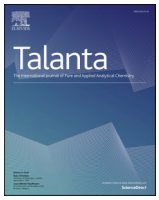
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CRISPR/Cas and Argonaute-based biosensors for nucleic acid detection

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ABSTRACT

Nowadays, nucleic acid detection technology has been applied to disease diagnosis, prevention, food safety, environmental testing and many other aspects. However, traditional methods still have shortcomings. Therefore, there is an urgent need for a simple, rapid, sensitive, and specific new method to supersede traditional nucleic acid detection technology. CRISPR/Cas(Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) system and Argonaute (Ago) system play an important role in microbial immune defense. Their targeting specificity, programmability and special *trans*-cleavage activity make it possible to develop some new platforms for nucleic acid detection in combination with a variety of biosensors. We introduce the origins of these two systems and the biosensors developed based on CRISPR/Cas system and Ago system, respectively, especially the prospects for the future development of Cascade Amplification biosensors. This review is expected to provide useful guidance for researchers in related fields and provide inspiration for the development of Cascade Amplification biosensors in the future.

1. Introduction

Nucleic acid detection methods have played a crucial role in various fields, including disease diagnosis, treatment, prevention, food safety and other aspects, making them indispensable for safeguarding public health and ensuring product safety [1–3]. Nucleic acids are biological macromolecules that carry and store genetic information in organisms. Their species specificity is one of the important reasons why they can be used in detection [4,5]. In practical application scenarios, since the number of specific nucleic acid sequences targeted is usually very low, most nucleic acid assays need to be combined with some amplification modality, which is based on the principle of exponentially expanding a trace amount of the target nucleic acid sequences, and then subsequently presenting them negatively or positively using probes or fluorescent labels, etc., so as to visualize the results of the assay [6,7]. Nucleic acid amplification is divided into two categories of variable temperature amplification and isothermal amplification according to the different reaction temperatures. Variable temperature amplification, such as polymerase chain reaction (PCR), the DNA double strand undergoes three steps of denaturation, annealing and extension, with different temperatures for each step, the temperature of the denaturation stage is generally 94°C–95°C, the annealing temperature is 50°C–65°C, and the extension temperature is raised to 72°C. Isothermal amplification, on the other hand, includes recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), etc., the whole reaction is carried out at a constant temperature, generally set at 37°C–45°C [8,9]. These traditional nucleic acid assays have been widely adopted, especially PCR-based nucleic acid assays which are the gold standard technology for most nucleic acid diagnostics, however, it usually requires relatively advanced infrastructure and professionally trained technicians, and although isothermal amplification-based nucleic acid assays reduce the equipment requirements, their results are not sufficiently accurate due to non-specific amplification [10,11]. These limitations mentioned above restrict the scenarios and scope of use of traditional detection methods, which are not very friendly to developing countries and some underdeveloped and remote areas. Therefore, there is an urgent need to develop a new method that is highly accurate, specific, easy to operate, and does not require complex instrumentation. If this new method also has the ability to detect single-base mutations, it is of great significance for the detection of drug-resistant mutations such as viral mutations, tumor mutations, and drug-resistant mutations caused by the misuse of antibiotics

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[12].

In order to solve the problems raised above, biosensor detection methods based on CRISPR/Cas system and Ago enzyme system are beginning to emerge. In order to adapt to the changing environment, microorganisms have evolved defense systems that can resist foreign invasions, and CRISPR/Cas system and Ago system are two of them, and their Cas proteins and Ago proteins are the nucleases that play an important role in the defense system, which can protect the host from invasive species [13,14]. The detection principles of the above two types of nucleases are shown in Fig. 1, and the detailed mechanism of action of each nuclease will be described in Section 3. The CRISPR/Cas system includes guide RNA (sgRNA) and Cas proteins [8], when a virus invades, the host can recognize foreign gene fragments, which are subsequently cleaved and integrated into its genome. This allows the host to quickly recognize these fragments during subsequent viral invasions. Consequently, Cas enzymes can act as nucleic acid endonucleases, accurately cleaving target viral genes and thereby facilitating immune defense [15,16]. Although CRISPR-based nucleic acid detection methods have begun to find applications in various fields, they continue to face challenges. For example, the cleavage of DNA by Cas9 [9] and Cas12 [10] depends on specific PAM sequences [11], which significantly restricts their detection flexibility [17]. Argonaute proteins represent another class of nucleic acid endonucleases with targeted cleavage capabilities and are regarded as promising next-generation nucleic acid detection platforms due to their similarity to the CRISPR/Cas system and their potential to overcome challenges associated with CRISPR/Cas [12,18]. Biosensor is an instrument that is sensitive to biological substances and detects them by converting these substances into other signals, such as physicochemical signals, through a series of specific reactions. A biosensor comprises a sensing element, an interface, a conversion element, and other electronic components. It utilizes biologically active substances as the sensing element—such as enzymes, antibodies, antigens, microorganisms, cells, tissues, and nucleic acids—to recognize the target biomass, subsequently converting the signals from this biological activity into physical or chemical signals for easier detection through the conversion element [19,20]. The advantages of biosensors, including high sensitivity, stability, rapid analysis speed, and high accuracy, provide them with broad application prospects in areas such as food, pharmaceuticals, biomedicine, and environmental monitoring [21].

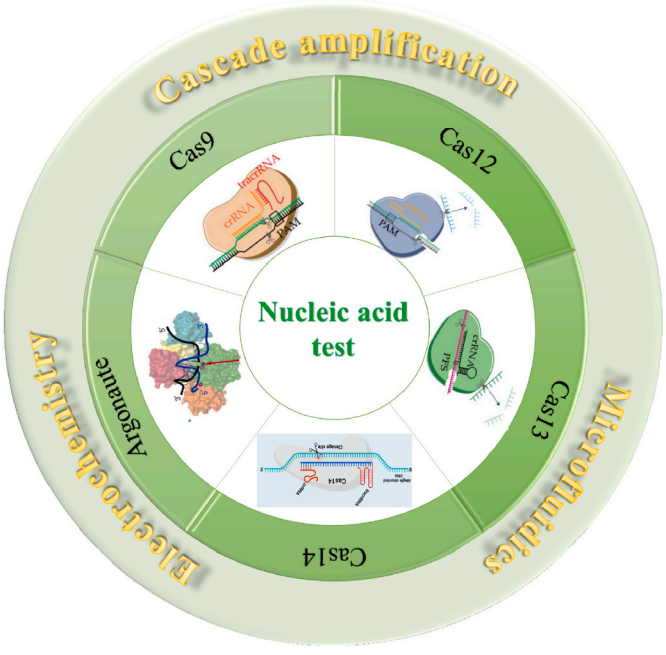


Fig. 1. Nucleic acid detection biosensor platform based on the CRISPR/Cas system and Ago system [30–32].

Biosensors derived from CRISPR/Cas system and Ago system play a very reliable role in pathogen detection, disease prevention and food safety [22–25]. With the advancement of the CRISPR/Cas system and the Ago system, a viable approach to achieving the ultimate goal of amplification-free nucleic acid detection has emerged. For example, these two systems can be integrated with microfluidic platforms, smart devices, electrochemical sensors, and autocatalytic biosensors to create a next-generation nucleic acid detection platform [26–28]. Among them, autocatalytic biosensors are valuable in multi-throughput nucleic acid detection due to their simplicity and high efficiency, and the research on autocatalytic biosensors based on the CRISPR/Cas system is relatively mature, but the Ago system is at the early stage of the research on autocatalytic biosensing, and the relevant domestic and international application examples are not yet many [29].

In this review, we summarize recent advances in the CRISPR/Cas system and the Ago system, as well as their applications in nucleic acid detection, with a focus on biosensors and amplification-free autocatalytic pathway constructs based on these systems. We begin by outlining the origins and operational mechanisms of both the CRISPR/Cas system and the Ago system, detailing the biosensors developed from different enzyme systems. We also enumerate existing nucleic acid autocatalytic sensing strategies aimed at achieving highly sensitive, amplification-free nucleic acid detection, highlighting how examples from the CRISPR/Cas system can inspire future developments of the Ago system. Finally, this paper envisions that future nucleic acid detection platforms should be continuously updated across several aspects, including portability, intelligence, targeted modification of more effective nucleases, and advanced signal processing methods, to establish more comprehensive biosensors for nucleic acid detection.

2. Origin of the CRISPR/Cas and Ago systems

In changing environments, the survival of bacteria and archaea is threatened by phage viruses and other organisms, prompting the evolution of various adaptive immune defense strategies, such as the CRISPR-Cas system and the Argonaute system, to protect themselves [30,31].

CRISPR/Cas. One of the components of the CRISPR/Cas system is a repetitive DNA sequence known as clusters of regularly interspaced short palindromic repeats (CRISPR), which scientists discovered while analyzing a gene that regulates the conversion of alkaline phosphatase [32], whose role in bacterial immune defense was only discovered in subsequent studies. CRISPR is interspersed with highly variable sequences derived from the genomes of invasive phage viruses, enabling host microbes to retain segments of the invading species and thereby prepare their defenses for subsequent invasions by the same species [33,34].The general principle by which the CRISPR/Cas system functions as an immune defense involves the following three steps [33,35,36]:(1) Adaptation: in this stage the DNA of the foreign invader is acquired and inserted into CRISPR to become the new spacer region. (2) Expression: In this stage, CRISPR is transcribed into pre-crRNAs, while genes encoding Cas proteins are also transcribed to participate in the process of post-transcriptional processing of pre-crRNAs, and then these crRNAs form complexes with one or several Cas proteins. (3) Interference: When the next round of invaders contain nucleic acid sequences complementary to crRNAs, they are recognized by the system, and then Cas proteins play the role of nucleic acid endonucleases to destroy the foreign organisms. Currently, CRISPR/Cas system is mainly divided into two categories and six types [37], the first category includes types I, III, and IV, which mainly utilizes a variety of Cas proteins together to realize the interference effect of the effector complex, and the second category includes types II, V, and VI, which is different from the first category in that it relies on a single Cas protein to play a cutting role only, such as Cas9, Cas12, Cas13, and Cas14, etc [38]. Fig. 2A demonstrates the characteristic genetic architecture of Class 1 and Class 2 CRISPR-Cas

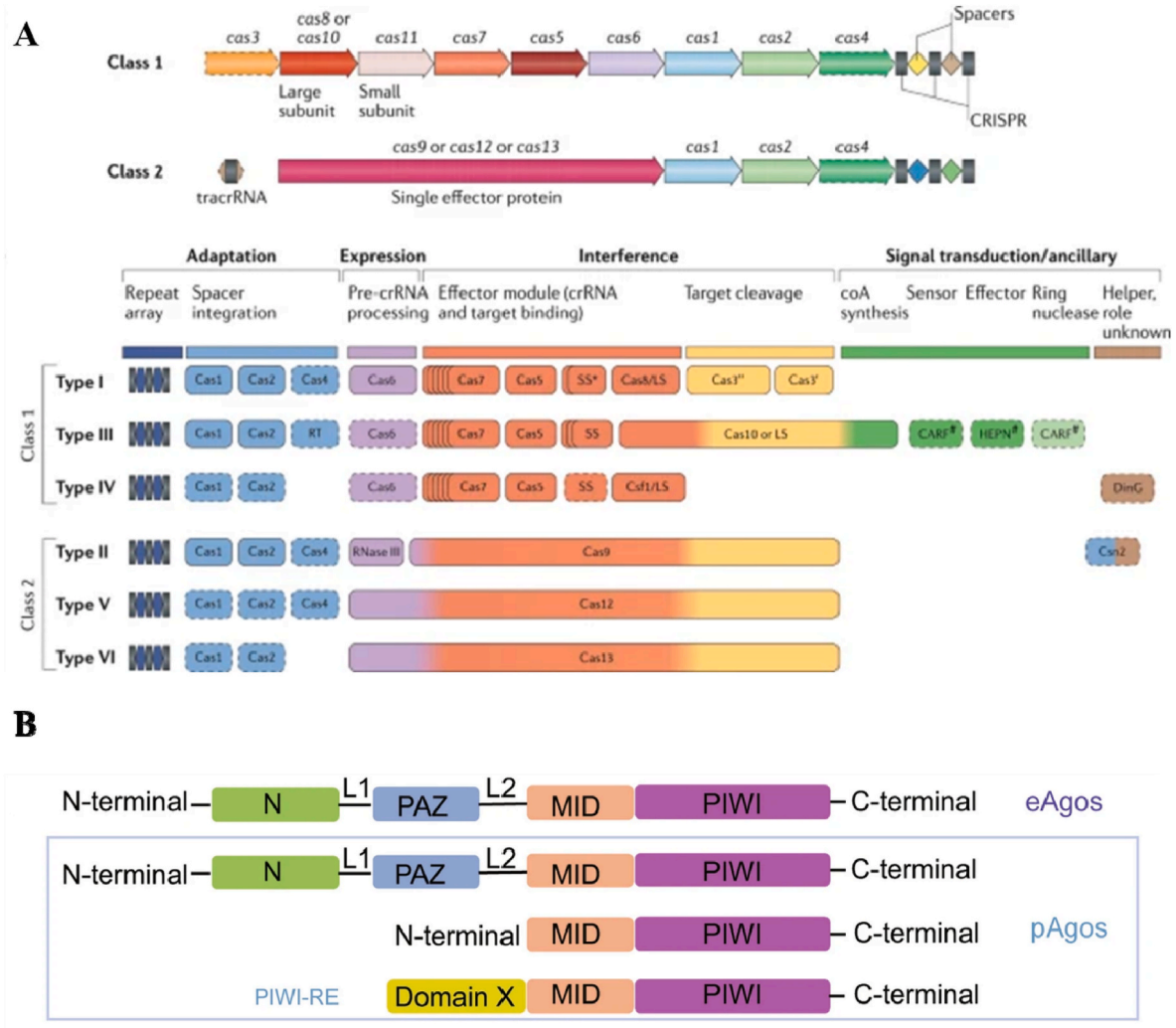


Fig. 2. CRISPR/Cas and Ago system types and structures.(A) The two classes of CRISPR–Cas systems and their organization [37].(B) The structure of Argonaute proteins [43].

loci.

Argonaute. Bohmert et al. first discovered the Argonaute protein while studying Arabidopsis mutants [39]. Due to the resemblance of the leaf curls in mutant plants to squid tentacles, the gene that regulates curling and the protein it encodes were named after the octopus *Argonauta argo* [40]. Argonaute protein is highly expressed in almost all organisms and is called eAGO in eukaryotes and pAGO in prokaryotes [41]. The structures of the two are shown in Fig. 2B. The structure of all eAgos contains an N-terminus, a C-terminus and two linkers L1 and L2 [42].There are three major classes of pAgos, namely, long pAgos, short pAgos, and PIWI-RE, of which the structure of long pAgos is very similar to that of eAgos [43]. The long pAgos consists of an N-terminal structural domain and a PAZ structural domain, which are connected to each other through L1, and these structures at the N-terminal end are then connected to the intermediate structure MID as well as to the C-terminal PIWI structural domains through L2 to achieve targeted recognition of cleavage targets according to this specific binding pattern [44]. Short pAgos have only MID and PIWI structures, the PAZ structural domain is not necessary for guided binding, and the lack of PAZ structure can still play a role in targeted cleavage [43]. PIWI-RE may function as an RNA-directed restriction system [45],structurally similar to short pAgos, with MID and PIWI domains, and differing from short pAgos by a new "structural domain X″ at its N-terminus [46].

3. CRISPR/Cas and Ago system-based biosensors

CRISPR/Cas. Numerous biosensor platforms based on the CRISPR/Cas system have been reported to achieve ultra-high sensitivity in nucleic acid detection, integrating the targeting specificity of the CRISPR/Cas system with the signal sensitivity of biosensing [47]. Given the well-established structure and function of Cas effector proteins in the CRISPR/Cas system, particularly the second class of nucleic acid endonucleases (Cas9, Cas12, Cas13, Cas14), which are widely employed in biosensors, we will describe each of the four Cas proteins separately and discuss their roles in conjunction with biosensors for nucleic acid detection(Fig. 3).

Cas9. The CRISPR/Cas9 system comprises three components: crRNA, tracrRNA, and the Cas9 protein. crRNA and tracrRNA form a double-stranded RNA through partial complementary base pairing, resulting in gRNA. This gRNA then associates with the Cas9 protein to direct it in recognizing and cleaving the target DNA sequence [48].In order to make the above process easier and to improve the stability of gRNA, Jennifer Doudna and Emmanuelle Charpentier fused crRNA and tracrRNA into a single RNA, called sgRNA, which facilitates the widespread use of the CRISPR/Cas9 system and has become a tool of choice for researchers [49].

SgRNA is only responsible for guiding the Cas9 protein to the target site, and subsequent modification of the target is continued by Cas9 [50]. Cas9 contains two specialized ribonucleic acid (RNA) domains that

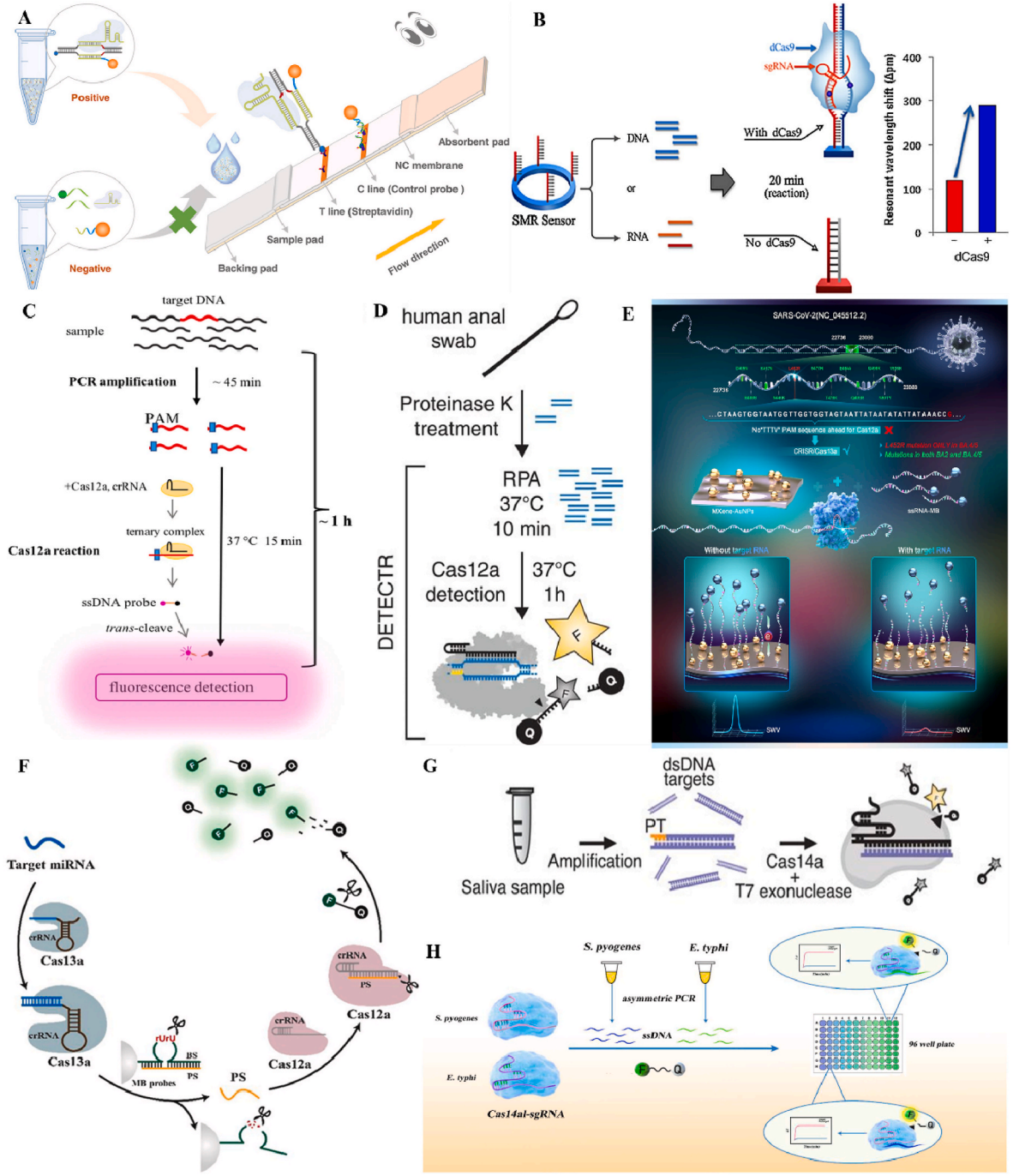


Fig. 3. Examples of biosensors based on CRISPR/Cas systems.(A) Schematic Diagram of CRISPR/Cas9-LFB for *Mycoplasma pneumoniae* Detection [59]. (B)CRISP-R/dCas9-mediated biosensor [60]. (C)An illustration of HOLMES [66]. (D)DNA extraction from human anal samples and HPV identification by DETECTR [62]. (E) SARS-COV-2 nucleic acid detection based on MoECS using the CRISPR-Cas13a system [73]. (F)Cas13a-12a amplified fluorescent biosensing strategy for detection of miRNA-155 [74].(G)Diagram of Cas14-DETECTR strategy and HERC2 eye color SNP [75].(H)Schematic diagram of CMP [79].

act on both strands of the DNA target to perform cleavage, a RuvC nuclease domain located at the amino-terminal end of the amino acid to cleave the DNA strand that is complementary to the sgRNA sequence, and a HNH nuclease domain located in the middle of the protein, which is responsible for the cleavage of the DNA strand that is the opposite of the complementary strand [51]. Cas9 hydrolyzes labile phosphates on double-stranded targets through a single metal ion mechanism in the HNH nuclease structural domain and a double metal ion mechanism in the RuvC nuclease structural domain [52–54].

It is worth noting that two conditions must be met for the Cas9 protein to successfully recognize and cleave the target sequence [52]: First, there is complementary base pairing between the 20 nt position at the 5′ end of the gRNA and the target DNA, and second, there is a base pair at the 3′ end of the target DNA. Corresponding PAM sequence, the PAM sequence is the specific recognition site of the Cas9 protein, and the Cas9 protein cuts in this region [48]. The common PAM sequence of Cas9 is 5′-NGG-3′, where N represents one of the four DNA nucleotides [55]. At this point, the CRISPR-Cas9 system completes the cleavage of the target nucleic acid and generates a site-specific blunt double-stranded break (DSB) [56], DSBs are the basis of all nucleic acid endonuclease-based gene editing, and changes in DNA sequence occur during DSB repair [57,58].

Qin et al. constructed a lateral flow biosensor (LFB) for the detection of Mycoplasma pneumoniae using the recognition specificity of CRISPR/Cas9, and its schematic diagram is shown in Fig. 3A. The researchers firstly used RPA to pre-amplify the target gene, and then combined with the CRISPR/Cas9 system to recognize the amplified target twice, so as to avoid false-positive results caused by non-targets.The high amplification efficiency of RPA and the appropriate temperature enabled the biosensor to have a detection limit of 3 copies at room temperature, and the accuracy of the detection was 100 % in the clinical trial, the whole process was completed within 30 min. The entire process can be completed in less than 30 min [59]. Yong et al. developed a novel molecular diagnostic tool based on the CRISPR/dCas9 system by combining nuclease-inactivated Cas9 (dCas9) and a single micro-ring resonator to achieve label-free and immediate detection of disease-causing nucleic acid molecules, and applied this biosensor to the detection of tick-borne diseases including scrub typhus (ST) and severe fever with thrombocytopenia syndrome (SFTS) (Fig. 3B). It should be noted that the above biosensor achieves single-molecule sensitivity of the two pathogens, with a minimum detection concentration of 0.54 aM for ST and 0.63 aM for SFTS. The entire assay is completed within 20 min, demonstrating 100-fold higher sensitivity than conventional Reverse Transcription PCR (RT-PCR) [60]. Cas9 stands as one of the most representative and widely utilized effector proteins, having been extensively applied in the development of various nucleic acid detection products. However, its lack of *trans*-cleavage activity imposes limitations when integrating with biosensing platforms for amplification-free detection.

Cas12. Cas12 is a second family of enzymes, an RNA-guided DNA nuclease, and Cas12a and Cas12b have been found to be the main classes [61]. The overall structure of Cas12 protein is similar to that of Cas9 protein, but it is worth noting that Cas12 protein has only one RuvC domain and lacks the HNH domain present in Cas9 protein. Only a single RuvC domain can cleave target dsDNA [62]. Unlike Cas9, Cas12 recognizes T-rich PAM sequences, such as 5′-TTN-3′, which facilitate self-catalyzed maturation of CRISPR RNA(crRNA). Following crRNA processing, Cas12 immediately initiates *cis*-cleavage of double-stranded DNA targets [62,63]. In addition, Cas12 also exhibits a "side--cutting"activity (*trans*-cleavage), non-specifically degrading nearby single-stranded DNA (ssDNA), and this property is now widely used in various assays by adding ssDNA probes, which are connected to fluorescent and quenching groups on both ends, to detect a fluorescent signal when Cas12 is cleaving the probe. A fluorescent signal can be detected when Cas12 cleaves the probe, and the fluorescence level can be analyzed to obtain data on the effectiveness of the assay [64].

Wang et al. found that Cas12a, once activated by the target ssDNA, would produce an additional cleavage effect, i.e., it would cut off other ssDNAs present in the system. Based on this, the team conducted various experiments to verify that both dsDNA and ssDNA can activate the *trans*-cleaving activity of Cas12a, and demonstrated that Cas12a from multiple species has *trans*-cleaving activity [65]. The team subsequently utilized the aforementioned conclusions to conduct in-depth research and developed HOLMES, a rapid point-of-care detection platform based on the *trans*-cleaving activity of Cas12a. By employing PCR to pre-amplify the target nucleic acid sequence, the Cas12a-crRNA complex performs specific recognition. This process first activates the collateral cleavage activity of Cas12a, which then exerts the *trans*-cleavage effect to sever the ssDNA probe for subsequent fluorescence detection. The detection sensitivity can reach as low as 10 aM [66] (Fig. 3C). Jennifer et al. also published a similar article at the same time. They also developed a detection system-DETECTR based on the *trans*-cleaving activity of Cas12a mentioned above. The difference is that it is combined with RPA to amplify the target sequence, and then The *trans*-cleaving activity of Cas12a is stimulated to cleave the ssDNA probe, converting the signal of the target sequence into a fluorescent signal to achieve the purpose of detection [62] (Fig. 3D). Finally, DETECTR was used to detect HPV16 and 18 subtypes as well as some other clinical samples, with a sensitivity reaching the atomic molar level.

Cas13. Cas13 is the main protein of type VI CRISPR-Cas system [64]. There are other members of the Cas13 family, including Cas13b, Cas13c, Cas13d, Cas13e and Cas13f [67]. Cas13 was also the first CRISPR/Cas system found to target RNA, and the initial effector complex, named C2c2, was found to have two types of ribonuclease (RNase) activity, one RNase responsible for pre-crRNA maturation processing, the other executes target RNA cleavage. Notably, the Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains are essential for Cas13's RNA-cleaving function. [68].Subsequent studies demonstrated that C2c2 is an RNA-targeted Cas endonuclease, which was later named Cas13a [69]. In the CRISPR-Cas13 system, Cas13 combines with crRNA to form a complex and then scans the target single-stranded RNA (ssRNA) in the system to find a position that is complementary to the crRNA spacer. This position is about 20-30 nt and is separated from the ssRNA by bases. complementary pairing [70]. After pairing is completed, the *cis*-cleaving activity and *trans*-cleaving activity of Cas13 are activated to cleave the target RNA and other existing RNAs [71]. In addition, similar to Cas9's requirement for neighboring motifs (PAM) in the prospacer region, Cas13 also has a certain substrate preference, requiring a less stringent prospacer flanking sequence (PFS) in the vicinity of the target, e.g., the PFS of LshCas13a is 3′H [72]. A number of biosensor platforms have also been developed for detection and diagnostic purposes based on the trans cleavage of ssRNA by Cas13, with a wide range of application scenarios.

Zhang et al. developed a highly sensitive and specific biosensor platform based on the CRISPR-Cas13 system and combined with MXene-AuNP (gold nanoparticle) composites as electrodes for the detection of single-base mutations in RNA viruses and clinical samples, which is important for the early diagnosis of new coronavirus variants as a complementary tool to RT-PCR. It plays an important role in the early diagnosis of new coronavirus variants as a complementary tool to RT-PCR. In the aforementioned study, MXene-AuNP composite materials (AuE-MXene-AuNPs) were first used to functionalize the working electrode (AuE), followed by the coverage of thiol and methylene blue-modified single-stranded RNA (SH-ssRNA-MB) on the electrode to form a reporter. When the target for detection is present in the system, Cas13 is activated to cleave ssRNA-MB from the surface of AuE-MXene-AuNPs, triggering relevant electron transfer events. This electronic change can be detected through electrochemical methods. The platform shows advantages in distinguishing different variants of the novel coronavirus, and its detection limit, stability, and specificity have also been further validated [73] (Fig. 3E). Xing et al. proposed a dual-amplification biosensing strategy using the CRISPR/Cas13a system to trigger Cas12a, called Cas13a-12a amplification. Cas13a and the corresponding crRNA form a complex that binds to the target in the system to activate the cleavage activity of Cas13a, which acts on the uracil nucleotide (rU) in the blocking strand (BS) structure, causing the primer strand (PS) on it to be released, and then the released PS goes to activate the cleavage activity of Cas12a to cut the single-stranded DNA probe, thus leading to the release of the PS on it. The released PS will then activate the cleavage activity of Cas12a to cut the single-stranded DNA probe, thus generating a detectable fluorescent signal. Finally, this strategy was applied to the detection of miRNA-155, and the detection limit of Cas13a-12a amplification was found to be as low as 0.35 fM, which provides a highly sensitive and specific miRNA biosensor platform for clinical applications [74] (Fig. 3F).

Cas14. Jennifer et al. discovered a new type of CRISPR effector protein in extreme enhanced archaea, named Cas14 [75], which only acts on ssDNA rather than dsDNA. This characteristic may have Virus enhancement and enhancement with sssDNA genome has important implications [76]. Cas14 is the smallest RNA-targeting endonuclease of all the CRISPR effector proteins identified to date, containing approximately 400–700 amino acids, making it easier to deliver to any tissue [76]. In addition, the binding between Cas14 and target sequences does not require special sequences similar to adjacent motifs in the pre-spacer region or PAM, unlike Cas9 and Cas12 which need to recognize a certain

base-rich PAM in order to exert cleavage activity [77], and thus can target a wider range of target genes. Similarly, the *trans*-cleavage effect of Cas nuclease is found on Cas14, which cleaves the ssDNA fluorescent reporter and emits a fluorescent signal when the Cas14/gRNA/ssDNA ternary complex is formed in the system [76]. Based on the above characteristics, researchers combined the CRISPR/Cas14 system with DETECTR to build a new DNA detection platform. DETECTR-Cas14 combines RPA isothermal amplification and the high specificity detection of Cas14 to record the target ssDNA and induce fluorescent reporter gene-coupled ssDNA. Parallel cleavage produces fluorescent signals for detection and diagnosis. Harrington et al. used the above platform to detect the human E3 ubiquitin protein ligase (HERC2) gene, which is associated with iris color variation. They designed gRNA to target the HECT and RLD domain-containing E3 ubiquitin protein ligase 2 (HERC2) gene in saliva samples of blue-eyed individuals with single nucleotide polymorphisms (SNPs), and then obtained saliva samples from blue-eyed and brown-eyed individuals. The ssDNA substrate of Cas14 was obtained using the phosphorothioate amplification method, and it was found that Cas14 has a stronger activity in identifying blue eye SNPs than Cas12. This method provides an effective way to screen disease-causing mutation genes [78] (Fig. 3G).

Wan et al. developed a highly sensitive and specific fluorescent tool for bacterial detection by utilizing the *trans*-cutting activity of Cas14a1, which was combined with molecular amplification to establish a CRISPR-Cas-based bioassay platform for rapid nucleic acid detection. The platform can accurately identify different pathogens present in milk samples, which is promising in the field of food safety [79] (Fig. 3H).

A comparison of the CRISPR/Cas systems in nucleic acid detection is reflected on their respective advantages, limitations, and potential applications. Different CRISPR/Cas systems are used in various nucleic acid detection fields. Cas12 is widely used for DNA detection due to its high sensitivity and fast response, making it ideal for amplification-free platforms in clinical screening and viral detection. Cas13 is specialized in RNA virus detection, directly recognizing RNA sequences, making it suitable for quick detection of viruses like influenza and coronaviruses. Cas14 focuses on single-stranded DNA detection and shows flexibility in viral detection, while Cas9 is primarily used for gene editing but also for gene variant detection.

The tolerance of CRISPR/Cas systems to target sites varies, affecting their applicability. Cas12 is limited by specific PAM sequences, restricting its use for certain targets. Cas13 requires specific RNA sequence structures, which may limit its accuracy in complex samples. Cas14 offers PAM-independent cleavage, providing more flexibility but is restricted to single-stranded DNA. Cas9 recognizes multiple PAM sequences, allowing for precise targeting, but its lack of *trans*-cleavage activity limits its use in amplification-free detection.

The sensitivity and speed of CRISPR/Cas systems differ, making them suitable for different applications. Cas12 provides high sensitivity and fast response, ideal for real-time and amplification-free platforms. Cas13 is highly sensitive but slower in detection, suitable for sensitive RNA detection. Cas14 performs quickly for single-stranded DNA detection, but its narrow target range limits multiplex detection. Cas9 has lower sensitivity and speed, primarily used in gene editing.

CRISPR/Cas systems face challenges in nucleic acid detection, with future research focusing on overcoming these limitations. Cas12's reliance on PAM sequences limits its target range, and engineering may expand its applicability. Cas13's dependence on RNA structure could affect accuracy in complex samples, requiring improvements in sensitivity. Cas14 excels in single-stranded DNA detection but has a narrow target range, potentially expanding to dsDNA. Cas9's lack of *trans*-cleavage activity restricts amplification-free detection but remains promising for precise gene variant detection.

Table 1 shows the information of the above common Cas effector proteins, with different advantages, researchers can choose the appropriate Cas effector proteins according to the experimental needs, in order to improve the success rate of experiments.CRISPR/Cas system can not only resist the microorganisms invaded by foreign pathogens, but also be used as a tool to build various types of nucleic acid detection related biosensors, generally the most widely used Class II CRISPR/Cas system, Table 1 also gives a brief example of these Cas effector protein-based biosensors, which are widely used in the field. Generally, two types of CRISPR/Cas systems are the most widely used, and Table 1 also briefly summarizes the biosensors based on these Cas effector proteins [81,82]. Recently, many autocatalytic biological reaction pathways have been introduced to construct various new biosensors for nucleic acid detection. For example, Nie et al. designed a feedback amplification system CONAN based on CRISPR-Cas autocatalytic drive for ultra-sensitive nucleic acid diagnosis. The entire CONAN system is divided into two parts: the first part is to detect the real target, similar to the traditional CRISPR reaction, with the purpose of activating the *trans*-cleaving activity of Cas12a to cleave a special DNA/RNA hybrid structure. Cas12a cuts the DNA and RNA in the hybrid structure to separate, forming free crRNA, which binds to another paired target in the system and Cas12a stimulates the *trans*-cleaving activity of the second Cas12a, which continues to cut to produce more free crRNA.Form a positive feedback loop to achieve the purpose of exponentially amplifying the signal [83].

Table 1
Comparison of class 2 CRISPR systems.

Cas protein	Cas9	Cas12	Cas13	Cas14
CRISPR/Cas system type [37]	II type	V type	VI type	V type
Molecular weight [22]	100−200 kDa	100−200 kDa	100−200 kDa	40−70 kDa
Guide RNA	crRNA and tracrRNA	crRNA	crRNA	crRNA and tracrRNA
PAM/PFS	PAM	PAM	PFS	\
Cleavage activity(cis/trans)	cis	cis and trans	cis and trans	cis and trans
Target	dsDNA	dsDNA、ssDNA	ssRNA	ssDNA
output signals [80]	fluorescence、colorimetry,、electrochemistry、lateral flow strips			
Examples of combination with biosensors [47,78]	NASBACC、RCH	HOLMES、DETERCTR、HOLMESv2	SHERLOCK、SHERLOCKv2	DETECTR-Cas14

Argonaute. Argonaute(Ago)proteins exist in both prokaryotes (pAgo) and eukaryotes (eAgo). Both are nucleases guided by small RNA or DNA and are related to the RNA interference process in organisms (RNAi) [84]. A more detailed comparison of these two classes of Ago proteins, including their guide molecule specificity, cleavage activity, and signal output characteristics, is presented in Table 2 below. RNAi refers to several types of small non-coding RNAs that regulate gene expression by specifically recognizing complementary RNA targets. It is crucial in host immune defense and transcriptional and post-transcriptional gene regulation in other eukaryotic organisms [85,86].The main role is played by an RNA-induced silencing complex (RISC). RISC is formed by a combination of Ago and small interfering RNA (siRNA). RISC recognizes complementary RNA targets, and then Ago exerts endonuclease activity for cleavage purposes. target [87]. Recently, there have been many studies on combining Ago with biosensors for nucleic acid detection, which is a prospective approach compared with the traditional CRISPR/Cas, and it is expected to be able to solve some of the problems in the CRISPR system, such as the limitations of the proximity of the original spacer to the adjacent motifs (PAM) and the difficulty of multiplexed pathway detection [88]. The eAgos exhibit a more uniform structural architecture and demonstrate high evolutionary conservation, functioning exclusively in RNA interference processes [89]. In contrast, pAgos display remarkable structural

Table 2
Comparison of ago systems.

Ago protein	pAgos	eAgos
protein family [110]	PIWI-protein superfamily	PIWI-protein superfamily
host type	prokaryote	eukaryote
Domain architecture [110]	Short pAgo:MID、PIWI Long pAgo:N、PAZ、MID、PIWI	N、PAZ、MID、PIWI
Guide	RNA and DNA [111]	RNA [90]
PAM/PFS	/	/
Cleavage activity [80]	sequence specific cleavage	sequence specific cleavage
output signals [80]	fluorescence	
Examples of major families [110]	*Tt* Ago、*Aa* Ago、*Mj* Ago、*Rs* Ago	Trypanosoma Ago family、WAGO family、Ago-like family、PIWI family
Examples of combination with biosensors	SARS-CoV-2 PAND [93]	PON [107]、Pf Ago-LFB [108]

and functional diversity, participating in host defense mechanisms against foreign genetic elements [90]. In addition, through the biochemical structure analysis of the identified Ago proteins, it was found that they have efficient catalytic properties in vivo and in vitro, including high recognition efficiency, good cleavage efficiency, etc [91]. In the process of nucleic acid detection, the target is first pre-amplified by variable temperature or isothermal amplification, Ago is guided by the 5′ phosphorylated auxiliary chain to complete the target recognition and cleavage, and the cleavage produces a new 5′ phosphorylated fragment, which serves as a new auxiliary chain for the fluorescent probe cleavage [92]. This target-specific cleavage activity is termed Ago protein's *cis*-cleavage, which represents the primary mechanism utilized in current Ago-based detection systems. Unlike certain Cas enzymes (e.g. Cas12, Cas13), Ago proteins generally lack *trans*-cleavage activity. Finally, signals are detected and analyzed through a series of molecular and biochemical methods, such as real-time quantitative PCR (qPCR), droplet digital PCR (ddPCR), and Sanger sequencing [92].

Ma et al. established a nucleic acid detection platform for large-scale screening of COVID-19 based on *Pyrococcus furiosus Argonaute* (PfAgo) and combined with RT-PCR. The method was named SARS-CoV-2 PAND (Fig. 4A). First, COVID-19 RNA is extracted from nasopharyngeal or oropharyngeal swabs as a template for RT-PCR amplification, mainly to obtain conserved sequences in the viral genome that can be used for detection. Then mix PfAgo, the corresponding crRNA, and the reporter probe with the appropriate buffer, and incubate at 95 °C for 20–30 min. Finally, the fluorescence signal is collected and detected to complete the virus detection process. The entire process is approximately Done in 1 h. In addition, SARS-CoV-2 PAND can also be used to detect COVID-19 mutants (D614G), and the detection results are 100 % accurate compared with RT-qPCR [93].

Unlike the CRISPR/Cas system, the Ago system has certain differences in guide types, target detection and cutting steps, and output signal methods [80]. Generally speaking, RNA is mostly used as a guide for Cas-based methods. For the Ago system, the guide can be DNA or a shorter RNA. It is worth noting that in addition to the existence of a guide, Targeted cleavage of target nucleic acids also requires 5′ phosphorylation modification [62,94]. Almost all of the Cas proteins in the CRISPR/Cas system have both *cis*- and *trans*-cleaving activities, such as Cas12a and Cas13a, etc. In particular, the *trans*-cleaving activity has the function of signal amplification, which enhances the detection sensitivity of the system [95]. Ago proteins do not have *trans*-cutting activity and rely only on recognition of specific sequences to cut nucleic acids [96]. Therefore, target detection based on the Cas method only requires one step of cleavage to output a signal to achieve the detection purpose, while the Ago-based method requires two steps of cleavage to generate a detection signal, that is, the first step of cleavage acts on the detection target, and the product as a guide for the second step of cleavage, both steps strictly follow base pairing rules to ensure specificity [80]. Finally, the signal output methods based on the Cas system include fluorescence, colorimetry, and electrochemistry; however, the Ago method typically utilizes only fluorescence as the output signal [80].

Furthermore, these two systems exhibit distinct differences in performance metrics, limitations, and applicability.

Specificity. Both CRISPR and Ago systems demonstrate high specificity in targeting nucleic acid sequences. However, CRISPR systems typically require a PAM sequence, which may restrict their detection versatility. In contrast, Ago systems operate without PAM sequence constraints, enabling enhanced specificity across a broader range of targets and providing researchers with a versatile detection platform.

Sensitivity. In terms of sensitivity, CRISPR-based biosensors typically demonstrate superior sensitivity, particularly when coupled with PCR or isothermal amplification techniques for detecting low-abundance targets. Additionally, the *trans*-cleavage activity of Cas12, Cas13, and Cas14 further enhances detection sensitivity to a significant degree [97]. In contrast, Ago systems also achieve high sensitivity when integrated with pre-amplification strategies and often exhibit more stable performance under low-copy-number conditions, attributed to the inherent stability of DNA guide sequences during detection processes. The RT-RPA-PfAgo system by Xu et al. detects RNA at 1 copy/μL in 35–45 min through sequential RT-RPA and PfAgo reactions, while maintaining high specificity [98].

Temperature Stability. Thermal stability is another important differentiating factor. Most Cas proteases employed in detection systems exhibit limited thermostability(room temperature), whereas Ago proteases demonstrate broader temperature tolerance ranges(60°C–80°C) [90]. This characteristic makes Ago-based systems potentially more suitable for extreme environmental conditions. Furthermore, this comparison suggests a promising direction for improving CRISPR/Cas detection technologies, developing novel thermostable Cas variants to enhance their adaptability to diverse detection environments.

Detection Time. Regarding detection time, both CRISPR- and Ago-based biosensors demonstrate relatively short detection times when coupled with nucleic acid amplification strategies. Wang et al. developed a portable CRISPR-RPA system for rapid SARS-CoV-2 detection, achieving 0.5 copies/μL sensitivity in 20 min using smartphone-based readout [99]. Kong et al. developed an exponential isothermal amplification-coupled TtAgo assay for miRNA detection, achieving attomolar sensitivity within 30–35 min [100]. While some amplification-free CRISPR/Cas detection methods also achieve reasonably rapid detection, few amplification-free Ago-based detection systems have been reported to date unless supplemented with specialized components to enhance performance. Hu et al. developed the NAPTUNE platform, which achieves attomolar-level nucleic acid detection within 45 min without pre-amplification, leveraging the synergistic enzymatic activities of APE1 and PfAgo endonucleases [101].

Off-target Effects and Application Range. Both systems, however, are susceptible to off-target effects, particularly when encountering highly variable target sequences or imperfect guide-target complementarity. However, Ago systems exhibit significantly lower off-target activity compared to CRISPR/Cas systems, attributable to their stringent Watson-Crick base-pairing requirement. The enhanced specificity is further reinforced by the inherent stability of DNA guide strands. Importantly, this precise pairing mechanism prevents non-specific cleavage, a distinct advantage over the *trans*-cleavage activity characteristic of Cas proteases, and makes Ago systems particularly suitable for multiplex detection applications. CRISPR-based biosensors have been widely utilized for detecting a variety of nucleic acids, including DNA and RNA. Argonaute systems are more versatile in certain applications, such as small RNA detection and target RNA interference, which CRISPR systems may not readily perform without additional components.

Nowadays, more and more biosensor platforms are developed based

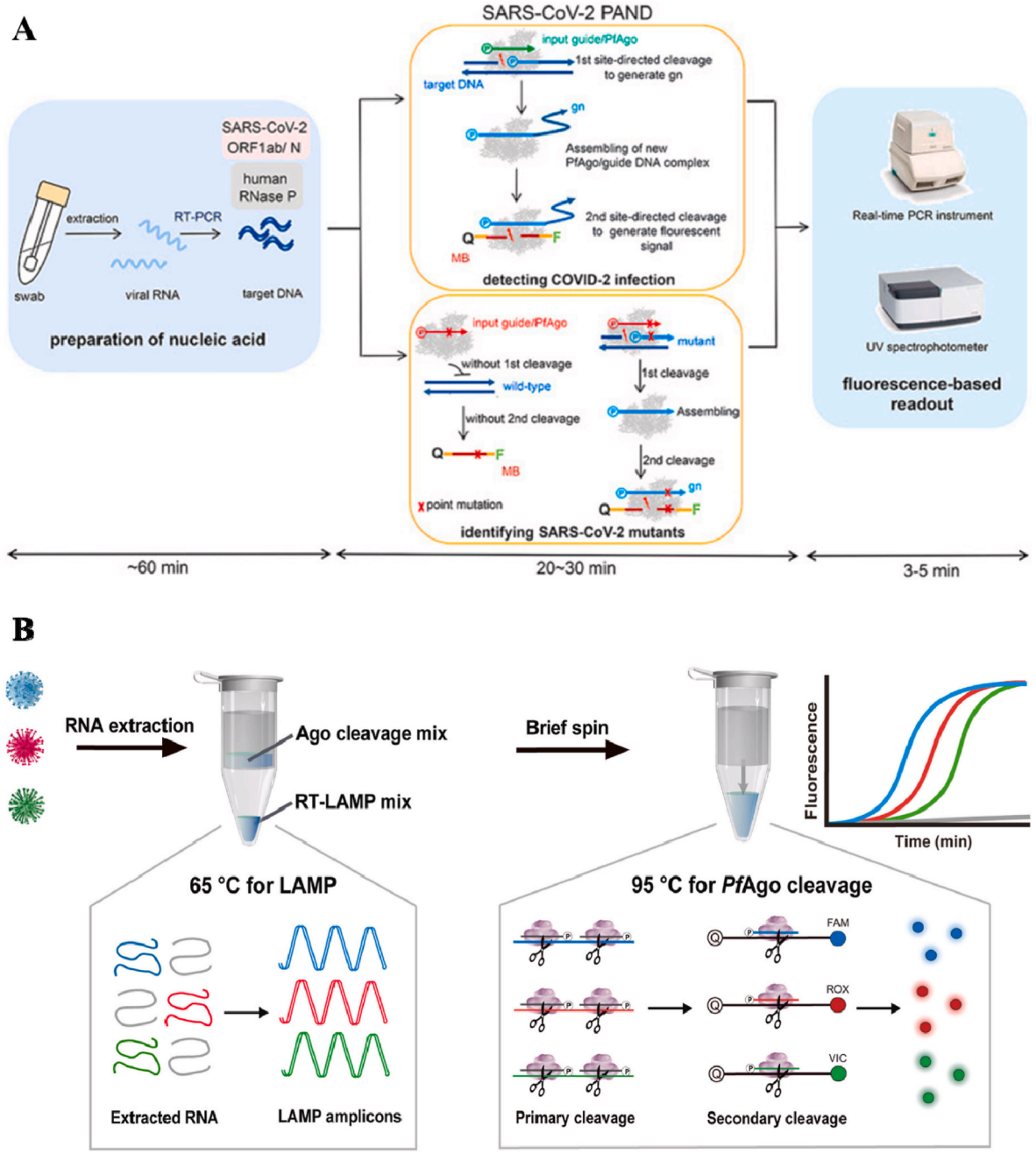


Fig. 4. Examples of biosensors based on Argonaute systems.(A)SARS-CoV-2 PAND workflow. MB: molecular beacon; gn: newly generated guide; Q: quencher; F: fluorophore [93].(B)The *Pf*Ago-based MULAN for simultaneous detection of three targets [109].

on CRISPR/Cas system [102]. The CRISPR/Cas system, combined with multiple signal output modes, converts the target nucleic acid sequence information into detectable signals, which can be used for pathogen detection, cancer mutation detection, and single nucleotide polymorphism identification, etc., and shows good prospects in nucleic acid detection [103–105]. Currently reported CRISPR/Cas biosensing systems can be divided into three categories according to different Cas effectors, including Cas9-based systems, Cas12-based systems and Cas13-based systems [47].Xu et al. combined a graphene self-assembled three-dimensional structure (3D GR) with noble metal nanomaterials (AuPtPd) to form a complex, and subsequently constructed an electrochemical sensor that triggers an entropy-driven strand displacement reaction (ESDR) by integrating the CRISPR/Cas9 system. This approach was the first to utilize this method for the detection of mutant epidermal growth factor receptor (EGFR) ctDNAs. The method primarily relies on the specific cleavage activity of Cas9 on the target DNA to generate the primer target strand necessary for initiating the ESDR. Specifically, the sequence produced by the initial target cleavage serves as the initiating sequence for the next cycle, thus amplifying the signal layer by layer. Furthermore, the low non-specificity of ESDR, the efficient cleavage activity of Cas9, and the excellent catalytic activity of metal nanomaterials contribute to making the CRISPR/Cas9-based 3D GR/AuPtPd hybrid nanoflowers an outstanding electrochemical sensing platform. This platform can be effectively employed as a multifunctional DNA detection method for bioanalysis and clinical diagnosis [106]. Liang et al. constructed an electrochemical biosensor with switchable signals to achieve dual detection of human immunodeficiency virus (HIV) and human papillomavirus (HPV-16) DNA using CRISPR/Cas12a, with detection limits of 30 fM and 0.32 pM, respectively. Moreover, the entire detection process

can be successfully completed within 16 h without the need for additional nucleic acid amplification methods. This method integrates the CRISPR/Cas system with spherical nucleic acids (SNA). The "on" signal is obtained by incubating the biosensor with the chemiluminescent group of HIV DNA and carbon dot (CD)-labeled SNA. The "off" signal is generated after the biosensor is incubated with auxiliary DNA, CD-labeled SNA, and the Cas12a/crRNA/HPV-16 dsDNA complex. This method has been successfully employed in the clinical diagnosis of HIV DNA and HPV-16 DNA [107].Gootenberg and coworkers developed a CRISPR/Cas13-based nucleic acid detection platform, called SHERLOCK (specific high sensitivity enzymatic reporter unlocking).This platform has been successfully used to detect Zika virus and dengue virus, antibiotic resistance genes and cancer mutations, etc [47,108].

The development of biosensors based on Ago proteins has been relatively slow, but there are still some examples that show excellent promise.Yan's team developed a Multiplexed Ago-based Nucleic Acid Detection System (MULAN) (Fig. 4B), which integrates RT-LAMP with the Ago system into a one-pot system, and used a portable device to successfully differentiate between SARA-CoV-2 wild-type and mutant individuals. In addition, this method successfully realized the triple detection of SARS-CoV-2, influenza A and B viruses in clinical trials, which demonstrated that this platform provides a rapid and sensitive strategy for nucleic acid detection [109].

The above biosensing platforms based on CRISPR/Cas system and Ago system combine isothermal amplification technology, electrochemical sensing technology and nanomaterials, etc., which can realize multi-detection and pass the examination of clinical samples, indicating that the biosensors developed based on the two systems have high research value and application value.

4. Autocatalytic biosensor strategy based on CRISPR/Cas and Ago systems

Artificial biochemical circuits based on nucleic acid chemistry, called cascade amplification pathways, exhibit excellent application prospects in detection-related scenarios due to their programmability and can serve as a significant approach to achieving the goal of avoiding amplification [112]. In these pathways, the designed nucleic acid molecules as input signals can be regarded as catalysts to make the specified oligonucleotides released, and the latter are both the output signals of the previous reaction and the input signals of the next reaction, thus constructing a nucleic acid autocatalytic cascade amplification circuit with exponentially increasing kinetics compared with the traditional signal readout method, which makes the detection more sensitive [113]. Current CRISPR/Cas-based amplification-free nucleic acid detection systems typically demonstrate detection limits at the picomolar (pM) level, which remains inadequate for meeting the sensitivity thresholds required in most clinical diagnostic applications. In contrast to conventional amplification methodologies such as PCR that depend on thermal cycling for nucleic acid amplification, cascade amplification systems operate through enzyme-driven signal enhancement mechanisms without requiring temperature modulation. This thermal-free amplification process can dramatically shorten reaction times, presenting distinct advantages for point-of-care (POC) diagnostic implementations where time-sensitive decision-making is critical - particularly in emergency healthcare scenarios. Notably, the elimination of thermal cycling requirements not only simplifies operational protocols but also reduces dependence on sophisticated instrumentation, thereby enhancing compatibility with resource-constrained settings including remote healthcare facilities and field-deployable diagnostic platforms. The CRISPR/Cas system and the Ago system have specific target nucleic acid sequence-targeted recognition functions and *trans*-cleavage enzyme activities related to signal output. The CRISPR/Cas system and the Ago system are integrated with the autocatalytic artificial biological circuit to construct The cascade amplification nucleic acid detection platform driven by CRISPR-Cas and Ago proteins can achieve rapid and accurate nucleic acid detection.

4.1. CRISPR/Cas system

Currently, autocatalytic biosensing platforms developed for the CRISPR/Cas system have been successfully realized and used for the detection of DNA or RNA targets with good overall sensitivity and specificity. In these autocatalytic biosensing platforms, the nucleic acid autocatalytic method utilizes a cascade or cyclic reaction to produce multiple outputs for a single signal input, which theoretically provides an advantageous pathway for signal amplification [114].Therefore, the autocatalytic pathway of nucleic acids based on the CRISPR/Cas system enables more sensitive target detection. This platform can achieve high sensitivity while ensuring detection accuracy, making it suitable for a wide range of detection scenarios. In the CRISPR/Cas system, the signal amplifier module is designed with a rational structure, fully utilizing the *trans*-cutting activity of the Cas protein to facilitate the autocatalytic generation of active Cas protein. This process constructs a positive feedback pathway, resulting in an exponential increase in the catalytic effect of the Cas protein. For example, as illustrated in Fig. 5A, Nie et al. designed a CRISPR-Cas driven autocatalytic nucleic acid circuit called CONAN, which can achieve ultra-sensitive nucleic acid diagnosis. The focus of CONAN is to design a switchable cage guide RNA (scgRNA) with self-reporting ability. scgRNA contains the gRNA sequence corresponding to the target and an inhibitory DNA sequence (iDNA) complementary to the gRNA. The switch is Cas12a. The reflex cleavage enzyme activity generated after activation destroys the structure of scgRNA, and the released gRNA combines with other Cas12a in the system to form a new active complex, which continues to participate in the target identification and detection process. CONAN has been successfully used to detect hepatitis B virus (HBV) infection in clinical samples and to detect single nucleotide mutations (SM) related to human bladder cancer. Especially in SM detection, this platform has significantly improved the sensitivity of detection [83]. In addition, Deng et al. established a cascade amplification nucleic acid detection strategy based on the CRISPR/Cas system called AutoCAR (Autocatalytic Cas12a Circular DNA Amplification Reaction) (Fig. 5B). This strategy allows a single nucleic acid target to activate multiple ribonucleoproteins, significantly enhancing the cleavage efficiency of each target on the reporter gene, enabling DNA detection at a level of 1 aM (<1 copy/μL) within 15 min at room temperature, without the need for additional amplification tools. In AutoCAR, the researchers designed a small DNA nanostructure called the "Cir-mediator" (Fig. 5B), which consists of a double-stranded region (dsDNA) and a single-stranded structure (ssDNA). The dsDNA region contains PAM sequences and sequences complementary to the gRNA in the Cas12a RNP. When the target is present in the system, the first round of the CRISPR reaction proceeds normally, activating Cas12a′s *trans*-cleaving enzyme activity to cleave the ssDNA region of the Cir-mediator. This results in the nano-DNA structure changing from circular to linear, with the newly formed linear dsDNA pairing complementarily with the gRNA of the RNP, completing the assembly of the RNP and activating a new round of the CRISPR reaction, thus forming a cascade within the AutoCAR system (Fig. 5B). Moreover, this strategy enables the detection of SNP mutations in circulating tumor DNA, *H. pylori* genomic DNA, and SARS-CoV-2 genomic RNA in plasma at levels down to 1 aM [115].

Zhou et al. developed a simple amplification-free DNA virus nucleic acid detection platform called substrate-mediated autocatalysis of CRISPR/Cas12a (DSAC) for the detection of African swine fever virus (ASFV) (Fig. 5C). The platform achieves a cascade cycle reaction of signal amplification by designing a DNA substrate containing a special structure. This DNA substrate contains a crRNA2 recognition sequence and a special closed-loop structure. The closed-loop structure prevents the Cas12a/crRNA2 complex from binding to the substrate DNA. Only After adding the target DNA and being recognized by Cas12a/crRNA1 and stimulating the *trans*-cleaving enzyme activity of Cas12a to act on

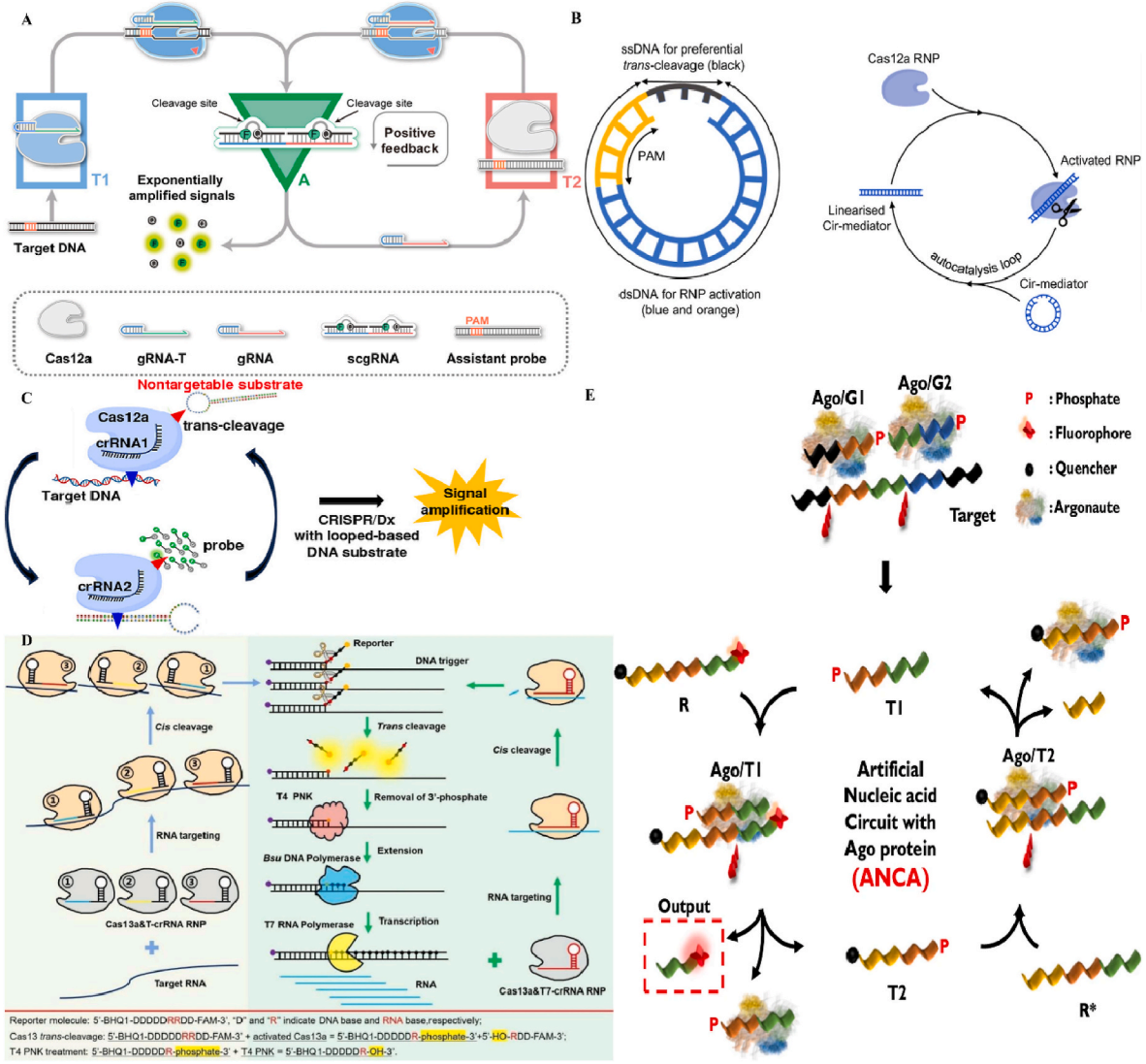


Fig. 5. Examples of autocatalytic biosensors based on CRISPR/Cas and Ago systems. (A)CONAN [83]. (B)AutoCAR [115]. (C)DSAC [116]. (D)The CRISPR-Cas13a autocatalytic system for one-tube target amplification-free RNA detection [117]. (E)ANCA [28].

the substrate DNA, it will cause a conformational change so that it can contact Cas12a/crRNA2, and then stimulate more active Cas12a, leading to more ssDNA detection. The needle and substrate DNA are cleaved, producing an exponentially amplified fluorescent signal. The DSAC system accurately detects African Swine Fever Virus (ASFV) in pig blood samples in less than 20 min with femtomolar sensitivity [116]. Compared to existing amplification-free CRISPR/Dx platforms, DSAC provides a cost-effective and easy-to-use assay.

Rao et al. proposed a CRISPR/Cas13a-based RNA biosensor system to address the challenges of cumbersome process and easy cross-contamination faced by current detection methods. The system assembled Cas13a, T4 polynucleotide kinase, Bsu DNA polymerase, T7 RNA polymerase, and a combination of DNA probes into a single tube, realizing one-pot detection of isothermal RNA. The combined DNA probe contains a complementary double-stranded region and two single-stranded DNAs of different lengths. The 3′ and 5′ ends of the short single-stranded DNA are modified with fluorescent groups and quenching groups, and the 5′ end of the long single-stranded DNA is modified. The end is the reverse non-complementary spacer sequence of T7-crRNA, the 3′ end is the reverse complementary sequence of the T7 promoter, and the 5′ end of the short single-stranded DNA is partially bound to the 3′ end of the long single-stranded DNA. When the RNA target is present in the system, the Cas13a/T-crRNA complex is activated by binding to it, which in turn activates the *trans*-cleaving activity of Cas13a to cleave the short single-stranded DNA, producing 3′-phosphate groups. Subsequently, T4 polynucleotide kinase transforms the 3′-phosphate terminus into a 3′-hydroxyl terminus, allowing Bsu DNA polymerase to synthesize a complete DNA double strand. This double strand then binds to the Cas13a/T7-crRNA complex, reactivating Cas13a′s *trans*-cleaving activity to act on the next substrate DNA, thereby initiating a cyclic cascade of reactions [117](Fig. 5D).

4.2. Argonaute system

Similar to Cas proteins, Ago proteins possess specific target recognition abilities and *trans*-cleaving enzyme activity, allowing them to function as endonucleases against ssDNA in the system. Based on these characteristics, Ago proteins can also be developed as a nuclease tool for novel autocatalytic cascade biosensor platforms.In particular, pAgo proteins can cleave nucleic acid targets without special sequence, unlike Cas proteins whose recognition is dependent on the prototypical spacer area neighboring motifs (PAMs), and thus have a wider applicability compared to Cas proteins. Moreover, the Ago protein, which originates from thermophilic microorganisms, exhibits high-temperature

resistance, making it a widely applicable prerequisite that enhances the feasibility of Ago-based nucleic acid diagnostic methods. Additionally, systems utilizing the Ago framework can employ DNA-guided probes, which are more stable and cost-effective than RNA, further improving the effectiveness of the detection method [28].

Recently, some Ago-based nucleic acid detection methods have also been reported, such as the realization of Zika virus detection based on *Pf Ago*, using which the diagnostic results of analyzing simulated clinical samples or Zika virus samples were in complete agreement with the qRT-PCR results [118], and isothermal amplification centrifugal microfluidic microchip detection bioreactor based on *Thermus thermophilus Argonaute* (*Tt Ago*), which has been able to sensitively detect the presence of *S. aureus*, and can simultaneously perform multiplexed detection, which is promising in practical applications [119]. However, these current nucleic acid detection methods based on Ago protein still need to be combined with amplification methods to function, such as PCR, LAMP, etc. Ma et al. After converting SARS-CoV-2 RNA into cDNA through reverse transcription, they used the cleavage enzyme activity produced by the activated Ago protein to act on the cDNA to generate a corresponding fluorescent signal, thereby achieving the purpose of detecting the target [93]. Feng et al. developed a multiplexed nucleic acid detection platform (MULAN) based on Ago protein, which combined isothermal amplification and Ago system to realize the simultaneous detection of SARS-CoV-2 and influenza viruses and the typing of mutated genes [109]. Although the methods described above can achieve target detection by utilizing the specificity of the Ago protein and the signal output strategy of its endonuclease activity, they require exploration of specific systems, and the reaction procedures are not sufficiently straightforward. Given the wide applicability and programmability of the Ago system, we anticipate that Ago-derived autocatalytic cascade amplification biosensors will become one of the future trends in nucleic acid detection. The development of Ago-based autocatalytic biosensors is still in its early stages and needs to draw on the successful experiences of CRISPR in this field. We believe that in the near future, Ago-derived autocatalytic biosensors will be capable of detecting any nucleic acid target.

Jiang et al. developed an artificial nucleic acid circuit with Ago protein (ANCA) to realize one-step amplification-free isothermal DNA detection, which was successfully applied to the detection of carbapenemase-producing *Klebsiella pneumoniae* (CPKP). In ANCA, the Ago protein first binds to guide DNA1 (G1) and guide DNA2 (G2) to recognize the target. The Ago protein exerts its cutting activity between the 10th and 11th bases at the 5′ end of each guide DNA, cleaving the target nucleic acid at the active site between them. Subsequently, a short DNA fragment (T1) is released from the target DNA, which can also serve as an additional guide DNA. The Ago/T1 complex then recognizes and cleaves the reporter gene R, releasing two short DNA strands; one of these outputs a fluorescence signal, while the other is a new trigger DNA (T2). T2 can combine with the Ago protein to form an Ago/T2 complex that recognizes and cleaves the complementary reporter gene R*, generating T1 to complete the positive feedback loop. Once the positive feedback pathway is established, as long as the target DNA remains present in the system, the subsequent cleavage reactions will continue, ultimately allowing for the detection of the target nucleic acid through fluorescence [28](Fig. 5E).

The emerging CRISPR/Cas and Ago-mediated biosensing systems demonstrate significant potential for nucleic acid detection through cascade amplification mechanisms. Despite their promise as amplification-free diagnostic tools, substantial technical barriers impede their translation into practical POCT and home-use applications, particularly in achieving clinical-grade performance metrics.

Specificity Limitations. A critical challenge lies in the inherent off-target effects exacerbated by multi-stage signal amplification. The sequential reaction architecture can amplify non-specific interactions between detection components and sequences bearing partial homology to the target, particularly problematic when analyzing complex biological matrices. This phenomenon manifests clinically as false-positive signals, fundamentally compromising diagnostic accuracy. Comparative analysis reveals distinct mechanistic constraints between CRISPR/Cas and Ago systems, CRISPR/Cas lack target conversion capability and exhibit restrictive PAM dependency, narrowing their applicability to nucleic acid targets with compatible PAM sequences. In comparsion, the PAM-independent target recognition mechanism of Ago proteins enables broader sequence compatibility, suggesting superior adaptability for multiplex target detection scenarios.

Signal-to-Noise Ratio Optimization. Background noise is a significant challenge in cascade amplification, as even small amounts of non-specific amplification can be magnified through successive reactions. This leads to increased signal noise, which can obscure the detection of target signals, especially in complex biological matrices. The cascading nature of the amplification process, where each step builds upon the previous one, means that any initial noise can be exponentially increased, leading to inaccurate or false results.

Temporal Constraints in Clinical Implementation. The temporal demands of cascade amplification introduce critical operational limitations. Low-abundance targets necessitate prolonged incubation periods to achieve detectable signal accumulation, conflicting with the rapid result requirements for emergency diagnostics and field-deployable systems. This temporal expansion directly correlates with increased nonspecific signal drift, creating an inherent trade-off between detection sensitivity and operational practicality.

With the increasing applications of these systems in biosensing and the ongoing depth of related research, we believe that Cas and Ago proteins will play an increasingly valuable role in next-generation detection technologies. Although cascade autocatalytic RNA detection sensors based on Ago proteins still face some challenges, we remain optimistic that improvements or new approaches will emerge in the future.

5. Summary and outlook

Nucleic acid detection technology has been used in a variety of scenarios such as medical care, food, and the environment [120–122]. Traditional nucleic acid detection methods have primarily relied on amplification techniques, such as PCR and qPCR. However, these methods have notable shortcomings, including the necessity for specialized training of personnel, complex operational procedures, the requirement for specific detection instruments, and insufficient specificity. In contrast, the CRISPR/Cas system and the Ago system, which evolved in microorganisms over time, play a crucial role in resisting foreign pathogen invasions and offer promising new avenues in amplification-free nucleic acid detection.

In order to meet the market demand for amplification-free nucleic acid detection, in addition to artificial targeted modification optimization for crRNA [97,123], specifically, we can develop a series of biosensor platforms based on the CRISPR/Cas system and the Ago system, which, in combination with, e.g., electrochemical platforms and autocatalytic cascade amplification pathways, can achieve more accurate detection results and higher signal output response. The ideal biosensor platform should have low cost, high sensitivity, short detection time and simple operation procedure, etc. Achieving these requirements is still challenging. In order to improve the sensitivity of the sensor, among the various signal amplification means, autocatalytic reaction is one of the most promising means due to its exponential signal growth. Although Ago-based autocatalytic biosensors are still in the preliminary stage of development, the successful application of CRISPR/Cas system in autocatalytic sensing provides experience for their development. Although autocatalytic biosensors based on CRISPR/Cas systems and Ago systems have great prospects, certain shortcomings need to be solved to make these technologies an important means of next-generation nucleic acid detection. For biosensors based on the CRISPR/Cas system, the target needs to have a specific PAM

sequence, which makes the detection have certain limitations. However, Ago protein can make up for this defect of Cas nuclease. Ago protein has catalytic activity in vivo and in vitro, has the advantages of not requiring a PAM sequence and high cleavage efficiency. However, current Ago-based nucleic acid detection methods mainly rely on thermophilic and mesophilic Ago proteins, so future research should focus on the development of biosensors with thermal stability and room temperature catalytic activity, such as *Clostridium butyricum Ago* (CbAgo) and *Myxobacterium Ago* (MbpAgo) [22]. The following is a list of issues requiring continuous improvement, which will serve as a reference for enhancing the autocatalytic biosensor platform based on the CRISPR/Cas system and the Ago system.

5.1. Economical

From a long-term development perspective, the production costs of the components of the CRISPR/Cas and Ago systems need to be considered. Cost reductions can be achieved through clever design of the nucleic acid circuitry in the CRISPR/Cas and Ago systems or the use of reproducible reaction materials. Future efforts may focus on the development of novel nucleic acid reaction materials and on reducing the cost of signal-collection devices to meet market demand.

5.2. Portable integrated

Considering the realistic application scenarios, autocatalytic biosensors based on CRISPR/Cas system and Ago system can be combined with some portable devices (e.g. microfluidic chips) [124,125]. Microfluidic technology can transfer nucleic acid detection from test tubes to microchips, which has the advantages of miniaturization and integration. Compared with traditional biological methods, microfluidic systems have shorter detection time, less contamination, lower cost, etc., and are expected to realize high-throughput detection [126]. At the same time, because of the small size of the microfluidic system, it is expected to meet the future demand for the development of portable nucleic acid detection instruments.

5.3. Intelligent

Signal processing methods combined with artificial intelligence can also have the result of improving the efficiency of detection, such as the use of machine learning-assisted fluorescence signal acquisition and data analysis, which can significantly improve the efficiency of biosensors in detection.Chen et al. developed a nucleic acid-free extraction and amplification biosensor for detection of *Salmonella typhimurium* in vivo by combining the assistance of machine vision and single microspheres. In this method, phage and lysis buffer were used to capture and lysed *Salmonella typhimurium* in vivo, and then Clostridium butyricum (Cb Ago) was used to lysed the bacterial nucleic acid to obtain the target DNA, which was then recognized by the corresponding fluorescent probes to produce the fluorescent signals accumulated on the single microsphere. Finally, the detection process and results were analyzed by a machine learning assisted algorithm with a detection limit of 40.5 CFU/mL and a linear range of 50–107 CFU/mL [73].

5.4. Translational challenges and barriers to Commercialization

At the level of large-scale production, CRISPR-Cas and Ago-based biosensing technology faces three major industrialization bottlenecks: **Core reagent production.** The efficiency of recombinant expression of Cas proteins and the synthesis purity of gRNA are far from meeting the IVD - level standards, resulting in relatively high detection costs. **Equipment integration challenges.** The integration of biosensors requires interdisciplinary collaboration in microfluidics and portable device engineering to ensure stability and accuracy in various usage scenarios such as at home or in the wild. **Difficulty in mass - production consistency.** Insufficient quality control and the lack of standardized production processes hinder the broader application of this technology in the clinical and diagnostic fields.

In terms of medical device approval, biosensing technologies based on gene - editing tools such as CRISPR and Argonaute proteins lack unified industry standards. Different from traditional diagnostic tools, CRISPR systems contain novel genetic components, making it difficult to classify them within the existing regulatory framework (such as FDA Class III medical devices or CE - IVDR standards). The absence of standardized guidelines leads to unclear regulatory pathways, significantly delaying the technology approval process.

In conclusion, the development of autocatalytic biosensors based on the CRISPR/Cas system and the Ago system holds great promise. This paper emphasizes the potential applications of CRISPR/Cas and Ago in nucleic acid detection and presents recent advances in the development of biosensors utilizing both systems. Furthermore, we aim to illustrate the advantages and disadvantages of CRISPR/Cas and Ago by analyzing and comparing their molecular and functional mechanisms, as well as their applications in biosensing. Future advancements should integrate autocatalytic biosensors with cutting-edge technologies such as microfluidics and machine learning to enhance detection efficiency and propel this nucleic acid detection strategy toward becoming the standard for next-generation molecular diagnostics.

CRediT authorship contribution statement

Qiangyuan Xiong: Writing – original draft, Investigation, Conceptualization. **Cancan Zhu:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Xueer Yin:** Investigation. **Ling Zhu:** Supervision, Resources, 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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Data availability

No data was used for the research described in the article.

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