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Advances in the Analysis of Foodborne Pathogenic Bacteria Based on CRISPR/Cas12a-Driven Electrochemical Detection

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Abstract

Foodborne pathogens can cause food poisoning and other illnesses, which have a significant impact on food safety and human health and require rapid and efficient early detection methods. Traditional laboratory techniques, such as culture and polymerase chain reaction methods, usually take a long time and do not meet the need for rapid detection, a background that has driven the development of rapid, efficient research strategies. The recent discovery of clustered spaced short palindromic repeats rule (CRISPR) and CRISPR-associated proteins (Cas) has thoroughly become a hotspot in the field of detection. The fast, efficient nature of electrochemical biosensors perfectly fits the requirements for rapid detection of foodborne pathogens in food and environment. This paper reviews the research progress of electrochemical biosensors constructed based on the CRISPR/Cas12a system for the detection of foodborne pathogens, evaluates the limitations and challenges of biosensors for the detection of foodborne pathogens, and discusses future possibilities.

Keywords: CRISPR/Cas12a; Gene Editing Technology; Foodborne Pathogens; Electrochemical Detection

Introduction

Pathogenic microorganisms are microorganisms that can cause metabolic disorders in the body and lead to disease, also known as pathogenic bacteria. Foodborne pathogens are bacteria that cause food poisoning or foodborne illnesses through the presence of a wide range of bacteria in meat, dairy, aquatic products, fruits and vegetables [1, 2]. Common foodborne pathogens mainly include Staphylococcus aureus (S. aureus) [3], Escherichia coli (E. coli) [4], Listeria monocytogenes (L. monocytogenes) [5], Salmonella [6], Clostridium botulinum [7], Vibrio cholerae [8] and so on. Once infected, they can lead to vomiting, nausea, diarrhea, and other symptoms [6, 9], which can be life-threatening in severe cases; therefore, foodborne pathogens pose a serious and potentially fatal risk to human health [10]. Common detection methods for foodborne pathogens include traditional bacterial culture assays [11, 12], enzyme-linked immunosorbent assay (ELISA) [13], and polymerase chain reaction (PCR) [14]. However, traditional bacterial culture detection methods have disadvantages such as long detection time and low efficiency. Although ELISA improves the detection efficiency, the antibodies used are expensive and not easy to store. While the PCR method can quantitatively detect foodborne pathogenic bacteria in food and environment, it requires complicated experimental steps and is prone to non-specific amplification leading to false positives. Therefore, there is a need to establish a rapid, sensitive, and accurate detection method to improve food safety and maintain human health as well as public health safety.

Advanced detection methods (biosensors as well as gene editing techniques) offer technological innovations for efficient, rapid, and easy detection of foodborne pathogens in food and the environment compared to traditional detection techniques. Among the many sensors, electrochemical biosensors are the most classical and widely used class of sensors [15]. Electrochemical biosensors are a class of biosensors that convert captured biological signals into electrochemical signals and output them in the form of current or potential (Figure 1). According to the classification of the output signal, they can be classified into Cyclic Voltammetry (CV), Electrochemical Impedance Spectroscopy (EIS), Differential Pulse Voltammetry (DPV), etc [16, 17]. Electrochemical biosensors have been widely used in the detection of biotoxins, diseases, and foodborne pathogens due to their advantages of rapidity, efficiency, high sensitivity, and portability in detection [18-20]. In addition, CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR) and its associated protein (Cas) gene editing system's unique way of recognizing sites and cutting them. This can improve the high specificity and sensitivity of the biosensor [21, 22]. For example, Lu et al. reported the research method of CRISPR/Cas system for SARA-CoV-2, with the detection time reduced to 30 min [23], which provided a research direction for the rapid detection of SARA-CoV-2. Also based on the CRISPR/Cas system, Tan et al. combined with recombinant enzyme polymerase amplification (RPA) to successfully construct a detection platform for 12 common respiratory pathogens [24]. The method does not cross-react with other bacteria or viruses, ensuring the accuracy and efficiency of the experiment. These studies demonstrate the advantages of the CRISPR method for high specificity and sensitivity in detection. Therefore, the selection of a highly specific research strategy in combination with the CRISPR/Cas system is crucial for the development of electrochemical biosensors for the rapid and efficient detection of foodborne pathogenic bacteria.

Foodborne Pathogen

Staphylococcus Aureus

Staphylococcus spp. is the most common group of gram-positive cocci among septic bacteria and is widely found in nature as well as on the surface of the skin of humans and animals [25, 26]. Most of these genera are not pathogenic, whereas Staphylococcus aureus (S. aureus) is more pathogenic, causing septic and toxoplasmic illnesses, and it is one of the most common foodborne pathogens [27]. In recent years, there has been a rapid increase in drug-resistant strains, especially methicillin-resistant Staphylococcus aureus (MRSA) has become one of the most common causative organisms of nosocomial infections [28, 29]. Once S. aureus invades the body, it can cause localized infections, visceral infections, sepsis, and septicemia [30, 31]. In addition, the toxins produced by S. aureus

can cause food poisoning and toxic shock syndrome [32, 33].

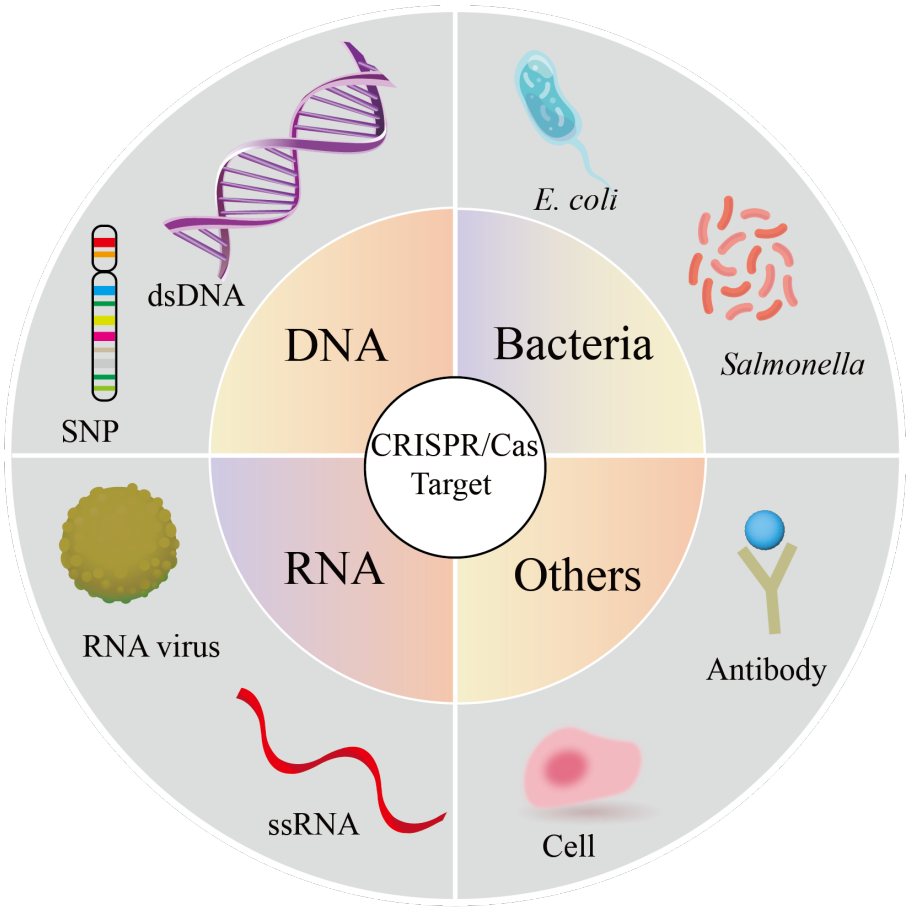

Figure 1: Schematic Diagram of CRISPR/Cas Detection of Bacterial Targets

Escherichia Coli

Escherichia coli (E. coli) is a common gram-negative bacillus that is a member of the intestinal flora [34]. Most strains of E. coli are part of the normal intestinal flora and are usually transmitted through food, water, or contact [35]. E. coli is categorized into two main groups according to whether they produce toxins or not: enterotoxin-producing E. coli and non-enterotoxin-producing E. coli. Enterotoxin-producing E. coli are important pathogens for infections in humans and a wide range of animals, and they can lead to gastrointestinal infections, causing symptoms such as diarrhea, vomiting, abdominal pain, and fever [36, 37]. Certain pathogenic strains can also cause urinary tract infections, respiratory tract infections, and other infections that can lead to death in severe cases [38], E. coli is also a safety indicator for water quality monitoring [39].

Salmonella

Salmonella is a group of gram-negative bacilli that parasitize the intestinal tract of humans and animals [40], and Salmonella spp. are pathogenic to humans, including Salmonella typhi, Salmonella typhimurium, and Salmonella enterica[41, 42]. Human infections due to the consumption of food contaminated with Salmonella, mostly meat products, are the most important factor in the development of gastroenteritis that re-

sults and are therefore of great importance in public health and food safety [43]. Its main pathogenesis is the invasion of the intestinal mucosa by Salmonella and enterotoxins, with a short incubation time and rapid onset, and the main symptoms are fever, nausea, and watery discharge [44]. Elderly people and those with weaker resistance will be rapidly dehydrated, leading to shock and in severe cases, death due to renal failure [45].

Listeria Monocytogenes

Listeria monocytogenes (Listeria), a group of gram-positive foodborne zoonotic pathogens, is widely distributed in nature [46]. There are 10 species in the genus Listeria, and only Listeria monocytogenes (L. monocytogenes) is pathogenic to humans, causing listeriosis, which is mainly manifested as meningitis and meningoencephalitis [47, 48]. The pathogenic mechanism relies mainly on its toxin, Listeria monocytogenes lysin O, which causes intestinal diseases and septicemia by contaminating foods such as cooked meat products and soft cheeses [49]. The main infected populations are neonates, elderly pregnant women, and immunocompromised individuals, resulting in preterm labor, stillbirth, or neonatal infections [50, 51].

CRISPR/Cas12-Based Electrochemical Biosensors

CRISPR/Cas Gene Editing System

The CRISPR/Cas gene editing system is a nucleic acid-based adaptive immune system present in numerous archaebacteria that defends against phages, plasmids, and other foreign DNA elements by invading nucleic acids, DNA, or RNA [52]. The CRISPR/Cas system functions in a three-step process (Figure 2): adaptation, expression, and interference. When a foreign virus or nucleic acid sequence invades, the CRISPR/-Cas system recognizes and edits the gene sequence attacking the invasion in a CRISPR array. When a secondary infection occurs, the integrated gene sequence is transcribed into a mature crRNA, which cuts the target sequence to target inactivation under the specific recognition of Cas [53, 54]. CRISPR systems can be classified according to structural composition into CRISPR systems can be categorized into six types based on their structural composition: Class I (types I, III, IV) consists of multiple Cas proteins and crRNAs that form a complex to perform cleavage in concert; Class II (types II, V, VI) consists of a single Cas protein that performs cleavage on its own [55]. Compared to class I, class II Cas proteins require only one protein to exert cleavage and accessory cleavage activities and play important roles in DNA or RNA editing, tracking, knockdown, and nucleic acid detection. The main ones widely used in the whole gene editing system are CRISPR/Cas9, CRISPR/Cas12, and CRISPR/Cas13, and this paper focuses on the overview of the electrochemical biosensor of CRISPR/Cas12a for the detection of foodborne pathogenic bacteria.

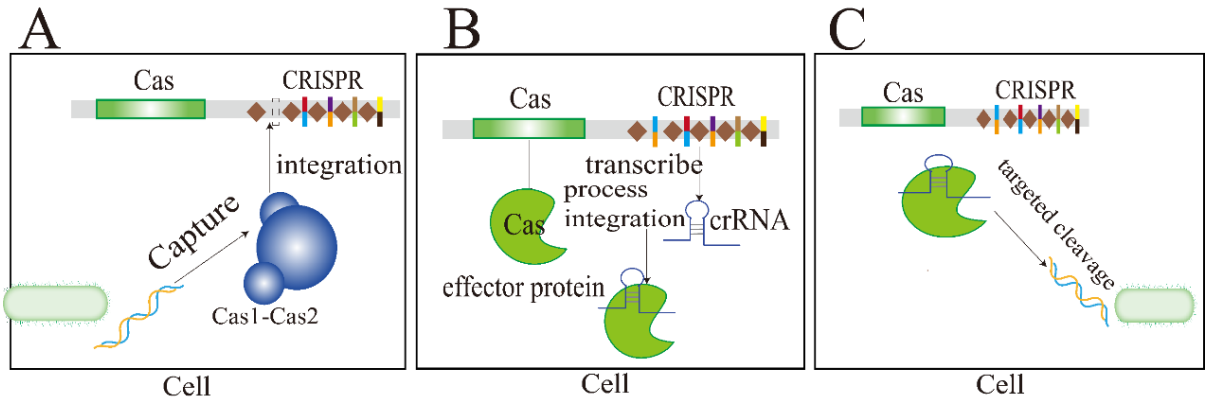

Figure 2: CRISPR/Cas System Adaptive Immune Response. A) Adaption, B) Expression, C) Interference

Crispr/Cas12

Cas12 is one of the prokaryotic deoxyribonucleic acids of the CRISPR/Cas protein family, which belongs to the class II type V RNA-guided CRISPR/Cas effectors [56] and is also an RNA-guided and DNA-targeted nucleic acid endonuclease. The family of Cas12 proteins includes the members of Cas12a - Cas12f, whereas the widely used ones are Cas12a and Cas12b, among these two proteins, the former is more widely used [57].

Trans-Cleavage and Cis-Cleavage of Cas12

Cas12a is also known as Cpf1 protein, unlike Cas9a which requires two nucleic acid structural domains to exert its cleavage activity, Cas12a can perform cis-cleavage and trans-cleavage [58]. Cis-cleavage: The crRNA-Cas12a binary complex scans double-stranded DNA and, upon finding PAM (typically 5′-TTTN-3′), the DNA proximal to PAM is locally unzipped to form an R-loop. The RuvC domain sequentially cleaves the non-target strand (NTS) and target strand (TS) approximately 18 nt downstream of the PAM, producing a 5′-sticky-ended dsDNA fragment. Trans-cleavage: After cis-cleavage is completed, Cas12a remains in a “highly active” conformation; at this point, the RuvC active site is fully exposed, enabling rapid and indiscriminate cleavage of any single-stranded DNA probe of length ≥8 nt in the reaction system [59]. Based on the trans-cleavage activity of Cas12a, Li et al. combined the activation of DNA-AuNPs (gold nanoparticles) nanotechnology with the CRISPR/Cas12a system to develop a sensitive Cas12a/crRNA-based nano-immunosorbent assay (Nano-CLISA) platform, which was successfully realized for the detection of carcinoembryonic antigen [60]. In addition, the cleavage activity of Cas12a maintains high efficiency at low temperatures, which may be beneficial for specific application scenarios (e.g., plant gene editing) [61, 62]. An et al. used the cleavage activity of Cas12a to knock out multiple targets of octahydroxylycopene desaturase gene 8 in poplar, a research strategy that provides a research direction for facilitating genetic studies in forest trees [63]. The CRISPR/Cas12a system is widely used in gene tracking [64], disease detection [65, 66], and biosensing [67, 68].

CRISPR/Cas12-Based Electrochemical Biosensors

The joint research strategy of the CRISPR/Cas12 system and electrochemical biosensors has been shown to improve the selectivity and specificity of detecting target analytes, which is mainly attributed to the CRISPR/Cas12-specific trans-cutting activity with crRNA design [69]. In CRISPR/Cas12-based electrochemical biosensors, an electrochemical signaling molecule, e.g., ferrocene (Fc), methylene blue (MB), labeled at one end of the ssDNA and immobilized on the surface of the electrode, acts as a bioreceptor, called a signaling probe. This probe cuts after the activation of CRISPR/Cas12 trans--cutting activity is activated, the electrochemical signaling molecule moves away from the electrode surface, and the electrochemical signal is altered as a way to achieve detection of the target.

In addition, the use of amplification technology can significantly amplify the biological signals, improve the sensitivity of the assay, and reduce the detection limit. Qing et al. reported the research strategy of rolled-circle amplification (RCA) in combination with CRISPR/Cas12a, which was realized by square-wave pulsed voltammetry (SWV) for the detection of prothrombin [70]. This method combines the advantages of efficient amplification of RCA and rapid specificity of CRISPR/Cas system to achieve precise detection of thrombin with a detection limit as low as 1.26 fM. Also in the strategy of RCA with CRISPR/Cas12a, Qing et al. constructed a DNA logic circuit detection platform with DNA input, and the detection limits of microRNA, fine virus B19 DNA and adenosine-5'-triphosphate with detection limits of 0.83 aM, 0.52 aM, and 0.46 pM, respectively [71]. In photoelectrochemical biosensors, the CRISPR/Cas12a system also performs well, and Shen et al. constructed a photoelectrochemical fluorescence strategy for miRNA detection by utilizing the "cis--cutting activity" and "trans-cutting activity" of Cas12a [72]. The following year, Shen et al. combined CRISPR/Cas12a with a multi-amplification system and reported a photoelectrochemical colorimetric strategy for miRNA detection [73].

CRISPR/Cas12a-Based Driven Electrochemical Detection of Foodborne Pathogenic Bacteria

The advantages of CRISPR/Cas12a-based electrochemical biosensors in improving selectivity and specificity and their applications in disease markers, environmental health, and food safety have attracted the attention of many researchers around the world, especially for the detection of foodborne pathogens. For example, Huang et al. developed a novel electrochemical biosensor based on jumping rolled-ring amplification (SRCA) coupled with CRISPR/Cas12a system for accurate detection of *S. aureus* [74]. The strategy uses methylene blue as the electrochemical signaling molecule and -SH modified signaling reporter probe (SH-ssDNA-MB) immobilized on the surface of a glassy carbon electrode modified with gold nanoparticles via Au-S bond. When the solution to be tested contains *Staphylococcus aureus*, the double-stranded DNA obtained by SRCA can be specifically recognized by Cas12a/crRNA complex and in this way activates the Cas12a trans-cutting activity, which specifically recognizes and cleaves SH-ssDNA-MB, resulting in the MB moving away from the electrode surface and the decrease of the electrochemical signals (Figure 3). Under optimal conditions, the detection limit for *S. aureus* was 3 CFU/mL, respectively.

Figure 3: The Principle of "An Electrochemical Biosensor for the Highly Sensitive Detection of *Staphylococcus Aureus* Based On SRCA-CRISPR/Cas12a"

In contrast, Bonini et al. combined the CRISPR/Cas12a system with electrochemical impedance spectroscopy (EIS) measurements to develop a label-free biosensing assay for the detection of E. coli and S. aureus [75]. This research strategy provides a research idea for the construction of a biosensing device based on CRISPR/Cas12a label-free impedance measurement. Similarly for E. coli, Bu et al. used CRISPR/Cas12a cascade signal amplification and primer exchange reaction (PER) to detect E. coli O157:H7 [76]. The functional DNA aptamer triggers the PER hairpin structure only when the target pathogen is detected, extending the primer to long single-stranded DNA (ssDNA), which then activates the cleavage activity of Cas12a on ssDNA modified on the Au electrode, resulting in a reduced electrochemical signal with a limit of detection of 19 CFU/mL. To reduce the limit of detection for E. coli. Chen et al. proposed a CRISPR/Cas12a combined with Immuno-Rolling Circle Amplification research strategy [77]. The strategy is a sandwich immunoassay on magnetic beads, Immune - RCA generates E. coli O157:H7 specific aptamers and long ss DNA targeting repetitive sequences. Thus, in the presence of E. coli O157:H7, CRISPR/Cas12a trans-cleavage activity is activated, and the MB-hairpin DNA probe on the surface of the modifying electrode is cleaved by cutting and cleaving. The peak current was altered. The detection limit of

E.coli was successfully reduced to 10 CFU/mL by the amplification system.

Liu et al. on the other hand, developed an electrochemical biosensor for the detection of the pathogenic bacterium *Salmonella typhimurium* (*S. typhimurium*) by combining the hybridization chain reaction (HCR) with CRISPR/Cas12a (Figure 4) [78]. Autonomous crossover opening of the functional hairpin DNA structure of HCR produces a single length of ds DNA consisting of several ss DNAs DNA, the ds DNA can then activate the trans-cutting activity of CRISPR/Cas12a through multiple binding sites to cleave the signaling probe modified on the electrode surface, resulting in the transfer of electrochemical signaling molecules and a change in electrochemical signaling. Polymeric ds DNA of HCR is immobilized on Dynabeads (DBs) via *Salmonella* typhimurium nucleic acid aptamers and is released from DBs released from DBs. The established method can selectively and sensitively quantify *Salmonella* typhimurium in samples with a detection limit of 20 CFU/mL. In addition to this, Zheng et al. proposed a research strategy based on the combination of Jumping Rolling Circle Amplification (SRCA) and CRISPR/Cas12a system [79]. Signal amplification was achieved by rapid SRCA amplification and the trans-cutting activity of Cas12a. As a result, non-specific amplification is eliminated, reducing the false--positive rate of the assay. With this strategy, the biosensor showed a linear range of 5.8 fg/μL - 5.8 ng/μL based on the ratio of Fc and MB current signals (IFc/IMB), with detection of *Salmonella* as low as 2.08 fg/μL.

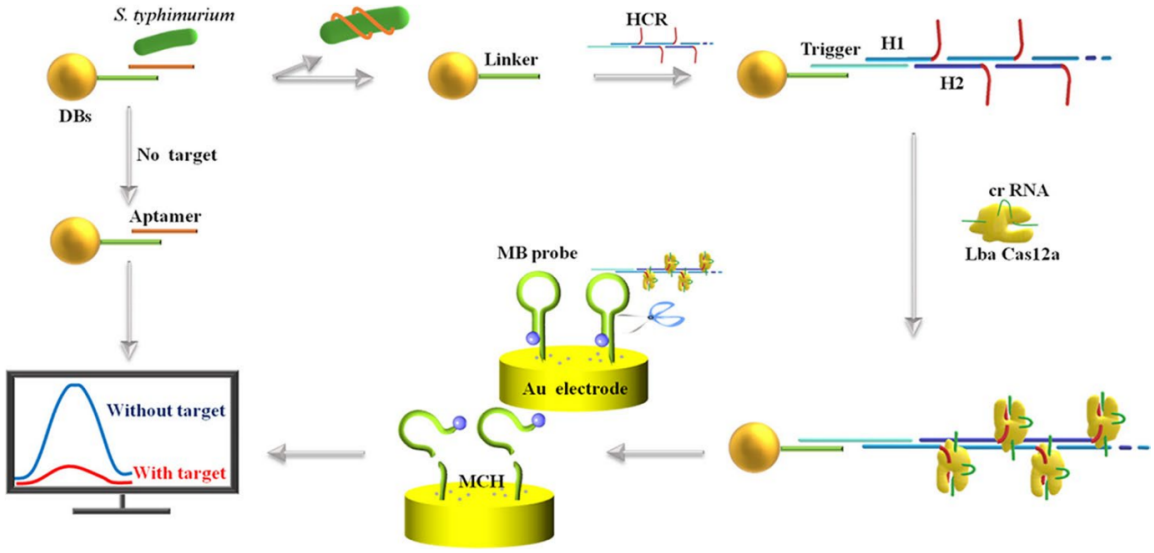

Figure 4: Schematic Illustration of the Electrochemical Biosensor for Detecting S. Typhimurium

Li et al. introduced the trans-cutting activity of CRISPR/-Cas12a into an electrochemical biosensor (E-CRISPR) and combined it with recombinant enzyme-assisted amplification (RAA) to establish a cost-effective, specific, and ultrasensitive method [80]. As shown in the schematic diagram of Figure 5A: *Listeria monocytogenes* DNA was extracted for RAA amplification to generate a large amount of ds DNA, which was subsequently combined with CRISPR/Cas12a-crRNA to activate the trans-cutting activity of CRISPR/Cas12a. When *Listeria monocytogenes* was present in the solution to be tested, the trans-cutting activity of CRISPR/Cas12a was activated to cut ss DNA on the electrode surface, and the MB modified on the tip was far away from the electrode surface, and the electrochemical signals were significantly altered, thus realizing the detection of *Listeria monocytogenes*, which was detected at a detection limit of 26 CFU/mL for this biosensor.

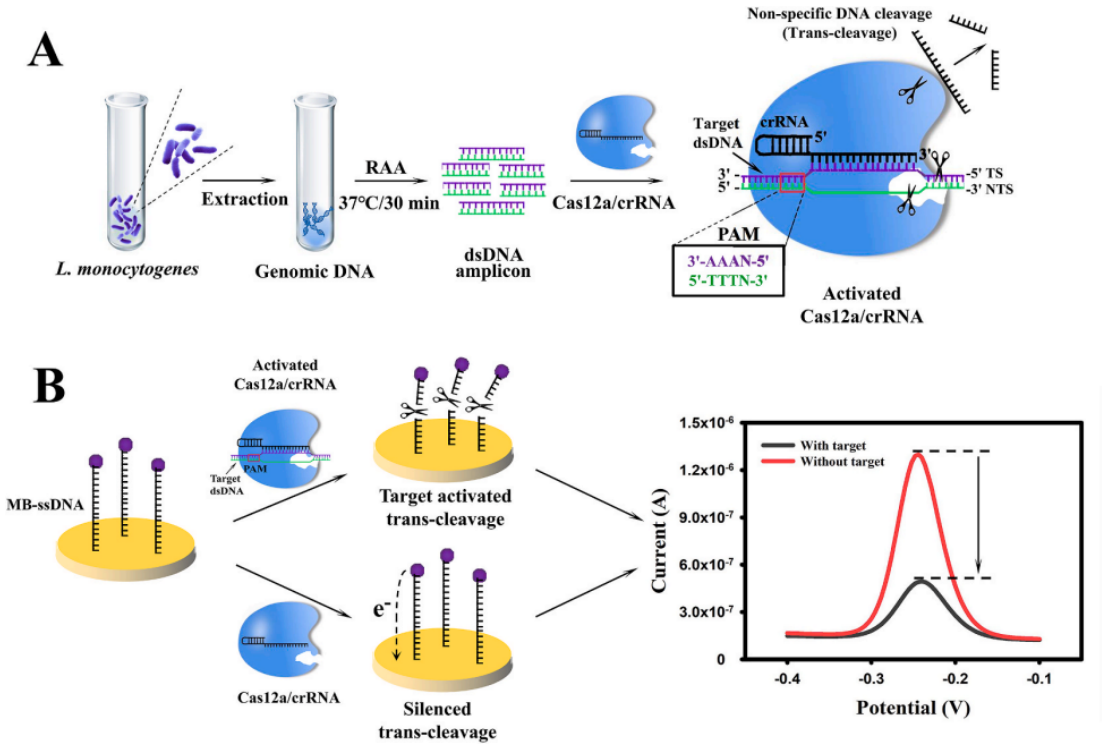

Figure 5: Principle of the RAA-Based E-CRISPR Biosensor for Detecting L.Monocytogenes

In addition to the sensors described above, there are research strategies based on the CRISPR/Cas system for the detection of foodborne pathogens.

Table 1: Biosensor based on the CRISPR/Cas system for the detection of foodborne pathogens

Detection Method	Target	CRISPR/Cas	LOD	Reference
Electrochemical biosensor	E. coli	Cas12a	5.02 CFU/mL	[81]
Electrochemical biosensor	S. typhimurium	Cas12a	55 CFU/mL	[82]
Electrochemiluminescence	Salmonella	Cas12a	37 CFU/mL	[83]
Fluorescence	S. aureus	Cas12a	1.50 CFU/mL	[84]
Photothermal	S. aureus	Cas12a	1 CFU/mL	[85]
Colorimetric	S. aureus	Cas12a	5 CFU/mL	[86]
Fluorescence	S. aureus	Cas12a	4 × 103 fg/μL	[87]
Fluorescence	S. aureus	Cas12a	10 copies	[88]
Flow test strips	S. aureus	Cas12a	10 - 100 copies	[88]
Single-tube detection	S. aureus	Cas12a, Cas13a	5 copies	[89]
Fluorescence	E. coli	Cas9	40 CFU/mL	[90]

Fluorescence	E. coli	Cas12a	1 CFU/mL	[91]
Lateral flow	E. coli	Cas12a	100 CFU/mL	[91]
Lateral flow	E. coli	Cas12a	1 CFU/mL	[92]
One-pot	E. coli	Cas12a	1 CFU/mL	[93]
G-Quadruplex	S. enterica	Cas12a	20 CFU/mL	[94]
Fluorescence	S. enterica	Cas12a	24.9 CFU/mL	[84]
Fluorescence	S. enterica	Cas12a	5 CFU/mL	[95]
Fluorescence	S. enterica	Cas12a	6 CFU/mL	[96]
Fluorescence	Salmonella	Cas12a	8 CFU/mL	[97]
Fluorescence	Salmonella	Cas12a	20 CFU/mL	[98]
Fluorescence	Salmonella	Cas12a	50 CFU/mL	[99]
PGMs-CRISPR	Salmonella	Cas12a	5 CFU/mL	[100]
One-pot	Salmonella	Cas12a	1 CFU/mL	[101]
Magnetic nanoparticles	Salmonella	Cas12a	130 CFU/mL	[102]
Fluorescence	S. enterica	Cas13a	1 CFU/mL	[103]
One-pot	S. enterica	Cas13a	100 copies	[104]
Fluorescence	L. monocytogenes	Cas12a	2.3 CFU/25g	[105]
Fluorescence	L. monocytogenes	Cas12a	33.7 CFU/mL	[106]
Cas12a-MA	L. monocytogenes	Cas12a	33.7 CFU/g	[107]

Inclusion

CRISPR/Cas12a-based electrochemical biosensors provide rapid and precise results in the detection of foodborne pathogenic bacteria, which will help maintain food safety. However, electrochemical biosensors based on the CRISPR/Cas system still have shortcomings, such as the simultaneous detection of multiple bacteria and their typing cannot achieve accurate detection. And the biggest challenge is whether it has good accuracy for clinical sample detection, which has little data in the known literature. In future research, photoelectrochemical research strategies can be developed to enhance the detection of other foodborne pathogens, biotoxins, and other pathogenic substances, and the application of the CRISPR/-Cas system for targeted detection needs to be further expanded.

CRISPR-Based Electrochemical Biosensors Are Still In the "Laboratory-To-Pilot" Transition Phase and Have Not Yet Entered Large-Scale Regulatory Approval or Industrial Implementation. This Can Be Summarized In The Following Three Points:

1. The regulatory framework is not yet fully established, but the core platform has already taken shape as a "reviewable" prototype: Although there are currently no approved cases of CRISPR-E (electrochemical) sensors in China, the National Medical Products Administration (NMPA) has initiated a special discussion on CRISPR-related products under the "Technical Review Guidelines for the Registration of In Vitro Diagnostic Reagents," with the expected release of review criteria for CRISPR-POCT by the end of 2025.

2. Technical bottlenecks are being addressed one by one, and conditions for industrialization are becoming increasingly ma-

ture.

3. The first "near-commercialization" scenarios are targeting high-value niche markets.

CRISPR electrochemical biosensors have not yet been widely commercialized like lateral flow antigen test strips, but they have reached the "convergence point of technology, regulation, and commerce." Once stability, multiplex detection, and standardized quality control systems are fully established, the first approved CRISPR-electrochemical POCT product is expected to emerge by 2026-2027.

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