The functional dsDNA containing the PAM special sequence and aTF binding sequence specifically bonded to aTF, making it unrecognizable by Cas12a. The conformation of aTF changed in the presence of the target small molecule (uric acid, *p*-HBA), causing dsDNA to fall off the DNA binding domain of aTF. The free dsDNA was specifically recognized by CRISPR-Cas12a, which activated the cleavage of the non-specific ssDNA of Cas12a, and modified with a fluorescent group at one end and a quencher group at the other end (Liang et al., 2019). CaT-SMelor sensitively detected the final concentration of the uric acid as low as 10 nM, and quantitatively analyzed uric acid in the range of 25–500 nM. In addition, CaT-SMelor sensitively detected the concentration of the uric acid as low as 1.8 nM, and quantitatively analyzed *p*-HBA in the range of 9–180 nM. Among them, uric acid is a metabolite of high-purine foods, which can provide a good solution for high-purine foods for health risk assessment.

The determination of adenosine triphosphate (ATP) is an indicator to evaluate the hygienic conditions of the food industry (Poulis et al., 1993). Xiong et al. designed ssDNA complementary binding ATP aptamers based on a competitive binding mechanism, which promoted ATP specifically binding with ATP aptamers (Fig. 2D). The competitive combination between ATP and the aptamer led to ssDNA falling off in the presence of ATP. The shed ssDNA triggered the accessory cleavage activity of CRISPR-Cas12a, cleaved the fluorescent reporter, and generated a fluorescent signal (Xiong et al., 2020). Bin Qiao et al. designed three locking schemes based on the principle of targeting alligator to lock ssDNA activators (Qiao et al., 2021) by using the same strategy. The activator released the *trans*-cleavage activity of CRISPR-Cas12a in the presence of targets. The results showed that two of the three designed locking schemes can effectively detect melamine in the solution with a detection limit as low as 38 nM. In addition, the target substance can be analyzed in 20 min without sample pretreatment in the actual sample detection.

Zhang et al. established a detection method for citrinin (a mycotoxin). Use gold nanoparticles modified with antigen and ssDNA as probes (AuNP-Antigen-DNA Probe). AuNP-Antigen-DNA Probe competes with citrinin for binding to the antibody-encapsulated magnetic beads. When citrinin is present in the sample, the substituted AuNP-Antigen-DNA can be collected by simple magnetic separation. Then the AuNP-Antigen-DNA probe was washed with dithiothreitol solution to release ssDNA to amplify dsDNA containing PAM sequence for CRISPR-Cas12a activation (Fig. 2E). This method shows a lower detection limit (0.127 ng mL^{-1}) and a wide linear range (0.005 – $500 \mu\text{g mL}^{-1}$), and has a good recovery rate in actual sample detection (97–104% in oats, 105–111% in flour) (Zhang et al., 2021a).

Gram-negative bacteria in raw milk are the main source of endotoxins in dairy products. Due to heat resistance, endotoxin still remains in dairy products after processing. This can partly reflect the degree of microbial contamination of raw milk and dairy products during processing, whether it is for the traceability of raw milk quality or the control of the safety of dairy products. Sheng et al. established a CRISPR-Cas12a analysis technique that can be used to detect endotoxin and gram-negative bacteria. MXenes has a strong ability to adsorb ssDNA and its advantages in visible light absorption, and can be used to adsorb and quench ssDNA-FAM. The aptamer is designed into a double-stranded structure containing PAM sequence to activate CRISPR-Cas12a, cleave ssDNA-FAM, and make it far away from MXenes to produce fluorescence recovery. When the target is present, the aptamer specifically binds to the target, and the double-stranded structure opens and the fluorescence recovery decreases. At the same time, MXenes can

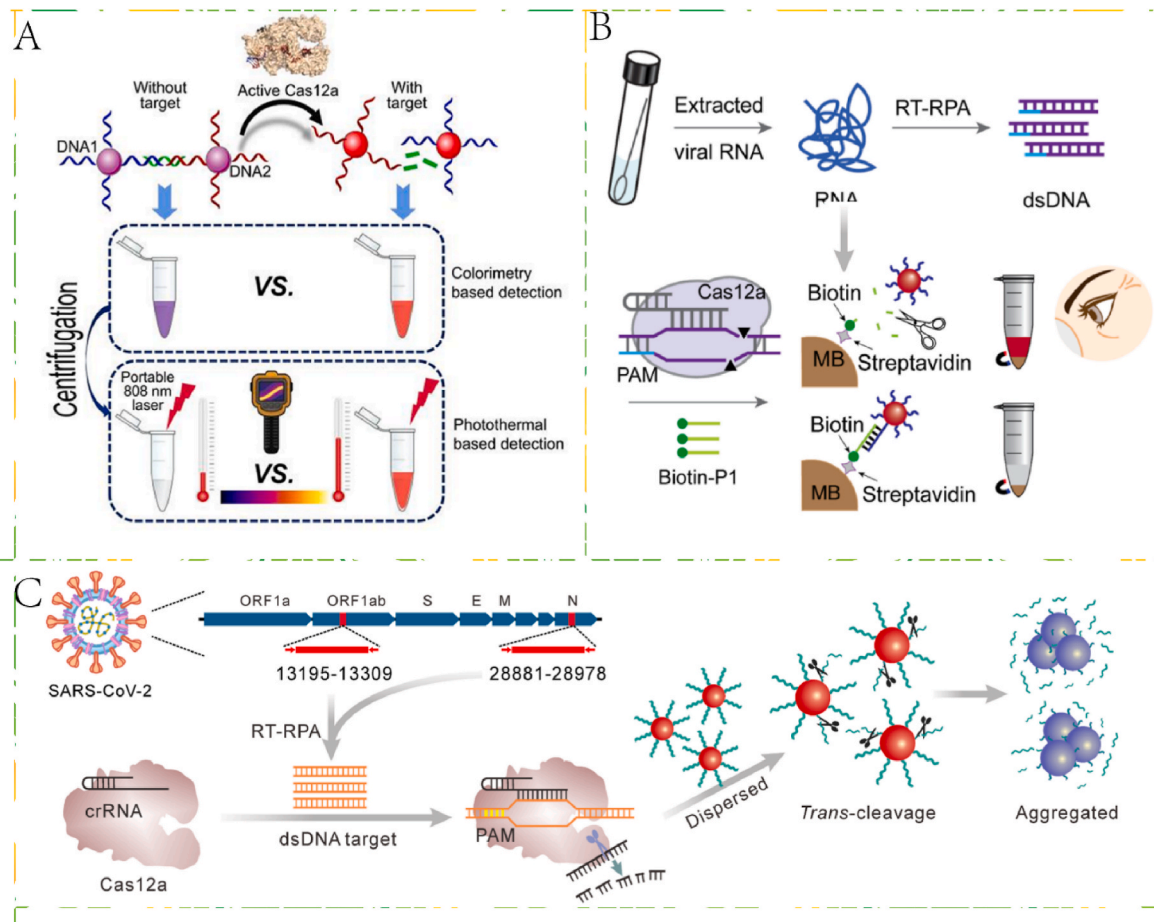


Fig. 3. (A) A gold nano-colorimetric method based on CRISPR-Cas12a for the detection of food-borne pathogens (Ma et al., 2021). Two gold nano-colorimetric methods for detecting SARS-CoV-2 based on CRISPR-Cas12a (B) and (C) (Jiang et al., 2021; Zhang et al., 2021b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

adsorb dissociated ssDNA and increase the release of ssDNA induced by the target (Fig. 2F). This method can quantify the selectivity and sensitivity of endotoxin and gram-negative bacteria in different samples, and the detection limits are 11 pg/ml and 23 CFU/mL, respectively (Sheng et al., 2021).

2.1.4. Detection of genetically modified crops

The safety of genetically modified food is always concerning to consumers, which is difficult to be widely accepted. Hence, some countries forced enterprises to label genetically modified ingredients that exceed the prescribed threshold (Broeders et al., 2012). The CaMV35S promoter widely exists in transgenic corn and soybeans (Becker & Ulrich, 2018). Wu et al. designed the corresponding crRNA to guide and activate the Cas12a protein by targeting the original spacer sequence rich in PAM motifs from the CaMV35S promoter sequence as the target, and visualized the fluorescent reporter by lamp amplification and CRISPR-Cas12a trans cleavage (Wu et al., 2020a).

2.2. Colorimetric biosensing

The color reaction as a sensitive color change reaction is widely used in the colorimetric detection of various molecules in the system, which is simple to operate, does not require complicated instruments, and is suitable for rapid result interpretation. Most commercial test strips and kits are based on colorimetric reactions, and the study of colorimetric reactions based on CRISPR-Cas12a has an important potential value.

2.2.1. Detection of virus

Zhang et al. established a reverse transcription recombinase polymerase amplification combined with CRISPR-Cas12a, highly sensitive colorimetric detection of SARS-CoV-2. The cleavage substrate of CRISPR-Cas12a is ssDNA, and ssDNA is modified to gold nanoparticles to prevent its aggregation effect. When CRISPR-Cas12a recognizes SARS-CoV-2, it produces ssDNA cleavage activity. The gold nanoparticles aggregate due to the cleavage of ssDNA on the surface, and the color changes. Gentle centrifugation can produce obvious visual inspection effects within 30 min. This method can achieve the sensitivity of 1 copy viral genome sequence/per test. In the actual clinical testing, it shows considerable credibility with the standard method (Fig. 3A) (Ma et al., 2021). Similarly, Jiang et al. established a colorimetric method for magnetically assisted separation of gold nanoparticles based on CRISPR-Cas12. The gold nanoparticles modified by ssDNA were used as the color developing solution, and the biotinylated ssDNA bridging the streptavidin magnetic beads and the gold nanoparticles was used as the enzyme digestion substrate. The detection performance of this method in 41 clinical samples was 95.12%, and the detection results were consistent with commercial clinical RT-qPCR diagnostic kits (Fig. 3B) (Jiang et al., 2021).

2.2.2. Detection of food-borne pathogens

Ma et al. used *Salmonella* (specific invA sequence) as a target model and used the CRISPR-Cas12a system to construct a gold nano-colorimetric sensor. The researchers designed the CRISPR-Cas12a enzyme cleavage substrate ssDNA to be able to connect two gold nanoparticle probe pairs (modified with DNA1 and DNA2) to induce

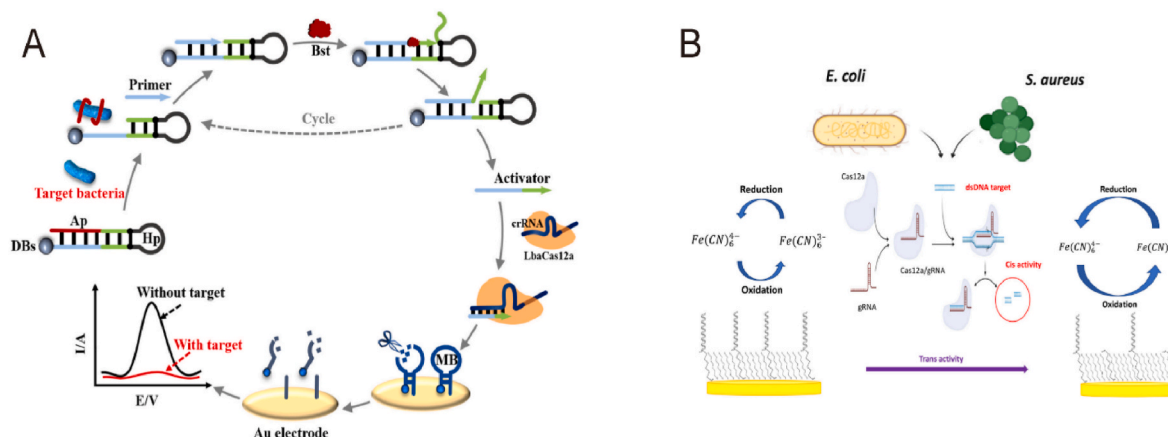


Fig. 4. (A) (B) CRISPR-Cas12a combined with electrochemical impedance spectroscopy (EIS) to detect foodborne pathogens (Bu et al., 2021). (B) CRISPR-Cas12a combined with primer exchange reaction (PER) for electrochemical detection of food-borne pathogens (Chen et al., 2021).

aggregation-dispersion changes. The supernatant can produce more obvious color changes under mild centrifugal conditions, which is recorded by the portable colorimeter. At the same time, the researchers used a thermal imaging camera to record the photothermal effects of gold nanoparticles under 808 nm near-infrared radiation. Combined with PCR amplification technology, the results show that the detection limit of the two measurement methods is 1 CFU/ml, and the dynamic linear range is 10^0 – 10^8 . The detection results of Salmonella in food samples are consistent with the traditional plate counting method (Fig. 3C) (Zhang et al., 2021b).

2.3. Electrochemical biosensing

2.3.1. Detection of nucleic acid

The electrochemical nucleic acid sensor with the advantages of simple structure, easy miniaturization, low cost, fast response, and high sensitivity can convert biochemical signals into electrical signals (Gu et al., 2018; Luo et al., 2019; Que et al., 2019), which is easy to integrate and assemble with electronic instruments to achieve convenience (Zhang & Liu, 2016). Bu et al. established a primer exchange reaction based on CRISPR-Cas12a to achieve ultra-sensitive detection of *E. coli* 0157:H7. The first is to design a DBs-AP-Hp PER structure locked by an aptamer. When the target bacteria are present, the aptamer specifically binds to expose the Hp chain. After the primer-strand binding, strand replacement extension, branch migration and transcriptional separation, it enters the next cycle. The new strand generated by the separation is used to activate CRISPR-Cas12a and cut the hairpin electrode (Fig. 4A). The detection limit of this method is 19 CFU/ml, and the linear range is 10^1 – 10^6 CFU ml⁻¹ (Bu et al., 2021). These results are basically consistent with the detection of *E. coli* 0157:H7 based on the combination of CRISPR-Cas12a and RCA extension by Chen et al. (Chen et al., 2021).

Andrea Bonini et al. established a label-free impedance biosensing method based on CRISPR/Cas12a for the detection of *E. coli* and *S. aureus*. Electrochemical impedance spectroscopy can sensitively and powerfully detect tiny physical and chemical changes on the electrode surface. Modifying ssDNA on the electrode can hinder the electron exchange between the electrode and the solution. When CRISPR-Cas12a recognizes the specific sequence of the target bacteria, it turns on the ssDNA cleavage mode, resulting in a decrease in charge transfer resistance (Fig. 4B) (Bonini et al., 2021).

2.4. Applications and products

2.4.1. Microfluidic device

Response materials activated by biochemical signals play an

increasingly important role in the detection of the analytes in the sensors (Spahr et al., 2017). The DNA response hydrogel is particularly suitable for binding with naturally occurring extracellular DNA or synthetic DNA structures (Iqbal et al., 2021). English et al. took advantage of the programmability of CRISPR-Cas12a to initiate the degradation of the DNA hydrogels and convert biological information into the changes of the material properties (English et al., 2019). The ssDNA molecules with fluorescent groups were introduced during the formation of polyethylene glycol hydrogel. The fluorescein was cleaved and released in the presence of the double-stranded targets *mecA* gene (*Staphylococcus aureus* specific sequence) and CRISPR-Cas12a. Furthermore, a gel was designed, in which the cleavage of ssDNA (for example, the cleavage caused by the amplification of Ebola virus genomic RNA by reverse transcription recombinant polymerase) changed the permeability or degradation of the gel. The changes demonstrated that the CRISPR-Cas12a gel embedded in a microfluidic-based device detected synthetic Ebola virus.

2.4.2. Smartphone reading strategy

Yin et al. used Salmonella as a model to design its specific *invA* gene as the amplification target to activate the *trans*-cleavage activity of CRISPR-Cas12a and trigger the ssDNA cleavage activity. Next, ssDNA rich in guanine sequences were designed, and K⁺ was added to form a stable G-quadruplex DNzyme. In the presence of hemin, DNase can catalyze the TMB-H₂O₂ reaction, the color changes, and the absorbance at 454 nm increases. This change can be easily recognized by naked eyes and smart phones. The detection limit of Salmonella by this method is 1 CFU/mL, which can be used for the detection of actual food samples (Fig. 5A) (Yin et al., 2021).

2.4.3. Flow-measuring device

Lateral flow immunoassay (LFIA) provides an easy-to-operate and portable tool for rapid self-inspection of food safety. Mukama et al. established an ultra-sensitive and highly specific lateral flow biosensor for the rapid detection of *Pseudomonas aeruginosa* (Fig. 5B) (Mukama et al., 2020). *P. aeruginosa* acyltransferase-specific genes were selected to design the corresponding crRNA and target nucleic acid pre-amplification primers. The test paper typically consisted of a sample pad, binding pad (gold nano-streptavidin complex), test line (single-stranded DNA complementary to the probe), and control line (biotinylated immobilized antibody), etc. The enzyme digestion system (CRISPR-Cas12 complex, biotinylated ssDNA reporter) was added for a 30-min reaction after the sample amplification by LAMP. Subsequently, the reaction solution was dropped on the test strip sample plate. In the presence of a target, the biotinylated single-stranded DNA reporter was *trans*-cut by Cas12a protease, making it impossible to hybridize with the

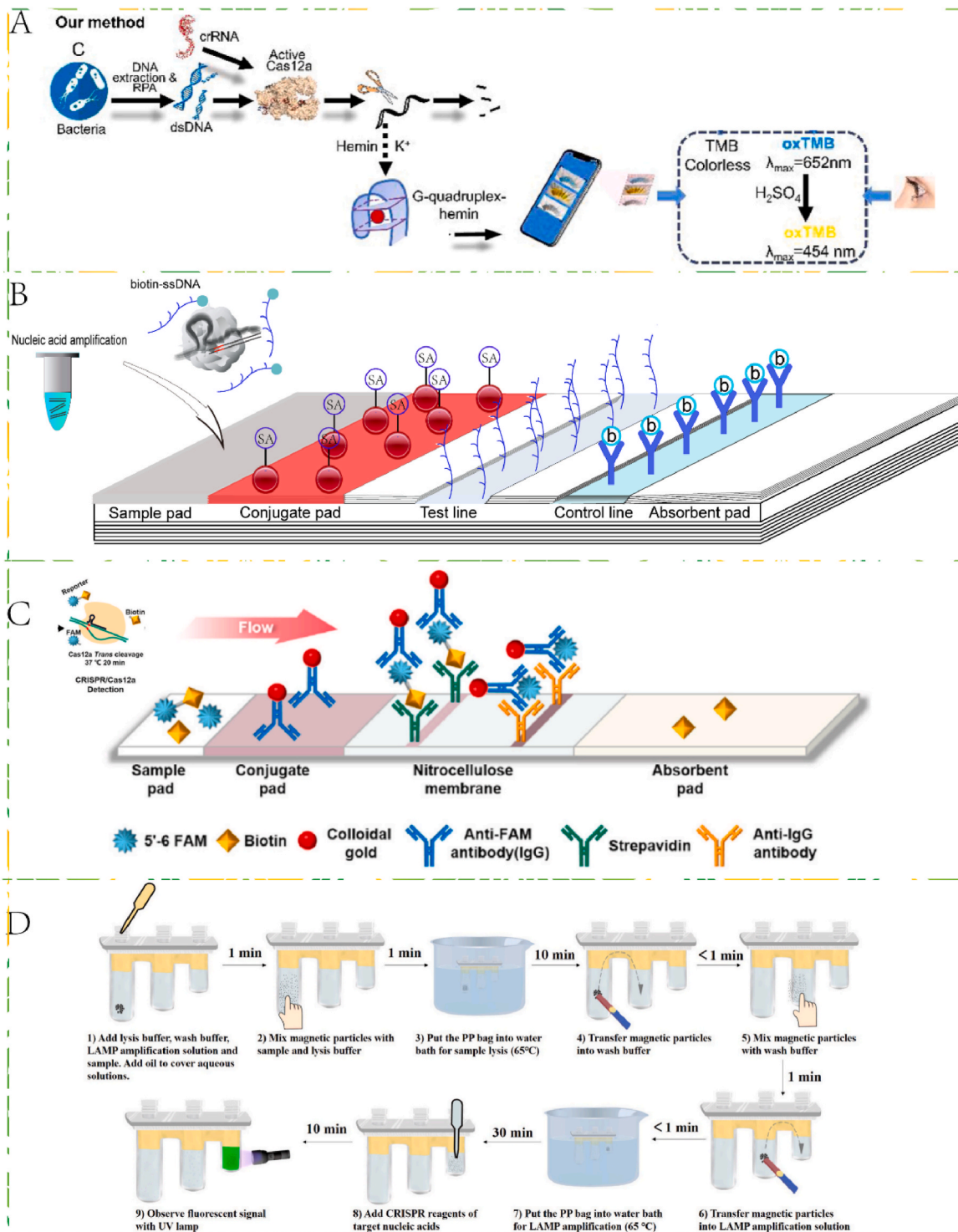


Fig. 5. (A) CRISPR-Cas12a biosensing based on label-free G-quadruplex combined with smartphone readings to detect food-borne pathogens (Yin et al., 2021). Amplification products used for the detection of test strips (B) (Mukama et al., 2020) and (C) (Qian et al., 2022). (D) The principle of portable PP bag for closed detection of pathogens (Wu et al., 2021).

complementary strand on the test strip. The gold nano-streptavidin complexes on the binding pad were captured by the biotinylated immobilized antibody on the control line, and the color was developed. Without a target in the reaction solution, the reporter molecule flowed through the binding pad, bonded to the gold nano-streptavidin complex, and it was fixed on the test line. Then, the test and control lines developed colors. Similar to the above method, Shao et al. reported a fluorescence-enhanced CRISPR-Cas12a-based flow measurement

biosensor. The main difference is that gold nanoparticles are replaced with functionalized quantum dots with higher fluorescence and more stability. After RAA amplification, the detection limit as low as 75 aM can be achieved under optimal conditions. This method can quickly and accurately detect *Staphylococcus aureus* in natural meat and vegetable samples (Zhou et al., 2022). Qian et al. modified the traditional fluorescence-quenching probe into a bio-ssDNA-FAM probe to make it compatible with commercially available test strips. The detection limit

Table 2
Summary of the main features of current CRISPR/Cas12a biosensing platforms.

Classification	Target classification	Detection	Amplification method	Detection steps	Isothermal	Dynamic Range	Detection limit	Multiple detection	Quantitative	Time	Refs
Fluorescence sensor	Nucleic acid molecule	HPV16 and HPV18	RPA	Two steps	Yes	–	–	No	NO	~1 h	Chen et al. (2018)
		COVID-19	RT-RAA	Two steps	Yes	–	10 copies	No	No	45 min	Wang et al. (2020)
		COVID-19	RT-LAMP	Two steps	Yes	–	10 copies	No	No	40 min	Ali et al. (2020)
		COVID-19	RT-LAMP	Two steps	Yes	–	10 copies	No	No	40–50 min	Broughton et al. (2020)
		Foodborne pathogens, genetically modified crops, and meat adulteration	RPA	Two steps	Yes	10^1 – 10^6 copies	10–100 copies	No	Yes	~30 min	Liu et al. (2020)
		Mycoplasma	RPA	Two steps	Yes	10^1 – 10^5 aM	10 aM	No	Yes	~30 min	Wang et al. (2019)
		CaMV35S promoter and Lectin gene	LAMP Rapid PCR	Two steps	Yes No	0.1%~10% 0.1%~10%	0.1% 0.1%	Yes	Yes	~30 min	Wu et al. (2020b)
		Swine Fever Virus	without	Two steps	Yes	100 fM~100 pM	100 fM	No	Yes	2–24 h	He et al. (2020)
		MRSA	RCA	Two steps	Yes	10^1 – 10^6 CFU/mL	10 CFU	No	Yes	80 min	Xu et al. (2020b)
		<i>Staphylococcus aureus</i>	PCR	Two steps	Yes	10^3 – 10^7 CFU/mL	10^3 CFU/mL	No	Yes	2 h	Peng et al. (2020)
	AmpR Bacteria	RPA	Two steps	Yes	10^3 – 10^5 CFU/mL	10^3 CFU/mL	No	Yes	~1 h	Chen et al. (2020)	
	<i>Vibrio parahaemolyticus</i>	PCR	One step	No	10^2 – 10^6 cfu/g	10^2 cfu/g	No	–	~1 h	Zhang et al. (2020)	
	Non nucleic acid molecules	Extracellular Vesicles	Dual-Cycle	One step	Yes	10^2 – 10^6 particles/ μ L	10^2 particles	No	Yes	~90 min	Zhao et al. (2020)
		Pb ²⁺	without	One step	No	1.1–10 nM	~0.053 nM	No	Yes	~1 h	Li et al. (2020b)
Acinetobacter baumannii miRNAs		without	One step	Yes	10 – 10^5 CFU/mL	~3 CFU/mL	No	Yes	~1 h	Li et al. (2020b)	
uric acid p-HBA melamine		without	One step	Yes	25–500 nM 9–180 nM 0–2.5 μ M	10 nM 1.8 nM 38 nM	No	Yes	~40 min 20 min	Liang et al. (2019) Qiao et al. (2021)	
Parvovirus B19	without	One step	Yes	100 nM–10 fM	10 fM	No	Yes	~1 h	Xu et al. (2020a)		
Electrochemical sensors	Nucleic acid molecule	<i>E. coli</i> and <i>S. aureus</i>	without	One step	Yes	3–18 nM	3 nM	No	Yes	1.5h	Chen et al. (2021)
		<i>Escherichia coli</i> O157:H7	PER	One step	Yes	10 – 10^6 CFU/mL	19 CFU/mL	No	Yes	1h	Bu et al. (2021)
Colorimetric sensor	Nucleic acid molecule	SARS-CoV-2	RT-RPA	Two steps	Yes	–	50 copies	No	No	50 min	Jiang et al. (2021)
		SARS-CoV-2	RT-RPA	Two steps	Yes	10 pM–100 nM	1 copy	No	Yes	50 min	Zhang et al. (2021b)
		Salmonella	without	One step	Yes	10^0 – 10^8 CFU/mL	1 CFU/mL	No	Yes	30 min	Ma et al. (2021)

of *Staphylococcus aureus* in the detection of artificially contaminated food samples is 2×10^1 (Fig. 5C) (Qian et al., 2022).

2.4.4. Portable inspection tool

Wu et al. developed a polypropylene nucleic acid detection bag that can be used at home. This PP bag contains three chambers (lysis chamber, washing chamber, amplification chamber/detection chamber), and the oil layer above the solution in the three detection chambers provides a closed space for nucleic acid detection. First, MPs and *Salmonella typhimurium* are added to the lysis chamber. The nucleic acid released by the lysis of *Salmonella typhimurium* is adsorbed by MBs and transferred to the washing chamber together under the action of an external magnet. After washing, it was transferred to the amplification room again for isothermal amplification in a water bath. Finally, CRISPR-Cas12a was added for specific recognition to generate fluorescent signals (Fig. 5D). For all operations, you can press the PP bag pressure chamber with your fingers to make the reaction solution fully mixed. All steps of this design are carried out in the enclosed space of the pp bag, avoiding aerosol pollution. In addition, this PP bag is used for the detection of inactivated new coronavirus samples, and the results are consistent with standard qPCR (Wu et al., 2021).

3. Challenges and prospects

With the continuous application of the CRISPR-Cas toolkit, new bright spots are emerging in the field of biosensors. This is an opportunity and a challenge for the food testing field. CRISPR-Cas12a is the most used in the CRISPR-Cas12 family, which offers several options for biosensor design due to the homeopathic and *trans*-cutting characteristics. Table 2 summarizes the current biosensing platforms based on CRISPR-Cas12a, which are mainly used for nucleic acid detection, and LOD can reach aM-level. Many methods require nucleic acid amplification before CRISPR detection to reduce LOD. The standard PCR amplification requires a bulky thermal cycler, cycling in multiple temperature steps between 60 °C and 95 °C, and a long reaction time (≥ 90 min), which is unsuitable for on-site detection. From Table 2, LAMP and RPA technologies are the most used amplification methods, which play a vital role in the clinical detection and field application of nucleic acids (Han et al., 2020b; Haque et al., 2021). Compared with conventional PCR, LAMP has the advantages of higher sensitivity, shorter reaction time, and simple operation (Gunasegar & Neela, 2021). The reaction temperature of RPA is between 37 °C and 42 °C (it can be used at room temperature, but this affects the reaction kinetics) for 5–60 min, depending on the initial nucleotide concentration. The reaction temperature of LAMP is 65 °C, and the reaction time is 15–60 min. The lower temperature easily caused the formation of the non-specific amplification products and primer dimers (Kojima et al., 2021). However, these extra products did not interfere with the sensing process and affect the results for the CRISPR-Cas12 complex with a high specificity of the target sequence. The combination of Cas12a nuclease, LAMP, and RPA can achieve ultra-sensitive detection of nucleic acids.

Pathogenic microorganisms, mycotoxins, and genetically modified crops are key challenges to food safety. The development of new detection technologies is essential to achieve and respond to potential food safety. CRISPR-Cas12a, biosensing analysis technology has a huge advantage in dealing with the harmful factors that target nucleic acids. However, the detection of non-nucleic acid substances needs further development. The biggest challenge in the detection of the non-nucleic acid molecules is how to convert non-nucleic acid signals into nucleic acid signals that can be recognized by the CRISPR-Cas12a binary. Currently, the emergence of the functional nucleic acids and bacterial allosteric transcription factors enables CRISPR-Cas12a to be used for the detection of the various non-nucleic acid targets. For example, Zhao et al. established a multi-functional biosensor platform based on aptamer (Zhao et al., 2021), and Liang et al. designed a multi-functional biosensor platform based on allosteric transcription factors, which were

used to detect different analytes. (Liang et al., 2019).

Due to the limitation of the receptor availability and practicability, the specificity and sensitivity of CRISPR-Cas12a-based biosensors need to be further improved. In addition, the use of evolutionary techniques to screen multiple functional recognition elements or improve various genome editing systems will play an important role in further promoting the research and application of the CRISPR-Cas12a biosensors (Su et al., 2021). Currently, the detection method based on CRISPR-Cas12a is still in the laboratory stage. The detection method represented by DETECTR achieves amplification and detection separation, but it introduces contamination and requires professional technical operators. Similar portable devices are only used in laboratories, and many technologies have a long way to go towards improvement and perfection for engineering and commercialization.

CRISPR-Cas12a can effectively identify the presence of specific nucleic acid targets and their small changes in sequence, which is particularly important for nucleic acid identification and pathogen detection. A similar novel coronavirus hazard has infiltrated every step of the food industry, from processing to packaging to transportation. CRISPR-Cas12a-based POC sensing has great potentials in the qualitative and quantitative analysis of the infectious pathogens. Moreover, the method can be adjusted and reconfigured within days to detect other viruses, providing equipment for future nucleic acid diagnostics in the field of food safety. The future work of multiple detection will focus on the development of the portable microfluidic devices. For example, Shao et al. used physical separation methods to separate Cas proteins in different microfluidic channels to achieve multiple detections, and each channel simultaneously detected different targets by adding crRNA with different spacer sequences (Shao et al., 2019).

Although CRISPR-Cas12a technology has outstanding advantages in detection, there are several technical barriers in the transformation from emerging technologies to practical applications. The newly developed CRISPR-Cas12a-based applications and methods promote the development of numerous diagnostic and detection solutions, and have great potential in medical diagnosis, environmental monitoring, and especially food detection.

Declaration of competing interest

The authors declare that no financial/personal interests or beliefs may affect their objectivity. Potential conflicts do not exist.

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